Chemical ecology and phenotype in lichens: examining some environmental factors that influence variability, species assemblages and chemical compounds

By

Mohanad Talal Zraik

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Biological Sciences

University of Manitoba

Winnipeg, Manitoba

Canada

ABSTRACT

The general goal of this thesis was to explore the chemical ecology and macromorphological variability in lichens that colonize soils in five locations in Manitoba with respect to the carbon-nutrient balance hypothesis (CNBH). The CNB hypothesize states that when a plant or lichen is growing in nutrient-poor conditions, the excess carbon may be shunted into biosynthesis of carbon-based secondary metabolites such as polyketides. Three specific goals were further examined in four chapters to investigate:1) the distribution of secondary metabolites and species assemblages with respect to soil characteristics (Ch. 2), which resulted in 2) a report on *Cladonia magyarica* and *C. humilis* in Manitoba, describing the habitat, and providing morphological and chemical comparisons to distinguish five species (Ch. 3), 3) the phenotype in lichen species showing plasticity with respect to soil type, temperature and precipitation (Ch. 4), and 4) the effect of temperature and moisture on the quantity of three secondary metabolites (atranorin, fumarprotocetraric acid, and usnic acid) produced by three lichen species (Ch. 5). Field collections of lichens, soil features and environmental data were made in five locations. Secondary metabolites were determined using Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC). Morphological and fecundity characteristics were podetium height, cup diameter, squamule diameter, apothecium diameter, number of apothecia on cup and presence of apothecia. Soil characteristics were pH, organic matter, sand content, and sand grain shapes. The results showed that usnic acid and atranorin supported the CNB hypothesis. Species as *Cladonia magyarica* and *C. humulis* were reported for the first time in Manitoba. The CNB hypothesis was not supported for all lichen species, which may have been complicated by variability in biotic and abiotic conditions. Temperature and moisture affected the stability of secondary metabolites and partial degradation

occurred. This thesis forms a foundation for further studies on the relationship between secondary metabolites with biotic and abiotic factors.

ACKNOWLEDGEMENTS

I would like to thank first and foremost my advisor Dr. Michele Piercey-Normore. Thank-you very much Michele for accepting me for the project in your lab. Her patient guidance, understanding and support were integral for the completion of this project. Working with you was a privilege and I could not have asked for a better supervisor.

I would like to thank my thesis committee of Dr. Jim Roth, Dr. Georg Hausner and Dr. John Sorensen for their guidance and advice regarding my thesis and during the candidacy exam and annual meetings. Thank-you Dr. Sorensen and your lab personnel for letting me work, and use your lab for a very long time to finish my HPLC analysis, and for all your guidance and valuable comments. My committee members were much appreciated and they taught me a lot about writing and data analysis.

I would like to thank my labmates from the former Buller Lab 509; special thanks to Jennifer Doering and Chris Deduke. Their friendship, help and laughter made work enjoyable. Lab discussions taught me how to better analyze and interpret scientific papers.

I would like to thank Dr. Tom Booth. He taught me a lot about efficient field work and data collection while being able to enjoy being outdoors. Thank-you Dr. John Markham, Dr. Bruce Ford, Diana Sawatzky and Dr. Sylvia Renault for providing your labs to finish my study.

I would like to thank the Biological Sciences faculty and office staff, particularly Maureen Foster, Jaime Stringer and Sylvia Lapointe. In addition, I thank the Faculty of Science, Faculty of Graduate Studies, University of Manitoba Graduate Student Association, Canadian Botanical Association, and National Science and Engineering Research Council (NSERC) for financial support offered throughout my project.

Finally, I would like to thank my family: my beautiful wife Iman, my three angels Shamel, Hamza, Yussif, and my parents, who are overseas and I have not seen them for seven years, but I feel their support. Their love and support really helped me complete this project.

DEDICATION

To all the innocent people who were killed in my country, Syria, many of whom were friends and relatives; to you I dedicate this thesis.

Thesis, the publication status of chapters, and the role of each coauthor in each chapter

Chapter	Author	Contributions of each author	Article status
1	Mohanad Zraik	MZ reviewed the literature, organized the chapter, and wrote the chapter. MPN edited the chapter.	Not to be published
2	Mohanad Zraik Tom Booth Michele D. Piercey-Normore	MZ collected the material, performed statistical analysis of the data and wrote the chapter. TB helped with field work, discussed results and recommended literature. MPN generated the original idea with me, guided the process and edited the chapter.	Published in Botany 96(4): 267-279
3	Mohanad Zraik Tom Booth Michele D. Piercey-Normore	MZ conducted the experiment, collected the data, performed the analysis of the data and wrote the chapter. TB helped with field work, discussed results and recommended literature. MPN generated the original idea with me, guided the process and edited the chapter.	Published in Evansia 33(3):136-144
4	Mohanad Zraik John Sorensen	MZ collected the material, performed HPLC analysis and statistical analysis of the data and wrote the chapter. JS helped with HPLC	Submitted to the journal

	Michele D. Piercey-Normore	analysis. MPN generated the original idea with MZ, guided the process and edited the chapter	Oecologia
5	Mohanad Zraik John Sorensen Michele D. Piercey-Normore	MZ conducted the experiment, collected the data, performed the analysis of the data and wrote the chapter. JS Reviewed, discussed the results and recommended literature. MPN generated the original idea with me, guided the process and edited the chapter	To be submitted
6	Mohanad Zraik	Final Discussion and Conclusions were written by MZ and edited by MPN.	Not to be published

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

growth, fecundity and secondary metabolites are the main carbon consumers that affect the CNBH in lichens. When an outer cortex is present, it forms a partial barrier to external conditions. The medullary hyphae maintain moisture and air spaces for photosynthesis by the algal layer. The stereome forms support for the podetium to stand erect from a basal substratum. See text for more details.

CHAPTER 1

General Introduction and Literature Review

1.1. General Introduction

Lichens are composed of fungal partners (mycobionts) and photosynthetic partners (photobionts; green algae and/or cyanobacteria), which form symbiotic associations (Honegger 1998; Richardson 1999; DePriest 2004; Nash 2008; Tehler and Wedin 2008). Lichens represent at least 17,000 species of fungi (Kirk et al. 2008) and have adapted to habitats in most ecosystems in the world (Nash 2008). One adaptation of lichens to extreme conditions includes the growth form of the thallus, which is important for conserving water, or special morphological structures for absorbing water quickly when it becomes available. The growth forms include crustose, foliose and fruticose forms, which are attached directly to, or within, the substrate, form a thallus with an upper and lower surface over the substrate, or extend from the substrate as upright or pendant thalli, respectively. The crustose growth form is more efficient at conserving water and avoiding abrasion damage in extreme conditions while the fruticose growth form is easily damaged but maximizes surface area for efficient water absorption in less extreme conditions. Special morphological structures may include extensions from the thallus to absorb water and variability in size and shape of these extensions depend on the environmental conditions (light, temperature and humidity) and exhibit phenotypic plasticity in some species (Gilbert 1977; Pintado et al. 1997; Heiðmarsson 2003; Kotelko and Piercey-Normore 2010; Perez-Ortega et al. 2012; Muggia et al. 2014). The development of special morphological characteristics, and the production of metabolites and pigments that have different colors for

light protection, are examples of strategies to tolerate changing environmental conditions. A better understanding of the changes in phenotypic features of lichens will lead to a better understanding of species variability.

Phenotypic features that provide a known function in the habitat may be considered functional features or traits. Matos et al. (2014) examined functional traits of lichens in arid areas and showed that crustose lichens were highly correlated with arid regions but foliose lichens were not significantly correlated with arid regions. They also reported a strong association between cyanobacterial lichens and arid regions suggesting that functional traits of lichens may be associated with aridity. Other features of lichens such as secondary metabolite production are hypothesized to play a variety of roles for the lichen thallus in protection from environmental conditions in the surrounding habitat. For example, evidence has been provided to support antiherbivory roles (Gauslaa 2005), protection against light levels (McEvoy et al. 2006; Nybakken et al. 2006; Armaleo et al. 2008), heavy metal uptake (Hauck and Huneck 2007), regulation of water content in the lichen thallus (Lawrey 1986), and a number of other hypothesized roles (Huneck 1999). However, there is little known about the cues that result in secondary metabolite production and the chemical ecology of the secondary metabolites. Lichens produce many secondary metabolites in relatively large quantities and some metabolites are constantly produced by species making them stable phenotypic markers. The ecological distribution of secondary metabolites can shed light on their significance in nature in terms of the known functions. There are different theories that explain the evolution and the production of secondary metabolites (Bu'Lock 1961). One of the key theories is the Carbon – Nutrient Balance hypothesis (CNBH; Bryant et al. 1983), which proposes that secondary metabolites may be produced when the organism is in a stage of slow growth and nutrients become depleted, so

carbohydrates may be shifted to produce secondary metabolites instead of thallus growth. The production of secondary metabolites in cultured mycobionts corresponded with stress conditions such as drought (Culberson and Armaleo 1992; Stocker-Wörgötter 2001) supporting the Carbon – Nutrient Balance hypothesis (CNB; Bryant et al. 1983). The CNB hypothesis is relevant to lichen secondary metabolites as the major class of metabolites, polyketides, are all carbon-based. A better understanding of the mechanism behind polyketide production in lichens may be beneficial for understanding polyketide function as well as manipulations for commercial harvest of the metabolite.

Many lichens tend to grow in nutrient poor locations, they are adapted to absorbing nutrients from the air, and they have slow growth rates. Nitrogen fixation by microorganisms, including cyanobacteria associated with some lichens, provides an important source of nitrogen for soils that are poor in nutrient availability and have low numbers of symbiotic nitrogen-fixing plants (Barger et al. 2013). The cephalodia, which are small gall-like structures found in some species of lichens, contain the cyanobacterial symbionts. Cephalodia provide a direct source of nitrogen, allowing early colonizers of soil or bare rocks to survive, such as *Stereocaulon* and *Placopsis* (Brodo et al. 2001). Nitrogen-fixation rates in biological soil crusts (BSC) have been wide-ranging and have been estimated at 2 to 365 kg of N/ha per year, but Belnap and Eldridge. (2003) report that the higher rates are more accurate. Vascular plants growing among the BSCs and the surrounding area show higher tissue concentrations of nitrogen than plants grown in bare soil ecosystems (Belnap et al. 1996, 2001; Su et al. 2011; Pietrasiak et al. 2012, 2013). Higher nitrogen content in the soil may imply higher plant colonization, more animal activity and higher levels of nutrients in general.

Approximately 40% of terrestrial ecosystems are composed of dry land (Baquerizo et al. 2013; Robinson et al. 2013), including the semiarid and arid environments throughout the world, with high light levels at the soil surface, a scarcity of water resources, and fluctuation of temperatures. In areas with large expanses of dry land, many non-succulent plants are rare or absent, and the open ground is generally covered by BSCs, which result from the association between soil particles and organisms such as algae, cyanobacteria, lichens, bryophytes, liverworts, fungi and bacteria (Honegger 1998; Richardson 1999; DePriest 2004)

These organisms can live together as a diverse community of organisms or they can live separately as a monoculture and cover large areas of soil and are adapted to extreme conditions of temperature and water availability. The soils present in boreal areas may also provide particulate substrata for attachment by organisms. Soil type is sometimes included with species descriptions but correlations among phenotypic features and soil type has not been examined, to the best of our knowledge, and will provide a better understanding of the role of soil in some of these lichens. Crustose lichens may become firmly attached to soil or rock with filaments weaving among the particles. The attachment may occur by soil anchoring fungal filaments or aggregates of filaments (rhizines), which intertwine among soil particles and polysaccharides (Schlech-Pietsch et al. 1994; Richardson and Smith 1996; Belnap and Lange 2003) to aggregate and bind the soil particles together, and harden the soil surface (Nash 2008) When the size of soil aggregates enlarge, they become heavier, and have a larger surface area, so they are more resistant to motion by wind or water. Terrestrial bryophytes are attached to the soil by rhizoids that may grow for more than 10 mm deep and form aggregates with the soil particles (Michler 2001). Crust-forming organisms tend to have low moisture requirements, and they have the ability to use the available water sources such as rainfall, snow, fog and dew, more efficiently

than other growth forms (Nash 2008). The ability for crustose lichens to stabilize soil and make use of moisture efficiently is an important adaptation for stabilizing disturbed areas.

Organisms that equilibrate their internal moisture levels to match that of the surrounding atmosphere are referred to as poikilohydric organisms, where most of the BSCs are poikilohydric and about 350 species of vascular plants have the ability for desiccation tolerance of vegetative tissues (Toldi 2009). Poikilohydric organisms generally become photosynthetically active and produce carbohydrates or sugars very quickly after water becomes available (Kappen 1988; Kappen and Valldares 1999). However, many species of biological crust communities still need high levels of hydration to optimally perform the physiological functioning. The threshold at which the moisture content allows for optimal physiological and metabolic activity is species specific and it helps to determine the geographic and microhabitat distribution of the various species that make up the biological crust community. Many of these organisms perform under a variety of light intensities and desiccate rapidly especially if they lack a waxy epidermis and they tend to leak nutrients into the soil upon wetting and drying (Nash 2008; Belnap et al. 2001). Some of these nutrients are sugars produced by lichen algae, the dehydrins, and other proteins that help to protect cells during desiccation (Lawrey 1984; Deduke et al. 2012; Millot et al. 2012). The poikilohydric nature of most lichens allows them to live in extreme conditions and habitats which cannot be inhabited by most vascular plants.

One of the ecological roles of BSC and lichen crusts includes the stabilization and protection of the soil surface from erosion by wind and water activity. Wind and water can have a significant destructive force in deserts and other ecosystems, as sparse vegetation causes large patches of bare, unprotected desert soil that can be displaced. BSCs also play an important role in building the soil by serving to hold the soil particles together and by increasing the biomass and

texture of biological material for trapping diaspores for further growth. The degree of protection of soil particles from the different kinds of BSCs varies and may form a surface layer of cyanobacterial and algal crust, lichen crust, and even more protection when fruticose lichens and acrocarpous mosses create a three-dimensional microhabitat allowing other organisms to grow (Belnap et al. 2001; Langhans et al. 2009; Robinson et al. 2013). Some lichen crusts can be very effective in retarding soil erosion because their thalli form a barrier and prevent the puncturing effects of raindrops on the soil. Rogers (1977) showed that the erosion of unprotected soil (with no lichen cover) was 10 cm greater compared with the nearby soil that was protected by lichen crusts growing on its surface (Rogers 1977; Mishler 2001; Bu et al. 2015; Chamizo et al. 2017).

The BSC and lichen crusts have good tolerance to arid conditions, but they are also sensitive to physical disturbances and to fluctuations in temperature or humidity, and it takes a very long time for their recovery after disturbance lasting from years to decades (Rajeev et al. 2013). Disturbance as an ecological term means changes in the average environmental conditions of a specific area or habitat, which causes a change in an ecosystem and/or community dynamics (Dornelas 2010). Disturbance such as fire, intense grazing, and drought can directly remove or damage biological crusts by reducing the cover and species composition, and reduce the efficiency of carbon and nitrogen fixation. Other disturbances caused by human activities such as air pollution and oil spills may also cause damage to biological crust communities. Disturbance can have an effect on the extent of the area of soil covered by the BSCs, loss of species composition, and reduction of carbon or nitrogen fixation. A better understanding of the mechanisms for survival by soil dwelling lichens will shed more light on species variability, secondary metabolite production and function, soil relationships, and potential stabilization of disturbed areas.

1.2. Thesis Goals and Objectives

The general goal of this thesis is to explore the chemical ecology and macromorphological variability in lichens that colonize soils in five locations in southern and northern Manitoba with respect to the carbon-nutrient balance hypothesis (CNBH; Bryant et al. 1983). The CNBH can be paraphrased to state that excess carbon, as a result of an imbalance in carbon and nutrients, would lead to an increase in secondary metabolites production. Even though this hypothesis has been tested repeatedly in the literature, it is still controversial (Koricheva 2002). Little is known about growth and chemical ecology of lichens in arid or sandy regions but it has been examined with respect to their more common secondary metabolites such as polyketides (Toni and Piercey-Normore 2013). If extreme conditions can trigger expression of some of the silent polyketide synthase (PKS) genes (Keller et al. 2008; Schroeckh et al. 2009; Armaleo et al. 2011), novel polyketides may be discovered that will improve our understanding of the ecology of lichens, which may ultimately be useful in the biotechnology or pharmaceutical industries. A better understanding of the environmental parameters on phenotype will improve our interpretations of evolutionary hypotheses and fungal plasticity in lichen fungi. Under more exposed disturbed or early successional conditions with low humidity and high light levels, we expect some morphological characteristics to be suitable to preserve moisture but under later successional habitats with more vegetation and more moisture retention in the community, the morphology might be more optimal for providing better conditions for reproduction and dispersal. If the lichen species assemblages and phenotype can be characterized that are suitable for exposed arid conditions, some lichens may be suitable to be relocated to these regions to minimize the damages of erosion of soil or sand dunes. The specific objectives are addressed in four chapters:

Chapter 2 examined the distribution of secondary metabolites and species assemblages with respect to soil characteristics, temperature, and precipitation in five locations in Manitoba. The differences between the soil composition and soil structure affect the water holding capacity of the soil and retention of the moisture around the lichen thallus, determining the species in the assemblage (Zraik et al. 2018). This chapter determined whether there were relationships among soil characteristics (pH, sand and organic matter) species, and secondary metabolite occurrence.

Chapter 3 reported the first occurrence of *Cladonia magyarica* and *C. humilis* in Manitoba and provides morphological and chemical comparisons to distinguish five species (Zraik et al. 2016). This chapter reports these two species for Manitoba, which were found in one of the total five locations for the larger study in Chapter 3.

Chapter 4 investigated whether phenotypic changes (secondary metabolites and morphology) in five lichen species (*C. cariosa, C. gracilis* subsp. *turbinata, C. chlorophaea, C. pyxidata,* and *C. arbuscula*) showed plasticity with respect to soil type, temperature and precipitation. This allowed for a close examination of the CNBH by examining phenotypic variability (including morphological characters, secondary metabolites, and fecundity) in selected lichen species as an indication of growth compared with the quantities of secondary metabolites. The hypothesis was that more thallus and tissue growth will lead to fewer secondary metabolites produced, supporting the CNB hypothesis.

Chapter 5 investigated whether temperature and moisture had an effect on the quantity of three secondary metabolites (atranorin, fumarprotocetraric acid, and usnic acid) produced by three lichen species (*Cladonia cariosa, C. chlorophaea*, and *C. stellaris*), respectively, and from commercial standards. The hypothesis for this study was that the high temperature and dry conditions will decrease the concentration of secondary metabolites over time by degradation.

Chapter 6 provided a general discussion with insights into the CNBH for lichens, expected potential directions of research, and future applications as a result of these findings.

1.3. Literature Review

1.3.1. Lichen classification and taxonomy

There are about 17,000 known species of lichen-forming fungi, which comprises 42% of all Ascomycetes (Kirk et al. 2008). Of the lichenized fungi, 98% belong to the Ascomycota (Kirk et al. 2008), only fifty species belong to the Basidiomycota (0.4%), and about 200 species belong to the Deuteromycota (1.5%) (Honegger 1992; Hawksworth and Honegger 1994; Kirk et al. 2001; DePriest 2004; Calcott et al. 2018), which likely belong to the Ascomycota. The family Cladoniaceae is one of the largest in the Order, Lecanorales (Ahti 2000; Stenroos et al. 2002; Miadlikowska et al. 2006). The genus *Cladonia* is a widely distributed genus of lichen-forming fungi in the Cladoniaceae, containing more than 400 species (Ahti et al. 2001). In North America, more than 168 species of *Cladonia* have been reported (Esslinger 2008). Species of *Cladonia* are found on a variety of substrates such as moss, wood, tree bark, rock, soil, and peat (Esslinger 2008) and most of them grow on the ground. *Cladonia* is divided to seven taxonomic sections (Ahti 2000) in addition to three sections in the former genus *Cladina* (*Crustaceae, Impexae,* and *Tenues*). The mat-forming lichens were formerly included in the former genus *Cladina,* which has been subsumed within the larger genus *Cladonia* (Ahti et al. 2001), and are a large and important group of lichens (Stenroos et al. 2002; Kotelko and Piercey-Normore 2010; Athukorala et al. 2016). They grow acropetally usually forming a carpet of thalli and the lower layer is dead in the older mats, which physically supports the upper layer (Crittenden et al. 2000). The mat-forming lichens (in the former genus *Cladina*) occupy about 8% of total

terrestrial surface on earth, and is an important vegetation component that provides 60% of a caribou and reindeer diet during the winter (Larson et al. 1987; Kytoviita et al. 2009); hence, they are often called Reindeer or Caribou lichens colloquially. Seven sections (*Cocciferae, Ascyphiferae, Cladonia, Helopodium, Perviae, Strepsiles,* and *Unciales*; (Stenroos et al. 2002)) comprise the species in the genus. *Cladonia* Section *Cladonia* is composed of the pixie cup lichens and a number of species of the *Cladonia chlorophaea* species complex including *C. cryptochlorophaea*, *C. grayi*, *C. pocillium, C. fimbriata* (Gilbert 1977; Piercey-Normore 2006; Kowalewska et al. 2008) *C. pyxidata, C. magyarica, C. humilis,* and *C. merochlorophaea* (Brodo et al. 2001; Stenroos et al. 2002; Kotelko and Piercey-Normore 2010). Members of the *Cladonia chlorophaea* species complex share very similar morphological characteristics but they are different in secondary metabolite content (Culberson 1986a; Culberson et al. 1988).

The photosynthetic partners comprise approximately 100 species of green algae and cyanobacteria (Friedl et al. 1996) and form photobionts for the 17,000 species of lichen fungi. *Trebouxia* is the most common genus of the lichenized algae (Honegger 1998) and it is associated with most of the Lecanorales (Helms et al. 2001). The genus *Cladonia* is mostly associated with a coccoid green algal genus called *Asterochloris* (Rambold et al. 1998; Miadlikowska et al. 2006). Lichen species that contain cyanobacteria as the main photobiont comprise only 10% of the total lichen species and the most common genus is *Nostoc* (Nash 2008).

1.3.2. Soil and geology in Manitoba

Soil is the upper layer of the earth surface, which formed from the parent rock material, climatic factors, activities of micro- and macro-organisms, including human activities, and from the effects of topology and erosion. Soil texture is a term used in the classification of the soil types based on the percentage contents of the three main components, which are sand, silt and clay. The size of sand particles ranges from 0.05 to 2.00 mm and can be classified into fine or coarse sand based on diameter size. The size of silt ranges from 0.05 to 0.002 mm, and clay is less than 0.002 mm (Soil management guide 2008). Sandy soils or coarse soil are unable to hold water so they lose water more rapidly than clay or heavy soil (Kuzyk 2013). Soil texture is also determined by the forming of aggregates by gluing the soil particles together using the organic matter as a clinging material. The type of soil is based on the type of original material (bedrock) and the method of deposition. In Manitoba, soil is comprised of a combination of limestone, granite, or shale (Bannatyne 1975). Weathering by abiotic and biotic methods over long time periods breaks the bedrock into pieces and forms the soil. Sand is formed from granite, clay soil is formed from shale, and limestone rocks breaks down into sand, silt and clay-sized particles (Soil management guide 2008). Bedrock comprises three types of rock, igneous, sedimentary, and metamorphic rock, from which all soils are derived (Manitoba Geology 2017). The variable geology provides substratum for many lichen species. In Manitoba, soil was formed by these methods as well as the result of large glacial lakes which deposited debris after the lakes disappeared.

Lake Agassiz, was a glacial lake, which replaced the Laurentian ice sheet that covered a portion of the prairies approximately 75,000 years ago (Andrews et al. 1978) extending from the southwest into Saskatechwan and Alberta, south into North Dakota and Minnesota, and southeast

into Ontario. Lake Agassiz was formed approximately 13,000 years ago and covered an area of 1,600,000 km^2 (Andrews et al. 1978; Teller et al. 2004). The lake fluctuated in size many times from the north, east and west because of the melting water that poured in from those directions (Huck et al. 2013). After the lake drained into the basin of Hudson Bay, outcrops of Ordovician and Silurian limestones and dolostones were left behind and became exposed from the Lake Agassiz (Teller et al. 2004). The exposed bedrock formed a wide area called the Western Canada Sedimentary Basin, covering about half the province of Manitoba forming the Precambrian Shield on a diagonal from south east to north west. The Trans-Hudson Orogen (THO) province was located in north of Manitoba in the early Proterozoic Era more than 1.7 billion years ago (Lewry et al. 1990). It was formed over exposed ridges of ancient volcanic lava flows (Brownell et al. 1935), and many kinds of rocks as basalts, quartz, intrusive granites, and gneisses (Kalliokoski 1953; Bailey 1989). These rocks enriched the soil with zinc and nickel (Brownell et al. 1935), and a mining source for north Manitoba.

The bedrock for Manitoba in general consists of granitic and mafic metavolcanic rocks. The Flin Flon area of the Precambrian Shield consisted of different and varied geological material of metavolcanic, mafic, felsic, granite, and gneiss rocks (Brownell et al. 1935; Kalliokoski 1953; Bailey 1989; Manitoba Geology 2017). The southern part of the province below the Precambrian diagonal consists of open and stabilized sand dunes and river bottom forest above limestone tables in the west. The southeast side of the province consists of glacial till regosols (mineral soil which developed very weakly by unconsolidated materials and formed a thin soil) (Chesworth 2008). The Sandilands till and the surrounding areas are comprised of sand and gravels that were formed from lobes of ice which eroded and were carried by Lake Agassiz waves (Huck et al. 2013).

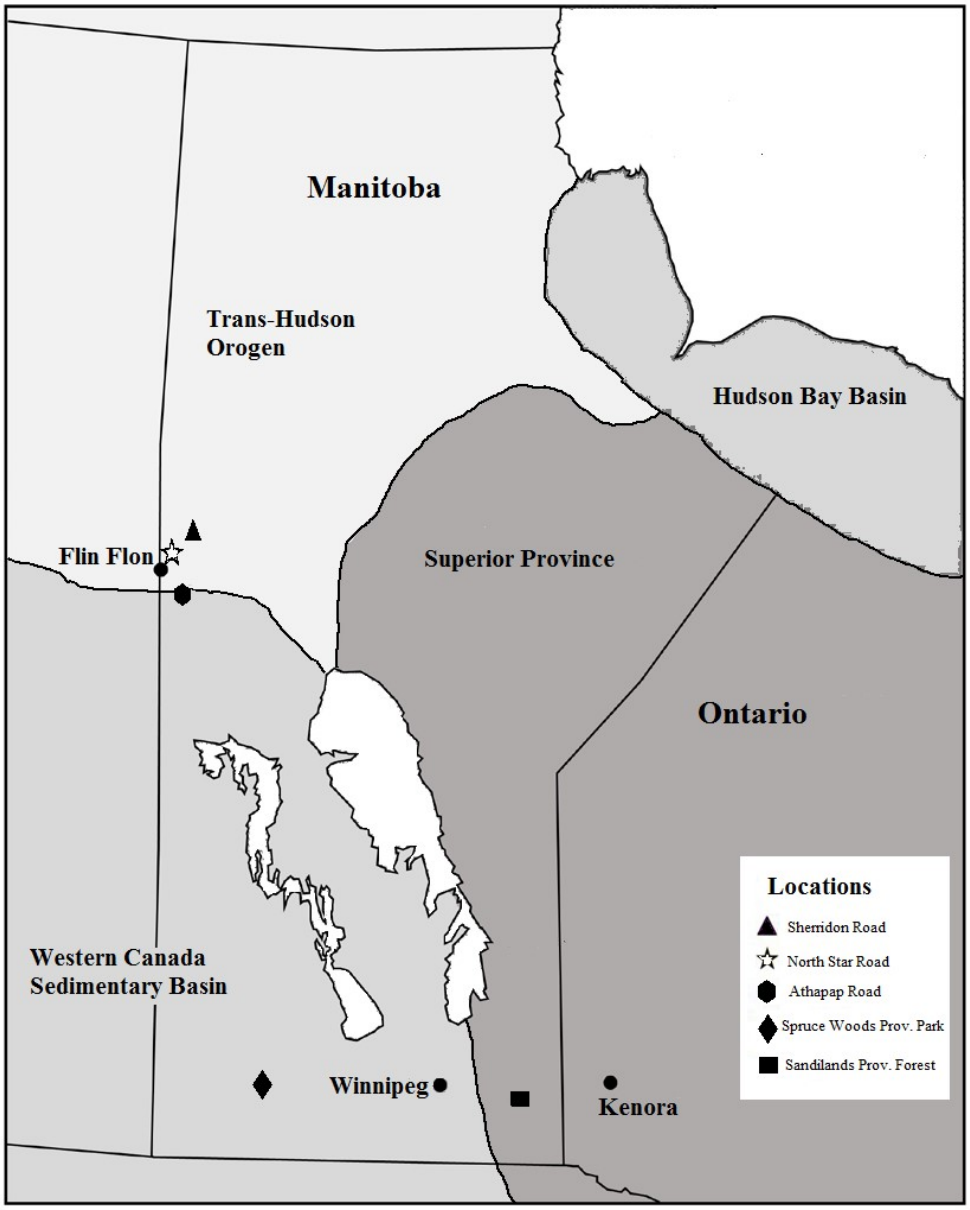


Figure 1.1. A map of Manitoba showing the five study locations (Sandilands Provincial Forest, Spruce Woods Provincial Park, Athapap Road, Sherridon Road and North Star Road) relative to the geological provinces. See text for further details. Map by Deduke (2015) and modified by M. Zraik. For a complete list of transects and location data, see Appendix B.

1.3.3. Chemical ecology in lichens

The ecology of secondary metabolites forms a large part of the field of study called chemical ecology, which has grown to be a broad discipline that deals with the interactions between biological organisms and the production and characterization of their secondary metabolites in an ecological context (Inderjit et al. 2002). Most of the literature involves plant-insect interactions and plant pathology, but fungal and lichen chemistry has had a number of studies and a longer history in the context of chemotaxonomy (Karunaratne et al. 2005). However, there have been a few studies on the ecological distribution of lichen secondary metabolites. Chemical ecology studies have mainly focused on the defensive role of lichen's secondary metabolites against consumers (Rundel et al. 1978; Lawrey et al. 1984, 1986). Some of these have suggested a correlation between the function of specific compounds and their occurrence in habitat types. For example, Toni and Piercey-Normore (2013) showed that environmental features are important key factors for secondary metabolite production and species distribution. Chemical ecology in lichens shows the correlation between the secondary metabolites and the habitat types (Huneck et al. 1999; Inderjit et al. 2002; Fox et al. 2008; Toni et al. 2013).

1.3.4. Lichen morphology and growth forms

In the symbiotic interactions between fungi and their photosynthetic partners, the lichen fungi absorb water and nutrients, and provide support for the photobionts (algae, cyanobacteria or both). The photobiont partner produces the sugars through photosynthesis which are transported to the mycobiont (Nash 2008). Lichens can grow on a wide variety of natural and man-made substrates and they exhibit a wide range of behaviours, secondary metabolite

production, and interactions with other organisms (Friedmann 1982; Nash 2008). They dominate the ground cover in northern environments and form a crusty carpet that stabilizes unstable soils in arid environments. The morphological characteristics are key factors for the adaptation of lichens in harsh conditions, such as the increase in the thickness of the thallus and the tough cortical layer (Stocker-Wörgötter and Turk 1987; Osyczka and Rola 2013). These are examples of some adaptations that develop in lichens and give lichens the ability for wide distribution in a wide range of climates especially in harsh conditions (Rogers 1977). Many of the phenotypic characters used in identification keys are assumed to be hereditary, but studies are suggesting that some of them may be influenced by environmental changes (Gilbert 1977; Behera 1997; Pintado et al. 1997; Kotelko and Piercey-Normore 2010).

Lichens that form crusts on the surface of substrates or grow within substrates are called crustose lichens. The thallus of crustose lichens has an upper side, the lower part of the thallus is attached directly to the substrate by fungal mycelia, and it is the most dominant growth form in arid areas, comprising 90% of total lichens (Galun 1988). Regions that are not extremely arid have about 60% crustose and about 40% fruticose and foliose lichens (Kappen 1977). The squamulose lichens are mostly *Cladonia* spp., which are lichens without podetia and composed of flat overlapping leaf-like scales of thallus (Brodo et al. 2001) attached directly to the substrate by a portion of their lower sides similar to other crustose lichens. Foliose lichens have an upper and lower side which is attached to the substrate by special attachment organs (rhizines or holdfasts). Fruticose lichens grow upright from the substrate or are tufted or pendant and hang from the substrate attached at a single point to the substrate. As a result, the thallus is round or nearly round in section and it has inner and outer tissues. Fruticose lichens are attached to the substrate by a single point, all sides of the thallus are exposed to the atmosphere, and they are

rare in arid habitats (Matos et al. 2015). Since foliose lichens have a lobed thallus and a lower and upper surface close to the substrate, they lose water faster than crustose lichens but not as fast as fruticose lichens. Fruticose and foliose lichens lose water because they have a higher surface area to volume ratio than crustose lichens. The crustose form is advantageous because the surface area to volume ratio of the lichen is small, allowing for preservation of water, and reducing evaporation during times of desiccation. The attachment of the crustose thallus to the soil substrate by fungal mycelia results in many mycelial hyphae extending throughout the soil and holding soil particles together by polysaccharides and other products of the hyphae (Eldridge 1998). In mature, undisturbed desert communities, terricolous lichens and mosses are important in stabilizing soil surfaces by their mycelia and rhizoids that weave among soil particles. Some lichens fix nitrogen, some of which may be transferred to the surrounding environment through leaching, loss of cyanobacterial cells, or by decomposition after death of the lichen. Saxicolous lichens and mosses frequently cover desert cliff sides with thalli of various shades of yellow, orange, green, brown and black (Shields et al. 1957) due to secondary metabolites which may serve as light shields or enhancers for algal photosynthesis (Nguyen et al. 2013).

In the dry sandy regions in Manitoba, the Carberry Sand Hills form stabilized dunes and rolling prairie with some dunes still active, and they have a humid continental climate. The average temperature in August is 25.4°C with the highest temperature reaching 37.4, and in January it is -24.7°C. Most precipitation falls as rain (371.1 mm) with the remainder as snow (101.4 cm) (Canadian climate data and information archives 2013). In contrast, the Sahara desert is the hottest and driest area on earth (Cook et al. 2015) and the average annual temperature for the Sahara desert is 30°C but during the hottest months temperatures can exceed 50°C. The highest temperature ever recorded was 58°C in Aziziyah, Libya, and in Port Sudan (Nicholson

2011) with low precipitation (251mm) yearly. In both areas lichens have evolved many morphological and chemical characteristics in order to survive. The size of the thallus may be reduced and the thickness of the cortex may be increased to avoid too much evaporation from the thallus because humidity is rare or absent. In the dry sandy regions in Manitoba, the Carberry Sand Hills, *Cladonia* species are dominant and wide spread (Piercey-Normore 2006). *Cladonia* spp. begin growth as a crustose or squamulose thallus (Ahti 2000), preserving water near the soil layer, and then produce podetia in sexual reproduction which are hollow allowing for water to be maintained within the hollow space. In the Sandhills of Manitoba, fruticose lichens can survive in moist microhabitats because higher humidity levels are found under trees and shrubs between the sand dunes. The highly branched nature of some fruticose lichens allows for water and humidity to be trapped between the branches. Humidity may also come from fog or dew. The fog lichens have to be adapted to the occasional high atmospheric moisture events and with their high surface area to volume ratio of the thallus, they can absorb moisture very quickly. The fruticose, pendulous, or tufted growth forms are usually highly branched or have cilia on the surface to increase the surface area of the thallus for absorbing water (Rundel 1978).

1.3.5. Secondary metabolites

Secondary metabolites are a wide range of compounds produced by many groups of organisms, which are not considered vital for the growth and development of the organism (Elix 1996; Keller et al. 2005) but they may be produced for other functions. There are different theories that explain the evolution and the production of secondary metabolites (Bu'Lock 1961). One theory suggests they may be considered as a waste secreted from the cell (Elix 1996) which may need further detoxification in the organism (Hartmann 2007). Secondly, there may be

specific ecological roles for secondary metabolites to allow adaptation of organisms and improve their tolerance to changes in the environment (Culberson and Armaleo 1992; Hartmann 2007). Some secondary metabolites like usnic, fumarprotocetraric, peratolic, and thamnolic acids are thought to increase the tolerance of lichens to acid rain and acidic substrates (Hauck 2008; Hauck et al. 2009b). Third, secondary metabolites may be by-products of primary metabolism that are transformed to secondary compounds (Hartman 1996). Fourth, they may be produced as a result of an imbalance in nutrients and carbon when the organism is in a stage of slow growth (Deduke et al. 2012). Secondary metabolite production may happen when nutrients become depleted and carbon acquisition by tissue growth slows down, so carbohydrates may be shifted to produce secondary metabolites. Studies have suggested important roles for secondary metabolites in adaptation of the organisms and improving their tolerance to changes in their environments (Culberson and Armaleo 1992; Hartmann 2007).

Many of the secondary metabolites produced by lichen-forming fungi are polyketides, which fall within two main classes: depsides and depsidones (Rundel 1978). Polyketides are formed by a multidomain Polyketide Synthase (PKS) enzyme, which is encoded by PKS genes that sometimes occur as large numbers of paralogs in fungal genomes (Sanchez et al. 2010). One of the earlier studies on the PKS genes in lichens has been with *Cladonia grayi* (Armaleo et al. 2011). Non-lichenized fungi, such as *Aspergillus nidulans*, are also known to produce fewer numbers of polyketides than there are PKS genes (Sanchez et al. 2010) suggesting plasticity in the PKS gene expression and biosynthesis of the polyketide. The many PKS paralogs present in fungal genomes are thought to be influenced by environmental conditions, but their individual functions are still unknown (Keller et al. 2008), and few studies have examined the ecological distribution of lichen secondary metabolites (Toni and Piercey-Normore 2013; Piercey-Normore

2007). Little is known about chemical ecology of lichens in arid or sandy regions. If extreme conditions can trigger expression of some of the silent PKS genes, novel polyketides may be discovered that will improve our understanding of the ecology of lichens and they may be useful in the biotechnology or pharmaceutical industries.

In lichens, secondary metabolites are chemical compounds secreted by the fungal partner of the lichen. More than 1000 compounds have been identified in lichens so far (Stocker-Wörgötter 2008; Lokajová et al. 2014). Most of these compounds (93%) are unique to lichens (Lokajová et al. 2014), and most lichens contain between 0.1 to 5% of total thallus dry weight in secondary metabolites (Fahselt et al. 1994) but exceptions exist. The widespread foliose lichen *Hypogymnia physodes* may contain 22% secondary compounds of dry weight (Solhaug et al. 2009) and it may sometimes reach 30% of dry weight (Huneck et al. 1973; Burkin et al. 2014). These fungal compounds are deposited on the surface of fungal hyphae, and they can be extracted by organic solvents without harming the lichen. Lichen substances are classified into groups based on their biosynthesis. First, polyketides are produced by the acetyl-polymalonyl pathway. Second, terpenes and steroids are produced by the mevalonic acid pathway. Third the cyclopeptides are produced by the shikimic acid pathway. Fourth, photosynthetic products are produced by the photobionts including sugar alcohols and polysaccharides (Rundel 1978; Schmitt et al. 2008), which are not considered secondary metabolites but may be used in the biosynthetic pathways.

Some of the secondary metabolites produced by lichen-forming fungi are thought to serve as protection against the sun light by acting as a sun screen layer for the UV light, which damages DNA and interferes with photosynthesis by the algae (McEvoy et al. 2006; Nybakken et al. 2006; Armaleo et al. 2008). Some secondary metabolites have allelopathic and antifungal

properties (Whiton et al. 1982; Lechowski et al. 2006; Hauck et al. 2009; Kowalski et al. 2011; Bačkor et al. 2013) while others may function in humidity control in the medullary hyphae (Culberson and Armaleo 1992), or as a deterrent against herbivory by animals and insects (Gauslaa 2005; Nimis et al. 2006). The production of some secondary metabolites is correlated with special environmental conditions such as the production of grayanic acid when the lichen culture begins to dehydrate (Culberson and Armaleo 1992), or larger number of secondary metabolites with more basic growth media (Timsina et al. 2013). The production of baeomycesic and squamatic acids in *Thamnolia vermicularis* var. *subuliformis* start only after the dehydration of the culture media and the exposure to high light conditions with a temperature of 15C° (Stocker-Wörgötter et al. 2001). A change in quantity of secondary metabolites with environmental conditions that influence nutrient availability may support the carbon-nutrient balance hypothesis (Bryant et al. 1983; Hyvarinen et al. 2003). If environmental conditions influence the type and amount of secondary metabolite produced, then there should be a relationship between the parameters in the habitats and the secondary metabolites. Secondary metabolites have a wide range of stability depending on their structure and chemical groups to which they belong to. Some secondary metabolites are unstable compounds, while some compounds such as anthocyanins are more sensitive to regular thermal treatment comparing to phenolic groups (Wilkes et al. 2014). Anothocyanins are significantly degraded between temperatures of 60°c to 80°c (Buckow et al. 2010; Wang et al. 2010), while a compound like atranorin is stable to a temperature of 200°c (Huneck et al. 1968).

1.3.6. Carbon-nutrient balance hypothesis

The carbon-nutrient balance hypothesis (CNBH) is based on the concept that an imbalance of nutrients will promote production of carbon-based secondary metabolites (Bryant et al. 1983, 1986; Waterman et al. 1989). The CNBH is considered an important hypothesis to understand the chemical and physiological responses to environmental changes. This hypothesis is studied with respect to plants (Bryant et al. 1983, 1986; Waterman et al.1989; Muzika et al.1993; Hyvarinen et al. 2003), but it is controversial because it represents a complex model with many interacting variables (Koricheva 2002). The CNBH suggests that when a plant or lichen is growing in nutrient-poor conditions, the carbon may be shunted into carbon-based secondary metabolites such as polyketides. When the plant or lichen is growing in nutrient rich and balanced nutrient conditions, the thallus growth use the nutrients and carbon because they are available at optimal levels, rather than carbon being used in the production of secondary metabolites. If the microhabitat has features that promote retention of humidity or changes in levels of nutrients, it may influence the balance between carbon and nutrients available to the lichen. Therefore, soil features and community structure may indirectly influence production of secondary metabolites (Bryant et al. 1983; Hyvarinen et al. 2003). Production of secondary metabolites may also be traded for other physiological processes such as thallus growth or fecundity (number of apothecia and ascospores) (Rhoades 1979; Deduke et al. 2014) or produced in a coordinated fashion with a combination of features (Marie et al. 2013). The investigation of many phenotypic variables over different habitats may allow the CNBH to be tested with lichens. Harsh and dry conditions in culture experiments have stimulated the production of secondary metabolites by mycobionts (Culberson et al. 1992; Stocker-Wörgötter 2001).

1.3.7. Water availability and phenotypic plasticity

While water is essential for all organisms, the limitation on the availability of water in arid regions restricts the species that can survive or requires special adaptations to tolerate low water levels. The hydration – dehydration fluctuation cycles of lichen thalli allows lichens to undergo more efficient transfer of carbohydrates between the fungal and algal partners (Nash 2008). The effect of water availability may be influenced by other environmental parameters such as temperature, soil type, and pH; and it may affect PKS gene expression after rehydration (Junttila et al. 2013) of the lichen. Water availability may also influence morphological features resulting in phenotypic plasticity of the lichen. Pintado et al. (1997) showed that *Ramalina capitata* had a slightly different morphology on the north and south facing aspects of a cliff, where thalli on the shaded, moist north side of the rock had higher chlorophyll content than exposed and sunny sides. The soil pH was thought to influence morphological variability in *Cladonia pyxidata* and *C. pocillum* (Gilbert 1977; Kotelko and Piercey-Normore 2010). The surrounding pH controls expression of a transcription factor, pacC, which mediates expression of genes and is involved in induction of the expression of other genes in salt tolerance (Caracuel et al. 2003) or controlling virulence (Bignell et al. 2005). Since pH can modify behaviours such as these, it might be expected to also modify expression of secondary metabolite genes that are often involved with stress tolerance and pathogenesis. The adaptation of lichens in arid areas where most vascular plants are absent, allows lichens to avoid competition with plants and to dominate these sites (Botting et al. 2006). A better understanding of these environmental parameters on phenotype will improve our interpretations of evolutionary hypotheses, fungal plasticity, and regulation of gene expression in lichen fungi.

Changes in phenotype and species assemblages would be expected in lichen-fungi with different stages of succession (Pugh 1980; Lan et al. 2012) resulting in changes in the structure of the surrounding vegetation. Under more exposed disturbed or early successional conditions with low humidity and high light levels, variation in some morphological characteristics of *Cladonia* such as the dimensions of podetia would be expected. In early successional conditions (high light and low moisture levels) the podetia might be short and wide with less surface area to preserve moisture but under later successional habitats with more vegetation and more moisture retention in the community, the podetia might be tall and narrow with less need to preserve moisture and more need to raise the apothecia higher in the air currents to provide better conditions for dispersal. Extensions of the podetia would raise the apothecia even higher, which are seen in many species of *Cladonia*. Because of the high levels of plasticity shown for some soil crust lichens, they may be the dominant vegetation in arid regions (Lalley et al. 2006). Since many species of *Cladonia* produce a primary thallus of squamules, they are often considered squamulose crustose lichens. Larger numbers of species within a small area may also increase the potential for competition for space and resources, and allelopathy by production of secondary metabolites (Armstrong et al. 2007; Rosentreter et al. 2014). If the lichen species assemblages and phenotype can be characterized that are suitable for exposed arid conditions, one application of this knowledge is that the lichen species can be relocated to these regions to minimize the damages of erosion of soil or sand dunes. Three volumes on lichens of the Greater Sonoran Desert region have been published (Nash et al. 2002, 2004, 2011), covering most of the lichen species in the Sonoran Desert region and provide a foundation for comparisons with other arid regions.

1.3.8. Techniques for studying lichen secondary metabolites

In lichens, chemical ecology can be complicated by chemotaxonomy. Chemotaxonomy refers to classification of the species depending on the production of specific major secondary metabolites as additional phenotypic characters. It has been used for lichen species that are morphologically similar with overlapping variation in morphological characteristics but differ in the secondary metabolites synthesized by the fungal thallus. These chemical forms are referred to as chemotypes or chemospecies (Hawksworth 1976; Lumbsch 1998a, 1998b). While chemotaxonomy can sometimes help with species determination, it is not always a means to differentiate species since the production of different types of secondary metabolites is generally not specific enough. Some of the first methods to use secondary metabolite characters in lichens involved the use of crystallization on glass slides (Evans 1943; Thomson 1967; Orange 1992). The crystalline reaction produced many different shapes and sizes of crystals that were easily distinguished under a microscope. Identification of substances depended mainly on the crystal shapes that have unique features for each compound. Later, spot tests became popular, which required a more simplified way to test for presence or absence of the compounds directly on the thallus by adding a tiny spot of reagent to different areas of the thallus and noting a change in the color on the thallus. Potassium hydroxide (10% KOH) is called the K-test and is one of the most useful reagents. Sodium hypochlorite solution or the C-test is another reagent commonly used and another is the alcoholic solution of *para* Phenylenediamine (abbreviated as PD) (Brodo et al. 2001; Orange et al. 2001). These reagents produce color changes on the thallus depending on the lichen compounds present in the medulla or the cortex. A number of other reagents for spot tests are listed in Orange et al. (2001). The use of the spot tests and with identification keys for lichen

species, aid the identification of some lichen species that are morphologically similar but different in their chemical substances.

Thin Layer Chromatography (TLC) is a technique used to detect the presence or absence of secondary metabolites and it separates secondary metabolites in a sample containing a mixture of compounds (Feige et al. 1993). The compounds in the mixture spotted onto a silica coated plate will migrate at different levels through the silica gel and the compounds separate (Culberson 1972, 1986a) depending on their differences in polarity. The Rf value is a calculation based on the separation of compounds and their distance from the original spot on the plate giving each compound a fairly unique number (Fair et al. 2008; Cheng et al. 2011). Thin-layer chromatography is a technique that provides a low-cost analysis of samples, and its preparation is relatively easy. TLC is the most suitable technique for surveying samples for presence or absence of the compound (Poole 2003).

High-Performance Liquid Chromatography (HPLC) is also another chromatographic technique, which provides more information than TLC. HPLC is used to separate the secondary metabolites in a mixture, to purify, collect and identify the compound through UV spectra, and to quantify the amount of each compound. Liquid samples are injected into a column filled with solid adsorbent material. Different kinds of columns are available depending on the nature of compounds tested (Nourish and Oliver 1976). Lichenologists have used HPLC for the separation, detection, and identification of secondary metabolites (Manojlovic et al. 2012). HPLC is replacing TLC methods as the technology becomes more readily available and the methods more feasible than in the past (Elix et al. 1988; Mietzsch et al. 1993). There are wide applications for detection and quantification of compounds by HPLC such as industrial applications in the pharmaceuticals, food and nutraceuticals, cosmetics, environment and ecology, forensic samples,

and industrial chemicals (Kupiec 2004). The structures of two new water-soluble heteroglycans, Ths-4 and Ths-5, from the lichen, *Thamolina vermicularis* var. *subuliformis* were further analyzed by 1D Nuclear Magnetic Resonance spectrometry (NMR) and showed that the heteroglycans Ths-4 and Ths-5 have similar structures, but they have large differences in molecular weight. The use of the NMR technique provides information on the differences between two molecules (Omarsdottir et al. 2006). Mass spectrometry (MS) is an important analytical technique to quantify known materials, to identify unknown ones in the sample, and to explain the structural and chemical properties for molecules. A mass spectrometer generates multiple ions from the sample, then separates them according to the ratio between the mass to the ion charge (m/z). The MS procedure could use liquid, gas, or solid samples, where the ions are divided depending on the mass to charge ion ratio, then the charged ions are detected by the electron multiplier (Kebarle 2000). The combination of chromatographic separation with mass spectrometric detection provides an important tool for solving analytical chemistry problems. Mass spectrometric detection added more information, complementary to the chromatographic process, improving the certainty of identification and the specificity of detection (Polle 2003).

Chapter 2

Relationship between lichen species composition, secondary metabolites and soil pH, organic matter, and grain characteristics in Manitoba

This chapter is published in the journal *Botany

Zraik, M., James T. Booth and Piercey-Normore, M.D. 2016. 33(3):136-144.

2.1 Abstract

Many lichen secondary metabolites have functions related to the environmental conditions of lichen habitats but few studies have compared soil characteristics with lichen species composition or their secondary products. The goal of this study was to investigate the relationship between soil characteristics with lichen species composition and secondary metabolites. Five locations were chosen in Manitoba, each with five sites (transects), and each transect with five quadrats (1 m × 1 m). All species were collected from each of the quadrats, presence of secondary metabolites was determined by thin layer chromatography, and soil characteristics were examined. The results revealed that rounded sand grains were significantly higher in southeastern Manitoba than in other locations, corresponding to a distinct species composition. Angular grains were significantly higher in northern locations, corresponding to a different group of species. Some of the significant relationships between soil characteristics and secondary metabolites include correlations of atranorin with pH, organic matter, and sand content; fumarprotocetraric acid with organic matter and sand content; and usnic acid with pH

and organic matter. A better understanding of the role of lichens with respect to soil characteristics will be important for improving soil stabilization in land reclamation.

2.2 Introduction

The effect of environmental conditions on individual lichen species has been shown through studies in which environmental variables were linked to morphological differences (Gilbert 1977; Goffinet and Bayer 1997; Pintado et al. 1997; Baloch et al. 2010; Kotelko and Piercey-Normore 2010; Cornejo and Scheidegger 2013; Muggia et al. 2014). Some of these studies supported the optimal defence theory (McKey 1974; Rhoades 1979; Asplund et al. 2010) in which secondary metabolite production was correlated with herbivory, and abiotic variables such as light (McEvoy et al. 2006; Armaleo et al. 2008), pH (Fox and Howlett 2008; Timsina et al. 2013), and humidity (Culberson and Armaleo 1992; Stocker-Wörgötter 2001). However, few studies have examined the relationship between substrate and the lichen phenotype or secondary metabolite production (Brodo 1973; Gilbert 1977). Additionally, the substrate has been examined with respect to water soluble nutrients available for lichen soil crusts (Fischer et al. 2014), substrate moisture in an Alaskan tundra (Williams et al. 1978), and as an important source of nutrients by fungal enzyme activity (Moiseeva 1961). The role of the soil in nutrient and moisture availability corresponded with species distribution (Jun and Roze 2005) and would be expected to depend to some degree on the extent of direct contact between the soil and the lichen thallus. To our knowledge, no study has examined the relationship between soil characteristics with lichen species composition or secondary metabolite production.

Secondary metabolites produced by lichen-forming fungi comprise diverse groups of metabolites (Elix and Stocker-Wörgötter 2008). A well-studied group includes the polyketides,

which are produced by the fungal partner in the acetyl-polymalonyl pathway. A large body of literature is available on polyketide production in nonlichenized fungi (Keller and Hohn 1997; Sanchez et al. 2010; Chen et al. 2014) and it is growing for the lichenized fungi (Stocker-Wörgötter 2001, 2008). While the biosynthesis of some polyketides from non-lichenized fungi is being unravelled (e.g., Chiang et al. 2009, 2010; Campbell and Vereras 2010; Weissman 2015), knowledge of the biosynthesis of the many unique compounds from lichenized fungi is lagging behind, with some progress made in usnic acid (Abdel-Hameed et al. 2016). Improved knowledge of the conditions required for culturing the lichen fungus to induce polyketide production would facilitate progress for studies on polyketide biosynthesis. Because lichen polyketides are produced in nature, it might be expected that a better understanding of the natural conditions, including the substrate, would facilitate culturing. The potential for the application of polyketides in industry or health related fields has increased the interest in this group of compounds.

Polyketide production has been hypothesized to be associated with slow growth of the fungus. For example, it has been suggested that overwintering stages, reproduction, and other life stages may cause slow growth (Bu'Lock 1961; Calvo et al. 2001; Timsina et al. 2013). Polyketide production may also be regulated by the carbon–nutrient balance (CNB) hypothesis (Bryant et al.1983; Hyvarinen et al. 2003), which is based on the concept that an imbalance of nutrients will promote production of carbon-based secondary metabolites. The hypothesis suggests that when a plant or lichen is growing in unbalanced nutrient and carbon conditions, the excess carbon may be shunted into carbon-based secondary metabolites such as polyketides. When the plant or lichen is growing in balanced conditions, the carbon would not be in excess and may be used in thallus growth rather than production of secondary metabolites. However, the

hypothesis is controversial because it represents a complex model with many interacting variables (Koricheva 2002). For example, soil features and community structure may influence the nutrients available to lichens and their growth rates; therefore, they may indirectly influence production of secondary metabolites in lichens.

Some lichens are extreme stress tolerators and able to adapt to harsh conditions on sandy soils in areas where plants are absent, reducing the competition on lichens and allowing them to dominate in these locations (Botting and Fredeen 2006). Jun and Roze (2005) found a significant relationship between the edaphic parameters (soil pH, water content, nutrient content) and the distribution of species for lichens and bryophytes in the sand dunes of the French Atlantic coast. The content of organic material and carbon also affected the occurrence of some species in sandy areas. For example, the occurrence of some species of *Cladonia*, such as *Cladonia arbuscula*, *Cladonia mitis*, and *Cladonia portentosa*, are favoured by the acidification of the substrate (James et al. 1977), and differences in the occurrence of *Cladonia pocillum* and *Cladonia pyxidata* corresponded with the pH of the soil on which they were growing where higher pH levels favoured *C. pocillum* (Kotelko and Piercey-Normore 2010).

The goal of this study was to investigate the relationship among secondary metabolites, lichen species composition, and soil characteristics in five locations in Manitoba. The soil characteristics were examined to determine which characteristics had the largest influence on species and secondary metabolite occurrence.

2.3 Materials and Methods

2.3.1 *Experimental design and sample collection*

Field collections were made in five locations in northern and southern Manitoba (Fig. 2.1), and were focused in areas with sandy soils (Table 2.1). The five locations have a continental climate and contain soils that vary in underlying geology (Table 2.1; Fig. 2.1). Samples of all species of lichens were collected from each of five quadrats (1 m × 1 m) in each of five sites (transects) in each of five locations (125 quadrats in total). The quadrats were placed every 5 m along a 25 m transect within each site and the upper right corner of the quadrat was placed over a lichen thallus. The percent ground cover of the species recorded as guilds and other biotic (vascular plants, bryophytes, and lichens by general categories) and abiotic (bare rock, exposed soil, and vegetative debris such as twigs, cones, leaves, etc.) factors were recorded by visual estimation to the nearest 5% using a 25 cm × 25 cm grid placed within the quadrat for estimating coverage. Percent canopy cover was estimated using the same grid and held directly above the quadrat to estimate the amount of light. Specimens were air dried and returned to the lab for identification using methods described by Ahti (2000), Brodo et al. (2001), Hinds and Hinds (2007), and Kowalewska et al. (2008). To determine the secondary metabolites present, thin layer chromatography was performed on all specimens using solvent A (185 mL toluene, 45 mL dioxane, and 5mL glacial acetic acid) (Orange et al. 2001). Briefly, about 200 uL of acetone was applied to the crushed thallus samples, which were left soaking for 20 min. Acetone extracts were applied to silica-coated glass plates, immersed within solvent A in a thin layer chromatography tank until the solvent front reached the top of the plate. After drying, the plates were observed under long (365 nm) and short (255 nm) wave UV light, sprayed with 10% sulphuric acid, and heated at 80 °C for 20 min. Standard secondary metabolites as positive

controls were prepared from herbarium specimens that had the chemicals previously identified for comparison. Known Rf classes and further spot comparisons were made with characteristics reported by Orange et al. (2001). Frequency of occurrence for secondary metabolites was recorded.

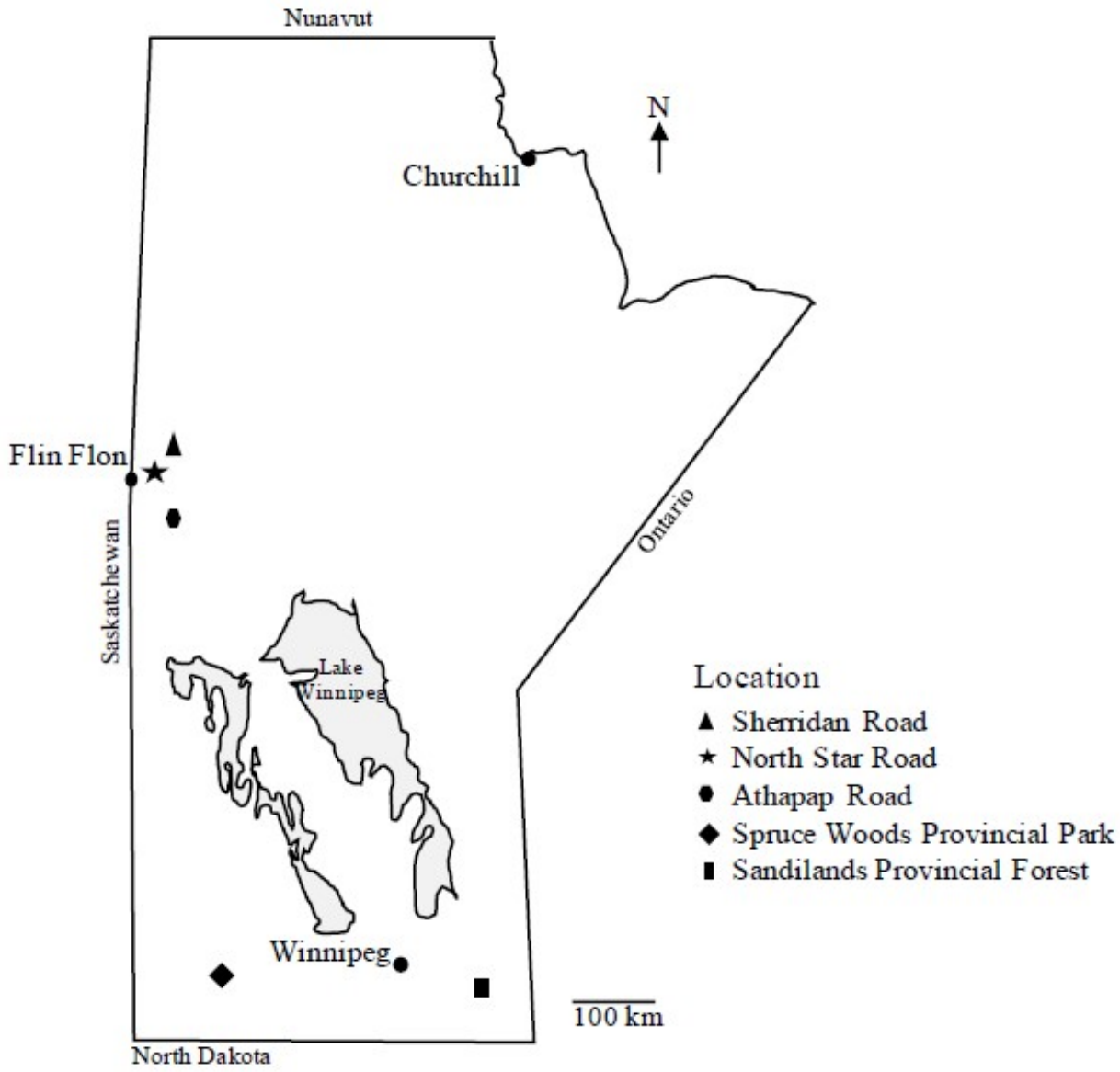


Figure 2.1. A map of Manitoba showing the five study locations (the Sandilands Provincial Forest (Southeastern Manitoba); Spruce Woods Provincial Park (Southwestern Manitoba); Athapap Road, Sherridon Road and North Star Road (Western middle Manitoba).

2.3.2 *Soil characteristics*

Two soil samples (multiple representative samples and a single bulk sample) were collected from each of the125 quadrats (25 in each of 5 locations). Multiple samples were collected from different places in the quadrat and placed within clean plastic bags (about 1 kg) to represent the surface soil in the quadrat (0 to 7 cm depth) and to determine soil characteristics. Bulk density samples were collected from the center of the quadrat using a standard bulk density cylinder (7.62 cm height × 7.62 cm diameter). Organic matter was calculated as loss of carbon on ignition by burning the soil samples at 650 °F for 18 h using a muffle furnace (Barnstead/Thermolyne). Percentage of the main soil components (sand, silt, and clay), and gravel as an additional soil component, were determined by sieving the soil samples with different soil sieves using the Canadian standard sieve sizes (Fisherbrand US Standard Brass Test Sieves; sieve number 10 (2.00 mm opening) to collect the silt and clay grains, sieve numbers 35 (0.50 mm opening) and 100 (0.149 mm opening) to collect the fine and coarse sand, respectively, and sieve number 200 (0.074 mm opening) for rocks and pebbles). Shape of the sand grains was also determined and divided into two main categories (angular and rounded) (Arculus et al. 2015) for 1 sample from each of 125 quadrats using a dissecting microscope (Leica MZ 6) at 10× magnification. The grain shape was determined from a soil analysis kit (Forestry Suppliers, Inc., Mississippi, USA). A distilled water mixture with soil was prepared for measuring the pH of the soil using a pH meter VWR sypHony SB70P). The pH measures were performed according to standard methods (Benton and Jones 1999).

2.3.3 *Statistical analyses*

Principle coordinates analysis (PCoA; Legendre and Legendre 1998) was used to test the similarities among lichen species composition using the R package "vegan" (Oksanen et al. 2015). The studied species were scored as present or absent. To examine whether soil characteristics influenced secondary metabolite frequency and species compositions, redundancy analyses were performed using the "rda" function in the R package "vegan". Monte Carlo permutation tests were performed to test the significance of redundancy analysis. The frequency of occurrence of secondary metabolites was tested by recording the number of quadrats in which each secondary metabolite was found. The soil pH levels were divided into two groups: high acidic soil (pH ranging from 4.2 to 5.7) and neutral to slightly acidic soil (pH ranging from 6.1 to 7.4) to compare frequency of secondary metabolites and lichen species. Significance tests for soil characteristics, biotic and abiotic factors, species frequency, and secondary metabolite frequency were performed using ANOVA by the JMP program 12.1.0 (64 bit), and comparison of the means was done by Tukey's honestly significant difference test. The frequency of secondary metabolites and species occurrence (number of quadrats in which each secondary metabolite or species was detected divided by the total number of quadrats for one location) was examined with location as a categorical variable. To test the relationship between soil features (organic matter, pH, and sand content) and occurrence of secondary metabolites as continuous variables, a correlation was performed using the JMP program 12.1.1. Statistical analyses were performed with the R program (R Core Team 2013), using a significance level of $\alpha = 0.05$ unless otherwise indicated.

Table 2.1. General characteristics of the locations of collection sites (transects) including latitude and longitude, prevalent parent bedrock material, maximum and minimum yearly temperatures, and location descriptions with dominant vegetation.

Characteristics	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Latitude and longitude	N54° 77' 72.9" W101° 47' 37.5"	N54° 69' 75.4" W101° 68' 12.3"	N54° 51' 58.8" W101° 53' 19"	N49° 43' 45.4" W99° 17' 29.2"	N49° 44' 31.9" W96° 39' 22.8"
Parent material	Granite	Gneiss	Calcaric regosols	Aeolian regosols	Glacial till regosols
Temp (°C) max/min	23.8/-24.7	23.8/-24.7	24/-28.9	26/-24.3	26/-23.2
Location description and dominant plant species	Precambrian Shield. Patches of soil in crevices of bedrock and among outcrops. Aspen, birch, white spruce, Jack pine, willow and alder.	Precambrian Shield. Patches of soil in crevices of bedrock and among outcrops. Aspen, birch, white spruce, Jack pine, willow and alder.	Shallow regosol on limestone bedrock. Mixed forest with aspen, white spruce, fir, white birch, and alder.	Active and stabilized sand dunes. Grasses and bluffs of white spruce, aspen, and juniper in depressions. Oaks on high ridges.	Rolling forest and sandy till. Jack pine, aspen, and white spruce interspersed with low lying black spruce stands.

2.4 Results

Soil characteristics for each of the five locations are described in Table 2.2. Gravel content was highest in the Sandilands Provincial Forest. The sand content in Spruce Woods Provincial Park was significantly higher than that of the other four locations. The finest sand grains were in the Sandilands Provincial Forest and Spruce Woods Provincial Park, and the coarsest sand grains were in North Star and Athapap. The percent of rounded sand grains was significantly higher in Sandilands Provincial Forest than other locations whereas angular grains were more abundant in North Star than other locations (though not significantly different from Sherridon). The highest rounded/angular (R/A) ratio was in Sandilands Provincial Forest. Clay and silt levels were significantly higher in Athapap than in other locations. Organic matter was significantly higher in Sherridon and Athapap than the other locations. The pH level was significantly higher in Spruce Woods Provincial Park than in North Star but there was no significant difference among the other three locations (Table 2.2).

Table 2.2. Soil characteristics in five sites (transects) across the five locations in this study.

Soil Characteristics	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Gravel (p = 0.0001)	11.7 ± 1.7^{ab}	9.8 ± 0.9^{b}	8.9 ± 1.1^{b}	6.4 ± 0.5^{b}	18.2 ± 3.2^{a}
Sand (p < 0.0001)	65.8 ± 3.9^{c}	60.2 ± 1.6^{cd}	54.8 ± 2.6^{d}	92.6 ± 0.5^{a}	78.7 ± 3.2^{b}
Fine sand (p < 0.0001)	61.6 ± 3.8^{b}	39.2 ± 1.4^{c}	46.3 ± 0.8^{c}	89.3 ± 1.4^{a}	87.1 ± 1.7^{a}
Coarse sand (p < 0.0001)	38.4 ± 3.8^{b}	60.2 ± 1.4^{a}	53.6 ± 0.8^{a}	10.7 ± 1.4^{c}	12.9 ± 1.7^{c}
Rounded grain (R) (p < 0.0001)	28.7 ± 4.0^{d}	15.2 ± 2.1^{e}	57.6 ± 3.3^{b}	40.0 ± 1.2^{c}	82.8 ± 2.1^{a}
Angular grain (A) (p < 0.0001)	71.3 ± 5.4^{ab}	84.9 ± 2.2^{a}	42.5 ± 4.6^{c}	60.1 ± 1.0^{b}	17.1 ± 3.4^{d}
R/A ratio (p < 0.0001)	0.62 ± 0.2^{b}	0.19 ± 0.03^{b}	1.77 ± 0.2^{b}	0.68 ± 0.03^{b}	8.22 ± 1.6^{a}
Clay and silt (p < 0.0001)	18.2 ± 3.4^{c}	27.3 ± 1.7^{b}	35.8 ± 2.0^{a}	0.8 ± 0.1^{d}	2.7 ± 0.4^{d}
Organic matter (p = 0.001)	28.6 ± 5.7^{a}	17.9 ± 3.5^{b}	27.9 ± 5.6^{a}	1.7 ± 0.3^{c}	5.4 ± 1.1^{c}
pH level (p = 0.001)	5.54 ± 0.4^{bc}	4.98 ± 0.3^{c}	6.62 ± 0.1^{ab}	7.00 ± 0.2^{a}	6.38 ± 0.2^{ab}

Note: Values (%) are mean ± Standard Error (SE) derived from five 1 m^2 quadrats from each of five transects for each of the five locations. The ratio of Rounded to Angular (R/A) grains are calculated as mean ± SE over the individual values of R/A for the 25 plots sampled in each of the locations. Organic matter values are the percentage of total mass). Lowercase superscript letters that differ indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.

The percent canopy and ground covers for the quadrats in each location are shown in Table 2.3. Briefly, Sherridon, and Athapap had a significantly higher percentage of canopy cover compared with Sandilands Provincial Forest and Spruce Woods Provincial Park. North Star showed a significantly higher percentage of ground cover of lichens than Athapap and Sandilands (Table 2.3). Bryophyte ground cover was significantly higher in Athapap. The cup-forming lichens were significantly higher in Spruce Woods Provincial Park (except North Star), whereas mat-forming lichens were significantly higher in Sherridon than other locations. *Stereocaulon* and crustose lichens were significantly higher in North Star than the other locations (except Sherridon), the red-fruited lichens were significantly higher in Athapap, but the foliose lichens showed no significant differences among locations. Other factors analysed included vegetative debris, which was high in Sherridon, exposed soil, which was high in Sandilands Provincial Forest, and bare rock, which was high in Sherridon and North Star.

Forty-six lichen species are reported in this study with greater than 10% frequency of occurrence (Table 2.4). Four species, *Cladonia arbuscula*, *Cladonia chlorophaea, Cladonia gracilis* subsp. *turbinata*, and *Cladonia pyxidata* were the most frequently occurring species being present in 21 or 22 out of 25 total sites (transects) and were present in all five locations. Three species (*Cladonia humilis*, *Cladonia magyarica*, and *Cladonia sulphurina*) were found in one of the five locations (Table 2.4).

Table 2.3. Percent ground cover within quadrats (mean ± SE) showing categories of canopy cover, vascular plants, vegetative debris, bryophytes, lichens (total and by general categories), unvegetated soil, and bare rock over five 1 m^2 quadrats for each of five sites (transects) in each of the locations. Lowercase superscript letters that differ indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.

Category	Sherridon Road	North Star Road	Athapap Road	Spruce Woods Provincial Park	Sandilands Provincial Forest
Canopy cover (p < 0.0001)	36±7.2^{a}	28±5.7^{ab}	34.4±6.1^{a}	3.8±2.5^{c}	11.6±4^{bc}
Vascular plants (p = 0.1000)	4.9±1.4^{a}	15.1±3.8^{a}	17.1±3.3^{a}	10.6±3^{a}	12.6±4.3^{a}
Vegetative debris (p = 0.0030)	26.5±5.8^{a}	14.4±4.1^{ab}	11.8±2.4^{b}	7.0±1.9^{b}	11.2±2.5^{b}
Bryophytes (p < 0.0010)	10.0±2.4^{b}	2.0±0.6^{b}	29.0±5.4^{a}	6.4±2.4^{b}	7.3±1.7^{b}
Lichens total (p = 0.0070)	49.8±4.8^{ab}	56.64±5.9^{a}	35.8±4.9^{b}	46.3±4.3^{ab}	34±4.5^{b}
Cup lichens (p < 0.0001)	18.7±5^{b}	30.6±5^{ab}	16.4±4.5^{b}	44.3±4.4^{a}	20.4±3.3^{b}
Red-fruited lichens (p < 0.0001)	0.28±0.09^{b}	0.44±0.1^{b}	14.1±4.4^{a}	0.2±0.08^{b}	0.42±0.9^{b}
Crustose lichens (p < 0.0001)	0.00±^{b}	13.8±3.7^{a}	1.1±0.6^{b}	0.00^{b}	0.00^{b}
Stereocaulon (p < 0.0001)	1.1±0.6^{ab}	3.4±1.3^{a}	0.2±0.13^{b}	0.00^{b}	0.00^{b}
Mat-forming lichens (p < 0.0001)	29.3±4.8^{a}	6.7±2.3^{b}	1.3±0.6^{b}	1.3±1^{b}	11.2±3.6^{b}
Foliose lichens (p = 0.052)	0.16±0.07^{a}	1.3±0.67^{a}	2.7±1^{a}	0.5±0.4^{a}	2±0.8^{a}
Exposed soil (p = 0.0005)	0.12±0.07^{b}	1.8±0.8^{b}	2.6±1.2^{b}	4.6±1.2^{ab}	10.3±3.1^{a}
Bare rock (p = 0.0011)	5.4±0.6^{a}	5.1±1.4^{a}	0.72±0.6^{ab}	0.00^{b}	0.00^{b}

Table 2.4. Frequency of occurrence (mean ± SE) of lichen species[a] over twenty-five 1 m^2 quadrats per site (transect) in each of the five locations in this study.

Location	Sherridon	North Star	Athapap	Spruce Woods	Sandilands
Arctoparmelia centrifuga ($p < 0.0001$)	0.00^{c}	0.64 ± 0.09^{a}	0.36 ± 0.09^{b}	0.000^{c}	0.00^{c}
Cladonia amaurocraea ($p < 0.0001$)	0.40 ± 0.10^{a}	0.24 ± 0.08^{a}	0.00^{b}	0.00^{b}	0.40 ± 0.10^{a}
Cladonia arbuscula ($p < 0.0001$)	1.00 ± 0.0^{a}	0.64 ± 0.09^{b}	0.44 ± 0.1^{bc}	0.16 ± 0.08^{c}	1.00 ± 0.0^{a}
Cladonia cariosa ($p < 0.0001$)	0.00^{b}	0.00^{b}	0.56 ± 0.10^{a}	0.36 ± 0.09^{a}	0.00^{b}
Cladonia chlorophaea ($p = 0.0196$)	0.52 ± 0.10^{ab}	0.68 ± 0.09^{a}	0.68 ± 0.09^{a}	0.28 ± 0.09^{b}	0.52 ± 0.10^{ab}
Cladonia cornuta ($p = 0.0450$)	0.48 ± 0.10^{a}	0.28 ± 0.09^{ab}	0.12 ± 0.06^{b}	0.32 ± 0.09^{ab}	0.48 ± 0.10^{a}
Cladonia crispata ($p = 0.0650$)	0.12 ± 0.06^{ab}	0.16 ± 0.07^{a}	0.00^{a}	0.04 ± 0.04^{a}	0.12 ± 0.06^{ab}
Cladonia cristatella ($p = 0.0010$)	0.24 ± 0.08^{ab}	0.44 ± 0.10^{a}	0.04 ± 0.04^{a}	0.04 ± 0.04^{b}	0.24 ± 0.08^{ab}
Cladonia decorticata ($p = 0.0494$)	0.00^{a}	0.04 ± 0.04^{a}	0.00^{a}	0.12 ± 0.07^{a}	0.00^{a}
Cladonia deformis ($p < 0.0001$)	0.40 ± 0.10^{a}	0.56 ± 0.10^{a}	0.00^{b}	0.00^{b}	0.40 ± 0.10^{a}
Cladonia gracilis spp. *turbinata* ($p < 0.0001$)	0.76 ± 0.08^{a}	0.68 ± 0.09^{a}	0.52 ± 0.10^{a}	0.12 ± 0.06^{b}	0.76 ± 0.08^{a}
Cladonia grayi ($p = 0.3144$)	0.12 ± 0.06^{a}	0.04 ± 0.04^{a}	0.00^{a}	0.04 ± 0.04^{a}	0.12 ± 0.06^{a}
Cladonia humilis ($p < 0.0001$)	0.00^{b}	0.00^{b}	0.00^{b}	0.44 ± 0.10^{a}	0.00^{b}
Cladonia magyarica ($p < 0.0001$)	0.00^{b}	0.00^{b}	0.00^{b}	0.96 ± 0.04^{a}	0.00^{b}

Cladonia merochlorophaea (p = 0.038)	0.16±0.07^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.16±0.07^{a}
Cladonia multiformis (p = 0.0008)	0.00^{b}	0.08±0.05^{b}	0.16±0.07^{ab}	0.00^{b}	0.00^{b}
Cladonia phyllophora (p = 0.0331)	0.20±0.08^{ab}	0.16±0.07^{ab}	0.08±0.05^{b}	0.04±0.04^{b}	0.20±0.08^{ab}
Cladonia pocillum (p = 0.0660)	0.00^{a}	0.00^{a}	0.04±0.04^{a}	0.12±0.07^{a}	0.00^{a}
Cladonia pyxidata (p = 0.0004)	0.16±0.07^{b}	0.64±0.09^{a}	0.60±0.1^{a}	0.36±0.09^{ab}	0.16±0.07^{b}
Cladonia rangiferina (p < 0.0001)	0.44±0.10^{a}	0.16±0.07^{abc}	0.00^{c}	0.04±0.04^{bc}	0.44±0.10^{a}
Cladonia scabruiscula (p = 0.0024)	0.00^{b}	0.04±0.04^{ab}	0.04±0.04^{ab}	0.28±0.09^{a}	0.00^{b}
Cladonia stellaris (p < 0.0001)	0.44±0.10^{a}	0.28±0.09^{a}	0.00^{b}	0.00^{b}	0.44±0.10^{a}
Cladonia stygia (p = 0.1876)	0.12±0.10^{a}	0.04±0.06^{a}	0.00^{a}	0.00^{a}	0.12±0.10^{a}
Cladonia sulphurina (p = 0.0138)	0.00^{b}	0.12±0.06^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Cladonia uncialis (p < 0.0001)	0.40±0.10^{a}	0.12±0.06^{b}	0.00^{b}	0.00^{b}	0.40±0.10^{a}
Cladonia verticillata (p = 0.0494)	0.04±0.04^{a}	0.00^{a}	0.00^{a}	0.04±0.04^{a}	0.04±0.04^{a}
Peltigera rufescens (p < 0.0001)	0.08±0.05^{b}	0.04±0.04^{b}	0.56±0.10^{a}	0.20±0.08^{b}	0.08±0.05^{b}
Sterocaulon tomentosum (p < 0.0001)	0.04±0.04^{b}	0.32±0.09^{a}	0.00^{b}	0.000^{b}	0.04±0.04^{b}
Umbilicaria deusta (p < 0.0001)	0.04±0.04^{b}	0.28±0.09^{a}	0.00^{b}	0.000^{b}	0.04±0.04^{b}
Vulpicida pinistari (p < 0.0001)	0.28±0.09^{ab}	0.40±0.10^{a}	0.08±0.05^{bc}	0.00^{c}	0.28±0.09^{ab}

Xanthoparmelia cumberlandia (p = 0.0365)	0.04±0.04[ab]	0.20±0.08[a]	0.04±0.04[ab]	0.00[b]	0.04±0.04[ab]

Note: Significant differences are represented by the given probabilities for each species as shown in the first column. Lowercase superscript letters indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.
[a]Only those species with >10% frequency of occurrence are included in the table. *Cetraria islandica*, *Cladonia acuminata*, *C. borealis*, *C. botrytes*, *C. cenotea*, *C. coniocraea*, *C. fimbriata*, *C. gracilis* subsp. *gracilis*, *C. mitis*, *C. pleurota*, *C. subulata*, *Nephroma helveticum, Parmelia saxatilis, Stereocaulon grande*, and *Umbilicaria muehlenbergii* were found at frequencies <10% and are not included in the table.

Table 2.5. Frequency of occurrence (mean ± SE) based on presence/absence of secondary metabolites[a] over twenty-five 1-m^2 quadrats per site (transect) for each of five locations in Manitoba.

Lichen acids	Sherridon Mean ± SE	North Star Mean ± SE	Athapap Mean ± SE	Spruce Woods Mean ± SE	Sandilands Mean ± SE
Zeorin ($p < 0.0001$)	0.48 ± 0.1^{a}	0.68 ± 0.09^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Barbatic acid ($p < 0.0001$)	0.52 ± 0.1^{a}	0.48 ± 0.1^{a}	0.04 ± 0.04^{b}	0.04 ± 0.04^{b}	0.32 ± 0.09^{ab}
Usnic acid ($p < 0.0001$)	0.96 ± 0.04^{a}	0.92 ± 0.05^{a}	0.2 ± 0.08^{c}	0.2 ± 0.08^{c}	0.6 ± 0.1^{b}
Thamnolic acid ($p = 0.0138$)	0.00^{b}	0.12 ± 0.06^{a}	0.00^{b}	0.00^{b}	0.00^{b}
Squamatic acid ($p = 0.0042$)	0.2 ± 0.08^{ab}	0.28 ± 0.09^{a}	0.04 ± 0.04^{b}	0.00^{b}	0.04 ± 0.04^{b}
Perlatolic acid ($p = 0.2757$)	0.16 ± 0.07^{a}	0.24 ± 0.08^{a}	0.04 ± 0.04^{a}	0.16 ± 0.07^{a}	0.08 ± 0.07^{a}
Fumarprotocetraric acid ($p = 0.2479$)	1.00 ± 0.00^{a}	0.92 ± 0.05^{a}	0.96 ± 0.04^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Atranorin ($p < 0.0001$)	0.36 ± 0.08^{bc}	0.32 ± 0.08^{c}	0.68 ± 0.08^{ab}	0.96 ± 0.08^{a}	0.4 ± 0.08^{bc}
Grayanic acid ($p = 0.3144$)	0.12 ± 0.06^{a}	0.04 ± 0.04^{a}	0.00^{a}	0.04 ± 0.04^{a}	0.12 ± 0.06^{a}
Merochlorophaeic acid ($p = 0.016$)	0.16 ± 0.07^{a}	0.00^{a}	0.00^{a}	0.04 ± 0.04^{a}	0.2 ± 0.08^{a}

Note: Significant differences are represented by the given probabilities for each species as listed in the first column. Lowercase superscript letters indicate significant differences within rows at $p < 0.05$ using Tukey's honestly significant difference test.
[a]Only those metabolites with >20% occurrence are included in the table. Metabolites with <20% occurrence include didymic, gyrophoric, stictic, norstictic, psoromic, salazinic, pinastric, lobaric, alectoronic, and vulpinic acids and are not included in the table.

Secondary metabolites with more than 20% frequency of occurrence among the five locations are shown in Table 2.5. Zeorin, barbatic acid, and usnic acid were significantly high in Sherridon and North Star. Squamatic acid and thamnolic acid were significantly high in North Star. The presence of atranorin was significantly high in Spruce Woods Provincial Park.

Fumarprotocetraric acid and perlatolic acid were recorded in high frequency in all five locations and there were no significant differences among them.

The relationship between location and each of the soil characteristics is shown in Fig. 2.2. In this PCoA, the horizontal axis explains 51.4% of the variation in the data, and the vertical axis accounts for an additional 21.6%. Spruce Woods Provincial Park clustered at the positive end of the horizontal axis with higher sand content and fine sand, whereas Athapap, North Star, and Sherridon clustered at the negative end of the horizontal axis. Sandilands Provincial Forest clustered at the positive end of the horizontal axis with rounded sand grains. The PCoA showed three main groups (Sandilands Provincial Forest, North Star, and Spruce Woods Provincial Park) that were separated based on the soil characteristics (Fig. 2.2). Athapap and Sherridon had more variable soil characteristics.

A highly significant relationship between the secondary metabolites and the soil characteristics is shown by a Redundancy Analysis (Monte Carlo permutation test, $p = 0.001$) (Fig. 2.3). The RDA axis 1 accounted for 62.2% of the variation in the data and axis 2 accounted for an additional 22.8% of the variation. Some of the trends show that fumarprotocetraric acid and atranorin were more commonly produced when lichens were growing in sandy fine soils. Grayanic acid, thamnolic acid and merochlorophaeic acid were weakly associated with more neutral pH levels (Fig. 2.3). Usnic acid, zeorin, and barbatic acid were associated with angular sand grains (Fig. 2.3). The sand content was negatively correlated with organic matter (Pearson's product-moment correlation $r = -0.89$, $p = 0.001$).

A significant relationship was found between species and soil characteristics (Fig. 2.4). The RDA axis 1 accounts for 44.81% of the variation and axis 2 accounts for an additional 19.93% of the variation in the data. The trends show that *Peltigera rufescens* was associated with

rounded sand grains whereas *Cladonia deformis*, *Cladonia phyllophora*, *Cladonia stellaris*, *Stereocaulon tomentosum*, and *Vulpicida pinastri* were more associated with the angular sand grains. The trend also suggested that *Cladonia cariosa* is associated with soil pH levels and *Cladonia humilis* and *Cladonia magyarica* are associated with fine sand and total amount of sand (Fig. 2.4).

The correlation between soil characteristics and secondary metabolites showed significant relationships. There were strong negative correlations between zeorin, usnic acid, barbatic acid and squamatic acid with pH of the soil but the correlation between atranorin and pH was strongly positive (Table 2.6). The correlation with organic matter was strongly positive with zeorin and usnic acid, but it was strongly negative with atranorin and fumarprotocetraric acid. The correlations between percent sand content and each of atranorin and fumarprotocetraric acid were strongly positive (Table 2.6).

The correlation between soil characteristics and species occurrence also showed significant relationships (Table 2.7). Some species (*Cladonia deformis*, *Cladonia amaurocraea*, *Cladonia gracilis* subsp. *turbinata*, *Cladonia stellaris*, *S. tomentosum*, and *V. pinastri*) showed a strong negative relationship with pH. A strong positive relationship was present between two species (*Cladonia humilis*, *Cladonia magyarica*) and the percent sand content in soil (Table 2.7).

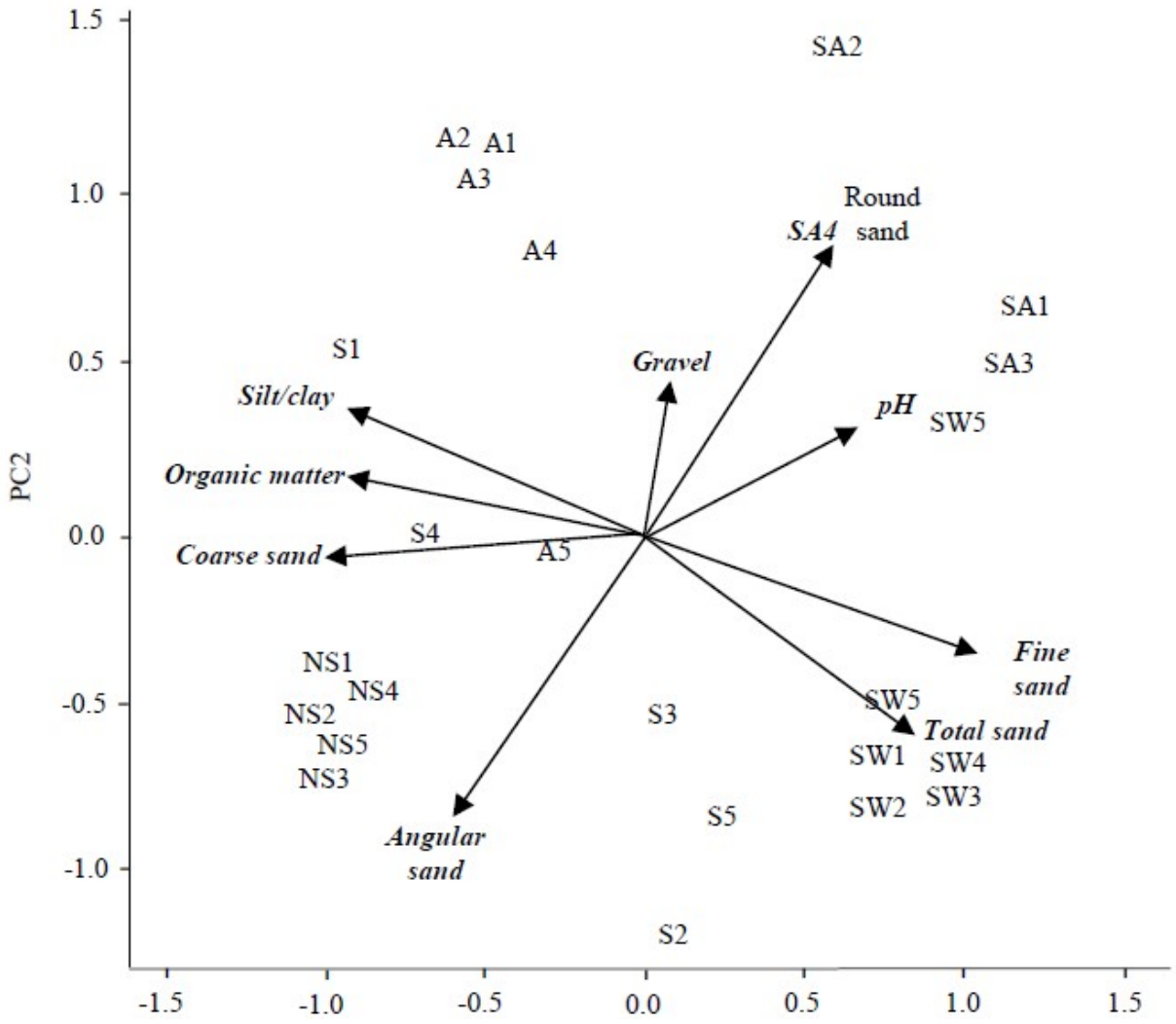


Figure 2.2. Principle coordinates analysis relating soil characteristics including gravel, sand (total, angular fraction, rounded fraction, and grain size), silt and clay, organic matter, and pH in each of the five sites (transects) in each of five locations (NS, North Star Road; S, Sherridon Road; A, Athapap Road; SW, Spruce Woods Provincial Park; SA, Sandilands Provincial Forest). The horizontal axis explains 51.4% of the variation in the data and the vertical axis explains 21.6% of the variation.

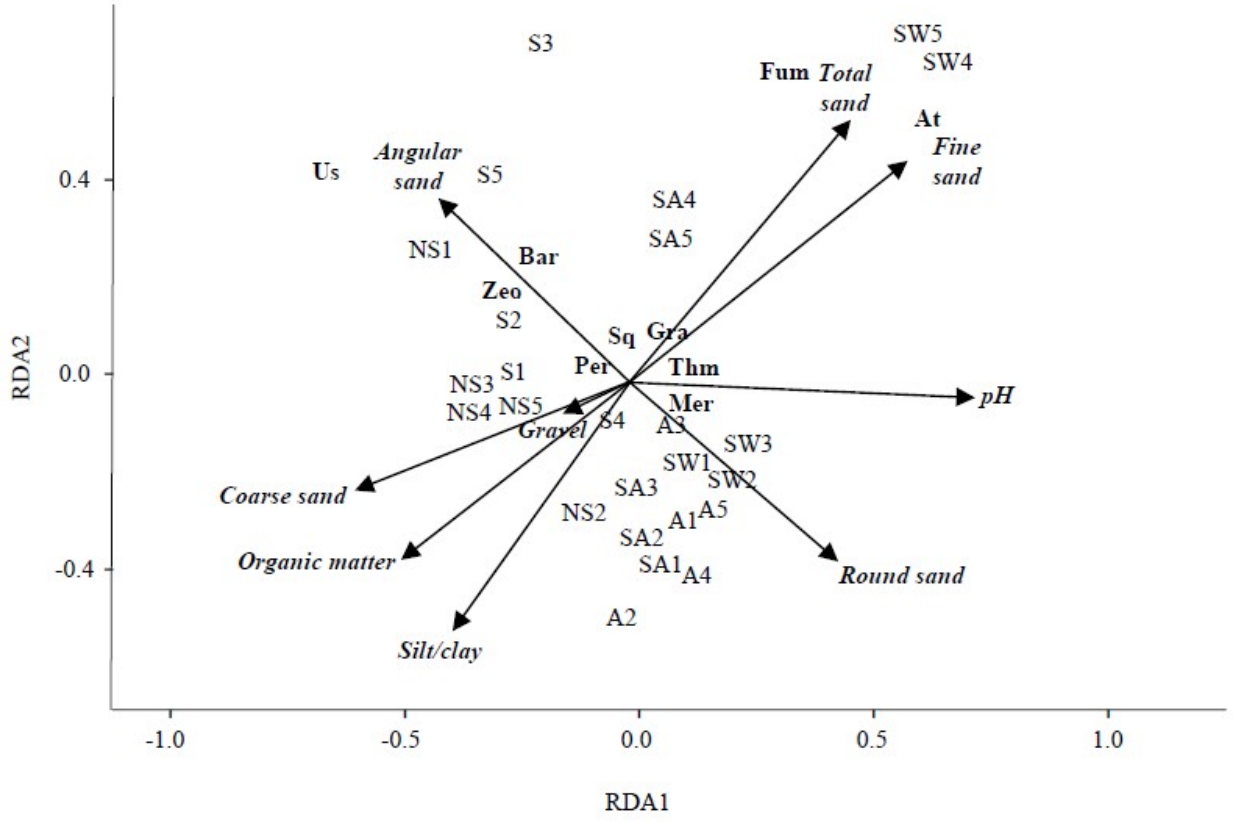


Figure 2.3. Redundancy analysis (RDA) of the Sherridon Road (S), North Star Road (NS), Athapap Road (A), Spruce Woods Provincial Park (SW), and Sandilands Provincial Forest (SA) locations and percent frequencies of the secondary metabolites, i.e., atranorin (At), barbatic acid (Bar), fumarprotocetraric acid (Fum), grayanic acid (Gra), merochlorophaeic acid (Mer), perlatolic acid (Per), squamatic acid (Sq), thamnolic acid (Thm), usnic acid (Us), and zeorin (Zeo), as constrained by soil features (silt/clay, sand (total, fine, and coarse), gravel, organic matter, soil particle shape (angular and rounded), and pH value). The secondary metabolites (didymic, gyrophoric, lobaric, norstictic, pinastric, salazinic, stictic, and vulpinic acids) were not found to be at significant levels in any of the five locations or were at frequencies <20% and are not included in the figure. The first RDA axis accounts for 62.2% of the total variation and the second axis for 22.8% of the variation.

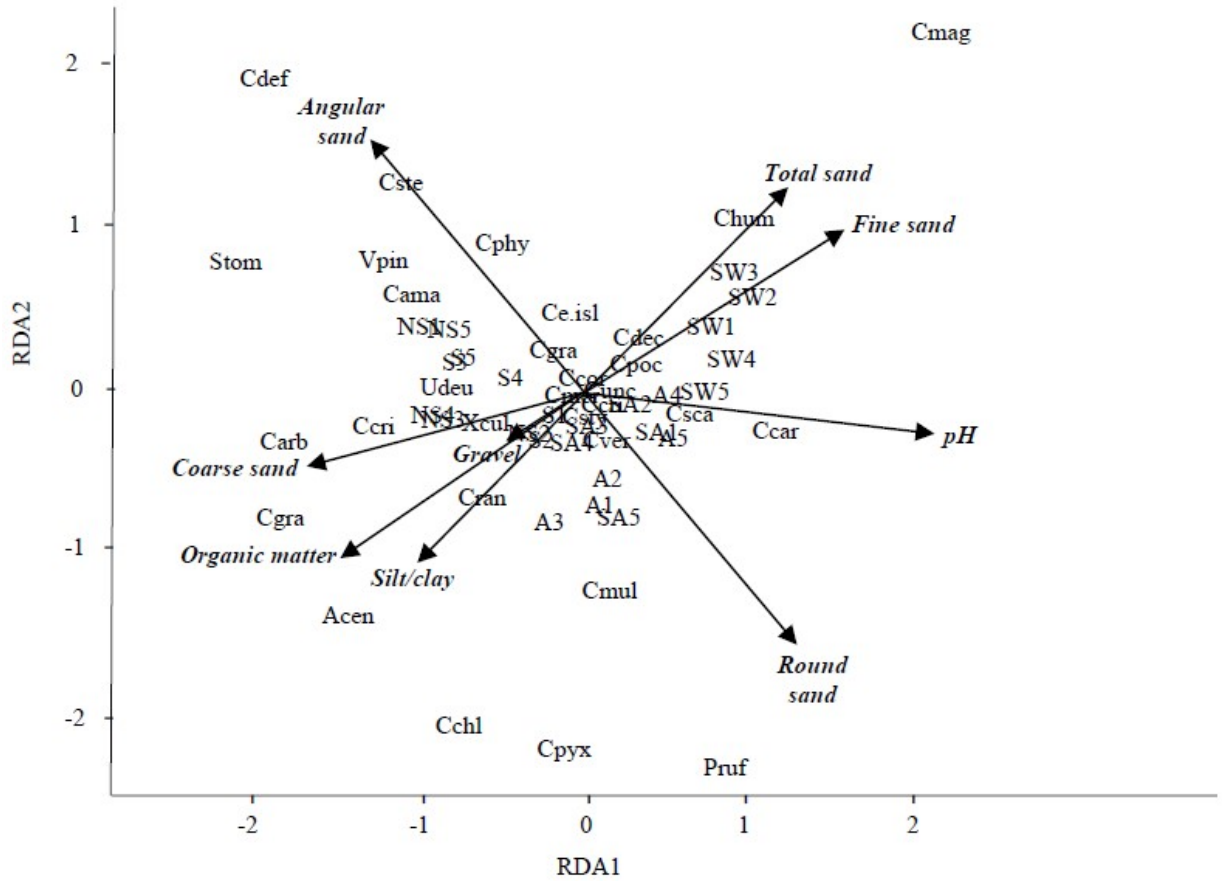


Figure 2.4. Redundancy analysis (RDA) of sites and lichen species constrained by soil features (silt/clay, sand (total, fine, and coarse), gravel, organic matter, soil particle shape (angular and rounded), and pH value). Species are represented by the first letter of the genus followed by the first three letters of the species epithet. See Table 7 for a list of the species names. The collection locations are abbreviated as in Fig. 2.3. The first RDA axis accounts for 44.81% and the second axis for 19.93% of the variation in the data.

Table 2.6. Relationship between three main soil characteristics (pH, organic matter, and sand content) and the total number of occurrence of secondary metabolites (n), ranged between 1 to 10 occurrences per quadrate showing results of Pairwise regression.

		pH			Organic matter (%)			Sand content (%)		
Secondary metabolite	**n**	**R^{2*}**	**F****	**P*****	**R^2**	**F**	**P**	**R^2**	**F**	**P**
Atranorin	141	0.155	22.53	**<0.0001**	0.730	9.63	**0.002**	0.116	16.10	**0.0001**
Barbatic acid	36	0.141	20.21	**<0.0001**	0.272	3.44	0.070	0.001	0.01	0.910
Fumarprotocetraric acid	432	0.240	3.05	0.083	0.110	15.20	**0.0002**	0.103	14.18	**0.0003**
Grayanic acid	8	0.058	0.72	0.390	0.130	1.60	0.210	0.299	3.79	0.054
Merochlorophaeic acid	13	0.007	0.09	0.760	0.027	0.34	0.560	0.001	0.02	0.890
Perlatolic acid	20	0.015	1.88	0.170	0.007	0.09	0.760	0.007	0.09	0.760
Squamatic acid	15	0.321	4.08	**0.045**	0.017	0.21	0.650	0.009	0.11	0.740
Thamnolic acid	3	0.015	0.19	0.670	0.150	1.88	0.170	0.002	2.80	0.090
Usnic acid	185	0.252	41.50	**<0.0001**	0.360	4.67	**0.032**	0.240	3.04	0.080
Zeorin	46	0.123	17.20	**<0.0001**	0.330	4.19	**0.042**	0.042	0.52	0.470

* Proportion of the variance in the observed values of the dependent variable that is explained by the regression model.

** The means between the two factors (secondary metabolites with pH, organic matter, sand content) are significant

***Significance is determined at $p < 0.05$, marked as bold

Table 2.7. Pairwise regression between three main soil characteristics (pH, organic matter, and sand content) and the frequency of occurrence of lichen species (n).

		pH			Organic matter (%)			Sand content (%)		
Species	**N**	**R^2**	**F**	**P**	**R^2**	**F**	**P**	**R^2**	**F**	**P**
Arctoparmelia centrifuga	25	0.235	7.0847	0.0139*	0.431	17.4422	0.0004**	0.169	4.7060	0.0406*
Cladonia amaurocraea	16	0.402	15.4861	0.0007**	0.225	6.6996	0.0164*	0.005	1.1611	0.2924
Cladonia arbuscula	70	0.243	7.4047	0.0122*	0.167	4.6230	0.0423*	0.132	3.4800	0.0749
Cladonia cariosa	24	0.165	4.5638	0.0435*	0.003	0.6901	0.4147	0.003	0.0671	0.7979
Cladonia chlorophaea	65	0.034	0.8168	0.3755	0.346	12.1679	0.0020**	0.235	7.1006	0.0138*
Cladonia cornuta	37	0.011	0.2406	0.6284	0.001	0.2907	0.5950	0.002	0.3320	0.5701
Cladonia crispata	8	0.135	3.5995	0.0704	0.003	0.0656	0.8001	0.001	0.0175	0.8960
Cladonia cristatella	26	0.195	5.6053	0.0267*	0.003	0.6652	0.4231	0.001	0.0020	0.9645
Cladonia decorticata	6	0.057	1.4035	0.2482	0.004	0.8741	0.3595	0.006	1.4948	0.2339
Cladonia deformis	24	0.459	19.5178	0.0002**	0.004	0.9173	0.3481	0.001	0.2874	0.5970
Cladonia gracilis ssp. *turbinata*	66	0.427	17.1879	0.0004**	0.232	6.9482	0.0148*	0.143	3.8526	0.0169*
Cladonia grayi	8	0.092	2.3289	0.1406	0.004	0.9832	0.3317	0.005	1.3291	0.2608
Cladonia humilis	11	0.185	5.2081	0.0321	0.150	4.0656	0.0556	0.377	13.9474	0.0011**
Cladonia magyarica	24	0.245	7.4652	0.0119*	0.167	4.6328	0.0421*	0.420	16.6716	0.0005**
Cladonia merochlorophaea	9	0.008	0.1852	0.6709	0.001	0.5679	0.5679	0.001	0.0735	0.7887
Cladonia multiformis	14	0.001	0.0033	0.9545	0.005	0.1247	0.7272	0.001	0.2831	0.5998
Cladonia phyllophora	11	0.234	7.0359	0.0142*	0.001	0.3001	0.5891	0.001	0.2111	0.6502
Cladonia pocillum	4	0.159	4.3614	0.0480*	0.002	0.4048	0.5309	0.003	0.8806	0.3578
Cladonia pyxidata	61	0.003	0.0687	0.7955	0.006	1.5001	0.2331	0.001	0.3497	0.5601
Cladonia rangiferina	24	0.021	0.4928	0.4897	0.001	0.0027	0.9588	0.001	0.2915	0.5945
Cladonia scabruiscula	15	0.029	0.6834	0.4169	0.008	2.0876	0.1620	0.167	4.6027	0.0427*
Cladonia stellaris	17	0.297	9.7325	0.0048**	0.004	0.8956	0.3538	0.001	0.2988	0.5899
Cladonia stygia	5	0.069	1.7141	0.2034	0.004	1.0196	0.3231	0.001	0.2586	0.6159
Cladonia uncialis	14	0.029	0.6786	0.4185	0.002	0.4546	0.5069	0.001	0.4096	0.5285
Cladonia verticillata	6	0.001	0.0033	0.9547	0.009	2.2149	0.1503	0.117	3.0625	0.0934
Peltigera rufescens	29	0.110	2.8648	0.1040	0.004	0.9922	0.3296	0.005	1.2226	0.2803
Sterocaulon tomentosum	31	0.031	10.5529	0.0035**	0.203	5.8556	0.0238*	0.008	2.0792	0.1628
Umbilicaria deusta	9	0.226	6.7020	0.0164*	0.231	6.9313	0.0149*	0.004	1.0273	0.3213
Vulpicida pinistari	19	0.431	17.4429	0.0004**	0.131	3.4801	0.0749	0.002	0.6744	0.4199
Xanthoparmelia cumberlandia	8	0.004	0.9856	0.3311	0.124	3.2572	0.0842	0	1.5151	0.2308

Note: The single asterisk represents significance at $p < 0.05$; the double asterisk represents Bonferroni correction of $p < 0.0005$. ($0.05/X$)

2.5 Discussion

2.5.1. *Soil characteristics may explain some species distributions*

The significant differences among the five locations were explained by differences in the soil characteristics and partially by species composition. This finding suggests that some species may not rely on the substrate for nutrients and moisture and have a wide tolerance for substrate characteristics whereas other species may be linked to soil characteristics. For example, *Peltigera rufescens*, *Cladonia cariosa*, *C. humilis*, *C. scabriuscula*, and *C. magyarica* segregated with rounded sand grains (Fig. 2.2 and Table 2.2). Furthermore, *Arctoparmelia centrifuga*, *Stereocaulon tomentosum*, *C. deformis*, *C. stellaris*, *C. uncialis*, and *C. amaurocraea* correlated more strongly with angular sand grains. The angular shape of the sand grains may minimize the spaces between sand grains by fitting together but the round shape may allow for more space between grains and, therefore, more water and air movement between grains (Lipiec et al. 2016). The shape of sand grains has been shown to affect the compaction, compressibility, and shear stress of soils and, therefore, also the aeration, water holding capacity, and temperature regulation (Horn 2011; de Bono and McDowell 2015). The thick mat of rhizines diagnostic of *P. rufescens* and the persistent basal squamules in *C. cariosa*, *C. humilis*, and *C. magyarica* would help to stabilize sand better than tall podetia with disintegrating basal squamules such as *C. gracilis* subsp. *turbinata*, *C. cornuta*, *C. amaurocraea*, or *C. uncialis*. This hypothesis would need further testing.

Cladonia phyllophora and *C. gracilis* subsp. *turbinata* are common boreal species (Brodo et al. 2001) that look very similar and were both present in all five locations, suggesting they may be tolerant of a wide range of soil conditions. They may receive their nutrients from a

combination of the soil (especially when they are young with squamules attached directly to the soil) and the atmosphere (especially as they age as the squamules disintegrate). *Cladonia phyllophora* has no cortex near the top of a tall podetium and a black mottled base whereas *C. gracilis* subsp. *turbinata* has a smooth cortex giving it an olive colour throughout the length of a tall podetium. The similar morphological features in these species, where both species produce tall podetia and the primary basal squamules disintegrate with time, suggests that the direct connection with the soil by the primary squamules may be severed after the podetia have been produced. It would stand to reason that after squamule disintegration, the colony of tall podetia must rely predominantly on an atmospheric source of nutrients and moisture or splash from the substrate or adjacent mosses. Few studies have been conducted on the source of nutrients; however, the substrate has been shown to be correlated with lichen growth for water-soluble nutrients in lichen soil crusts (Fischer et al. 2014), in an Alaskan tundra (Williams et al. 1978), and an important source of nutrients through enzyme activity (Moiseeva 1961). Whereas early squamule growth of these species may penetrate the soil particles and offer some stabilization properties for disturbed soils, the later podetial growth may be more independent of the soil characteristics.

High levels of organic matter may reduce the pH level of the soil, making the soil more acidic (Sparks 2003). Acidic soils seem to be more favourable for *Cladonia amaurocraea*, *C. arbuscula*, and *C. deformis*, whereas *C. cariosa*, *C. pocillum*, and *C. magyarica* are known to grow in calcareous substrata (Culberson 1969; Wetherbee 1969; Brodo et al. 2001). While *C. pocillum* was limited to the less acidic soils in Athapap and Spruce Woods Provincial Park (Table 2.4), *C. pyxidata* was more widespread showing no correlation with pH (Table 2.7), suggesting that acidic substrata may have been available or the species can tolerate less acidic

soils. Species such as *C. chlorophaea* and *C. rangiferina* were found in both acidic and neutral soils, suggesting they may also be more tolerant of pH changes in the substrata than some other species. Whereas some chemotypes of the *C. chlorophaea* complex (*C. cryptochlorophaea*, *C. grayi*, *C. pocillum* (Gilbert 1977; Piercey-Normore 2006), and *C. cariosa* (Culberson 1969)) prefer only calcareous substrata, *Cladonia merochlorophaea* and *C. pyxidata* are known to grow on acidic substrates (Brodo et al. 2001). Patches of soil or substrata with higher pH levels even in a location characterized by low pH levels may explain the presence of these species. For example, *C. merochlorophaea* was reported to grow directly on moss over calcareous soil where the moss provided the acidic substrate for the lichen (Piercey-Normore 2005, 2006, 2008a).

2.5.2. *Soil characteristics and the CNB hypothesis*

The CNB hypothesis (Bryant et al. 1983; Hyvarinen et al. 2003) was not supported by the results of this study. Lichens such as *Cladonia arbuscula* and *C. rangiferina* are mat-forming lichens because they grow acropetally forming a carpet of upright thalli in which the crustose primary thallus disintegrates over time and the upright thallus often grows well above the soil layer. The formation of this mass of vegetative thallus suggests that more carbon was needed to produce a larger mass of thallus (Crittenden 2000). If the CNB hypothesis was supported, the larger mass would have corresponded with fewer number of secondary metabolites, but this was not the case. Some species with this growth form were more abundant in locations with high organic matter in the soil such as *C. uncialis*, *C. stellaris*, and *C. amaurocraea*, which were predominantly in Sherridon and North Star. However, other species such as *C. arbuscula* and *C. rangiferina* were present in 4 or 5 of the locations, suggesting they could tolerate a wider range of organic matter in the soil. Only *C. arbuscula*, of these mat-forming species, was present in

Athapap, which also had significantly higher levels of organic matter than other locations. The larger amount of carbon in the soil with more organic matter may allow for more vegetative growth in these mat-forming species, but all species produced similar numbers of carbon-based secondary metabolites. While the numbers of different secondary metabolites in this study showed no support for the CNB hypothesis, it may be hypothesized that the quantity of each secondary metabolite may show more support for the hypothesis.

2.5.3. *Soil features may influence the occurrence of some secondary metabolites*

The distribution of usnic acid and atranorin, but not fumarprotocetraric acid, may be explained by soil characteristics. The presence of usnic acid was significantly higher in the more acidic sites of Sherridon and North Star (Table 2.6), where usnic acid may help the lichen tolerate acidic substrata (Hauck et al. 2009a). Usnic acid may also serve as an anti-herbivorous substance (Nimis and Skert 2006) or as a sun-screening metabolite (Hauck 2008). Lichen species that produce usnic acid were more dominant on acidic substrata such as *Cladonia amaurocraea*, *C. arbuscula*, *C. cristatella C. deformis*, *C. stellaris*, and *C. uncialis* (Table 3.7; Hauck et al. 2009b; Doering et al. 2016). The production of atranorin and fumarprotocetraric acid may also be affected by sand acidity levels. The presence of atranorin in lichen species in the less acidic sites, such as Spruce Woods Provincial Park and to a lesser extent Athapap (Table 2.5), may reflect the higher pH levels in the habitat rather than light or humidity levels in the environment. If light or humidity played a role in the distribution of thalli containing atranorin, the canopy cover should influence their distribution. However, Spruce Woods Provincial Park had the lowest canopy cover and Athapap had the highest canopy cover (Table 2.3), which does not support the light regulating properties of atranorin to make light available for the photobiont

(Melo et al. 2011). However, shrub or herbaceous canopy layers may have played a role in the amount of light reaching the lichens and, therefore, the presence of atranorin in the lichen thallus.

The pH of the soil alone does not seem to influence the type of secondary metabolite produced by lichens in this study. Species containing fumarprotocetraric acid were reported to be more common in acidic sites such as on peat soils (Purvis et al. 1992) or on the bark of conifers (Gauslaa et al. 1998) and the production of fumarprotocetraric acid was thought to increase the tolerance of lichens for acidic substrates (Hauck 2008; Hauck et al. 2009a). However, fumarprotocetraric acid was present in all five locations (in different species) with no significant difference in frequency of occurrence (Table 2.5). Culberson et al. (1977a) reported a correlation with the amount of fumarprotocetraric acid production in the *Cladonia chlorophaea* group with distance from the ocean in North Carolina. The frequency of occurrence of 13 other fumarprotocetraric acid containing species varied among locations with different pH levels, suggesting that pH was not affecting the production of this compound.

In conclusion, the physical and chemical components of the soil substrate have been previously assumed to be important factors in species assemblages but this is one of the first studies to closely examine soil characteristics relative to species and secondary metabolite occurrence. The finding that many of the species, with some exceptions, were not influenced by soil characteristics supports the concept that lichens receive their nutrients and moisture from atmospheric sources. However, the few species that showed relationships with soil, highlight valuable implications for stabilizing soils and provide further hypotheses for testing. The CNB hypothesis was not supported in this study because all species produced some secondary metabolites even when carbon was present in the soil. However, the quantification, rather than occurrence, of the secondary metabolites in lichen species may be used in future studies to test

the CNB hypothesis more directly. The sand content and shape of the soil particles seemed to have the greatest effect on species composition and the occurrence of some secondary metabolites in this study, but the correlations provide insights that need to be further tested. These novel findings are partially exploratory and draw upon relationships among variables that have not previously been examined.

2.6 Acknowledgements

The authors thank J. Doering and C. Deduke for assistance in the field and statistical analysis. This project was supported by a University of Manitoba Faculty of Science award to MZ, Graduate Enhancement of Tri-Council Stipends to MPN for MZ, a Natural Sciences and Engineering Research Council of Canada Discovery Grant to MPN, and collection permits from Manitoba Conservation and Parks Canada.

CHAPTER 3

Reports of *Cladonia magyarica* and *C. humilis* in Manitoba

This chapter is published in the journal *Evansia

Zraik, M., James T. Booth and Piercey-Normore, M.D. 2016. 33(3):136-144.

3.1 Abstract

The genus *Cladonia* is widely distributed around the world. Since the section *Cladonia* contains species complexes that are difficult to distinguish, the distributions of species within these complexes are even less well-known than others. The cup-forming species, *Cladonia magyarica,* is morphologically similar to *C. pyxidata* and *C. pocillum;* and *C. humilis* is morphologically similar to *C. conista* and *C. chlorophaea*. Both species, *C. magyarica* and *C. humilis,* have not been previously reported for Manitoba, but their distribution ranges fall very close to the province, and they grow in habitats that are present in southern Manitoba. The goal of this paper was to report the presence of *C. magyarica* and *C. humilis* in Manitoba representing range extensions for both species, and to show morphological differences among them and their close allies present in the same geographic locations. The morphological differences between *C. magyarica* and *C. pyxidata* include squamules inside the cup and a significantly taller podetium and wider cup diameter in *C. magyarica* than *C. pyxidata*. *Cladonia magyarica* also differs from *C. pyxidata* in possessing atranorin in addition to fumarprotocetraric acid, in every specimen we collected. Atranorin and fumarprotocetraric acid were also shown to be present in *C. humilis*, but *C. humilis* has soredia covering the podetium, which is not present in the aforementioned taxa.

3.2 Introduction

The genus *Cladonia* (Cladoniaceae) contains more than 400 species distributed around the world (Ahti 2000). *Cladonia magyarica* Vainio and *C. humilis* (With.) J. R. Laundon are two cup-forming members of *Cladonia* section *Cladonia* (Ahti 2000) or Supergroup *Cladonia* (Stenroos et al. 2002) and they are morphologically similar to *C. pyxidata* (L.) Hoffm. and other species in the *C. chlorophaea* complex. *Cladonia magyarica* was first collected on sandy soil in 1901 from the Danube island, Csepel in Hungary, and was called *Cladonia pyxidata* var. *neglecta*. In 1927, it was described as a new species, *Cladonia magyarica,* by Vainio, the lectotype being from the vicinity of the town Kecskémet in Hungary (Farkas and Lökös 1994). *Cladonia magyarica* is reported to be common only in arid parts of Hungary, with scattered localities in adjacent areas of Slovakia, Serbia, Austria, Germany, and Russia in Europe as well as in Iran and Kazakhstan in Asia (map: Litterski and Ahti 2004). Recent reports also include Ukraine, Turkey and the Caucasus, but they need confirmation (Teuvo Ahti, pers. comm.). In North America the distribution includes Ontario (Brodo 1981; Wong and Brodo 1992), New York, North Dakota, Minnesota, South Dakota, Wyoming, Nebraska (Egan et al. 2002; Litterski and Ahti 2004; Consortium of North America Lichen Herbaria 2016) and Wisconsin (James Bennett, pers. comm.). *Cladonia humilis* was long misnamed as *C. conistea* (Ahti 1980) then often treated as including the very similar *C. conista* (e,g., Hinds and Hinds 2007) until Pino-Bodas et al. (2013) demonstrated with molecular methods that they are different species. *Cladonia humilis* in the strict sense is considered to be present only in the western United States, Particularly California, in North America north of Mexico (Hammer 1995), not in Canada. However, neither *C. humilis* nor *C. magyarica* have been reported for Manitoba. *Cladonia magyarica* is an esorediate species which is similar in morphology to *C. pyxidata* and *C.*

pocillum, but it differs from both these species in producing atranorin in addition to fumarprotocetraric acid (Ahti 1966; Brodo 1981). The esorediate *C. pocillum* may also contain both fumarprotocetraric acid and very rarely atranorin (Kowalewska et al. 2008) or psoromic acid.

The main morphological character for *C. pocillum* is the large and thick basal squamules that form rosettes. *Cladonia humilis* may be confused with short podetia of *C. chlorophaea* or *C. conista* (Pino-Bodas et al. 2012), but *C. humilis* produces atranorin and fumarprotocetraric acid (accessory zeorin) with large basal squamules (Ahti and Stenroos 2013). *Cladonia humilis* is also corticated at the base of a greenish grey podetium (Hammer 1995; Ahti and Stenroos 2013). In this paper, we report the presence of *C. magyarica* and *C. humilis* in southwestern Manitoba and differentiate among *C. magyarica, C. pocillum, C. pyxidata,* and *C. humilis* using morphological and chemical features of the specimens collected.

3.3 Material and Methods

3.3.1 *Study location and experimental design*

Field collections were made throughout Manitoba (Fig. 2.1) but they were focused in areas with sandy soils at N49º 43’ and W99º 15’ in southwest Manitoba and N49º 36’ and W95º 32’ in southeast Manitoba. The collection sites are characterized by spruce and aspen parkland, mixed grass prairie with river delta sand dunes in southwest, Manitoba and glacial till with Jack pine dominated forests in southeast Manitoba. Previous collections deposited in the University of Manitoba Herbarium (WIN-C) (Department of Biological Sciences, University of Manitoba) were included in the study. Six additional specimens collected between 1950 to 1959 by J.W. Thomson, H.Crum, G.W. Scotter and W.B. Schofield from northern Manitoba and deposited in

the University of Wisconsin Herbarium (WIS) were also examined. Collected specimens were air-dried and brought back to the herbarium for identification and measurements. Morphological measurements chosen were based on those used in identification keys (Gallé 1968; Ahti 2000; Brodo et al. 2001; Hinds et al. 2007; Kowalewska et al. 2008) for this group of species and include podetium height, cup diameter, squamule diameter, apothecium diameter, pycnidium diameter, and presence of apothecia. Measurements were made using a dissecting microscope (Leica MZ6) with an eyepiece micrometer. Photographs were taken using a dissecting microscope (Olympus SZX10) at the University of Manitoba Herbarium (WIN). Thin layer chromatography (TLC) for compound identification and the K spot test as a preliminary test were performed on all specimens using Orange et al. (2001). Control compounds for TLC analysis were atranorin and fumarprotocetraric acid.

3.3.2 *Statistical analysis*

Statistical analyses were performed using JMP 10.0.2 (SAS Institute, Cary, NC). Oneway ANOVA of morphological characteristics (podetium height, cup diameter, squamule diameter, apothecium diameter, pycnidium diameter, and number of apothecia) compared the measurements among the four species using the post hoc Tukey's HSD tests.

3.4 Results

Seventy-one specimens were examined for morphological features. From these specimens, 31 were *Cladonia magyarica,* 10 were *C. humilis*, 20 were *C. pyxidata,* and 10 were *C. pocillum*. Six additional specimens were also examined that were previously collected in Manitoba and deposited in the University of Wisconsin Herbarium, but these were not included

in the analyses. All specimens analysed were deposited in the cryptogam division of the University of Manitoba Herbarium (WIN-C). All the *C. magyarica* and *C. humilis* specimens were found in southwest Manitoba where the soil is generally calcareous. Specimens of *C. pyxidata* were found in southeast and southwest Manitoba (Table 3.1) where there is a mix of acidic and calcareous soils.

Specimens of *C. pocillum* were collected from both southeast and southwest Manitoba and six others from northern Manitoba were included in the analyses. The podetial height and the cup diameter in *Cladonia magyarica* were statistically significantly larger than those of the other three species (Table 3.2). The mean podetial height and cup diameter in *C. magyarica* were 10.3 mm and 4.8 mm compared with 6.7 mm and 3.7 mm in *C. pyxidata,* respectively. The squamule size in *C. magyarica* was significantly larger than that of *C. humilis* and *C. pocillum* but not different from that of *C. pyxidata*. There were no differences in the size and number of the apothecia among the three species except that *C. humilis* lacked apothecia in this study. Pycnidial size in *C. magyarica* and *C. pyxidata* was significantly larger than in *C. humilis* but no significant differences were observed among pycnidia of *C. humilis* and *C. pocillum*.

A comparison of the morphology of *Cladonia pyxidata*, *C. pocillum, C. humilis* and *C. magyarica* was made (Fig. 3.2A, B, C, D, and E; Table 3.2). The podetia of *C. humilis* (Fig. 3.2 A, B) were 6.3 ± 2 mm tall and gray or gray-green in color; the non-proliferating cup diameter was 2.7 ± 1 mm; and farinose soredia covered the podetium except for 1-2 mm of cortex at the base. The basal squamules were 0.6 ± 0.2 mm diameter and rounded. Apothecia and pycnidia were rare or absent. Pycnidia were brown to dark brown in color and were 0.1 ± 0.01 mm diameter. The thallus was K+ yellow and TLC showed the presence of atranorin and fumarprotocetraric acid. This species was collected from dry, sandy, and disturbed soil in

southwestern Manitoba. The morphology of *Cladonia magyarica* is shown in Fig. 2C. The podetium height was 10.3 ± 2.0 mm high, the cups were goblet-shaped and they had thick squamules on the interior and margins of the cup. The cup diameter was 4.8 ± 2.0 mm. The thallus was light gray-green to gray-brown. The basal squamules of *C. magyarica* were round, 1.8 ± 0.2 mm in diameter and thick. The apothecia were 0.6 ± 0.1 mm in diameter and were brown to dark brown in colour depending on the maturity of apothecia but they were limited in number. Small dark brown pycnidia were 0.2 ± 0.01 mm in diameter and were present on the margins of the cups. The chemistry of the thallus was K+ yellow. The TLC showed both atranorin and fumarprotocetraric acid to be present in all specimens. *Cladonia magyarica* is distributed in open and exposed sandy areas in southwest Manitoba. A specimen (Scotter 2913; Canada, Manitoba, Fort Hall Lake) from WIS, which was collected in 1963 from a rocky cliff at Fort Hall Lake in northern Manitoba, was previously identified as *C. magyarica*. After performing TLC, the specimen was revealed to contain fumarprotocetraric acid alone. Additionally, the rosette form of the basal squamules, and the smaller cup diameter and height (even with the presence of bullate squamules inside the cup) revealed that this specimen is, in fact, *C. pocillum*.

Table 3.1. Specimens examined in this study showing collection numbers, secondary metabolites detected by TLC (AT-atranorin, FUM-fumarprotocetraric acid), and location of collection which were all within Manitoba, Canada. Specimen collection numbers are those of Zraik (Z) or Piercey-Normore (PN). All collections are from Manitoba in the Spruce Woods Provincial Park (SWPP) and the Sandilands Provincial Forest (SPF) with the exception of six specimens of *C. pocillum* previously collected from Wapusk National Park.

Cladonia species (collection number)	Secondary metabolites	Location of collection
C.magyarica Z 411-3	AT, FUM	SWPP, Epinette trail
C.magyarica Z 412-6	AT, FUM	SWPP, Epinette trail
C.magyarica Z 413-9	AT, FUM	SWPP, Epinette trail
C.magyarica Z 414-12	AT, FUM	SWPP, Epinette trail
C.magyarica Z415-16	AT, FUM	SWPP, Epinette trail
C.magyarica Z 421-2	AT, FUM	SWPP, Seton trail
C.magyarica Z 423-9	AT, FUM	SWPP, Seton trail
C.magyarica Z 424-13	AT, FUM	SWPP, Seton trail
C.magyarica Z 425-16	AT, FUM	SWPP, Seton trail
C.magyarica Z 432-2	AT, FUM	SWPP, Parsons Hill west
C.magyarica Z 433-6	AT, FUM	SWPP, Parsons Hill west
C.magyarica Z 434-11	AT, FUM	SWPP, Parsons Hill west
C.magyarica Z 435-15	AT, FUM	SWPP, Parsons Hill west
C.magyarica Z 442-4	AT, FUM	SWPP, Parsons Hill east
C.magyarica Z 443-13	AT, FUM	SWPP, Parsons Hill east
C.magyarica Z 444-1	AT, FUM	SWPP, Parsons Hill east
C.magyarica Z 44514	AT, FUM	SWPP, Parsons Hill east
C.magyarica Z 451-5	AT, FUM	SWPP, Road 47 north
C.magyarica Z 452-9	AT, FUM	SWPP, Road 47 north
C.magyarica PN 11325-1	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11325-3	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11325-4	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11326-1	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11326-2	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11326-5	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11326-6	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11327-1	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11327-2	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11304-1	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11304-3	AT, FUM	SWPP, Epinette trail
C.magyarica PN 11304-4	AT, FUM	SWPP, Epinette trail
C.humilis Z 413-10	AT, FUM	SWPP, Epinettetrail
C.humilis Z 422-7	AT, FUM	SWPP, Seton trail
C.humilis Z 423-12	AT, FUM	SWPP, Seton trail
C.humilis Z 433-8	AT, FUM	SWPP, Parsons Hill west
C.humilis Z 434-12	AT, FUM	SWPP, Parsons Hill west
C.humilis Z 441-1	AT, FUM	SWPP, Parsons Hill east
C.humilis Z 444-7	AT, FUM	SWPP, Parsons Hill east
C.humilis Z 452-10	AT, FUM	SWPP, Road 47 north
C.humilis Z 453-18	AT, FUM	SWPP, Road 47 north
C.humilis Z 454-12	AT, FUM	SWPP, Road 47 north

C.pocillum Z 412-7	FUM	SWPP, Epinette trail
C.pocillum Z 441-2	AT, FUM	SWPP, Parsons Hill east
C.pocillum Z 514-11	FUM	SPF, East end of Trail 17A
C.pocillum PN 1811	FUM	Wapusk National Park
C.pocillum PN 1802	FUM	Wapusk National Park
C.pocillum PN 4079	FUM	Wapusk National Park
C.pocillum PN 661	FUM	SWPP, Hwy 5 S of Hwy 1
C.pocillum PN 3835	FUM	Wapusk National Park
C.pocillum PN 1765	FUM	Wapusk National Park
C.pocillum PN 1788a	FUM	Wapusk National Park
C.pyxidata Z 411-2	FUM	SWPP, Epinette trail
C.pyxidata Z 412-7	FUM	SWPP, Epinette trail
C.pyxidata Z 421-1	FUM	SWPP, Seton trail
C.pyxidata Z 512-18	FUM	SPF, East end of trail 17A
C.pyxidata Z 513-5	FUM	SPF, East end of trail 17A
C.pyxidata Z 514-11	FUM	SPF, East end of trail 17A
C.pyxidata Z 523-8	FUM	SPF, south end of trail 20
C.pyxidata Z 524-11	FUM	SPF, south end of trail 20
C.pyxidata Z 525-13	FUM	SPF, south end of trail 20
C.pyxidata Z 531-14	FUM	SPF, trail 20 S of Hwy 210
C.pyxidata Z 532-6	FUM	SPF, trail 20 S of Hwy 210
C.pyxidata Z 533-11	FUM	SPF, trail 20 S of Hwy 210
C.pyxidata Z 534-12	FUM	SPF, trail 20 S of Hwy 210
C.pyxidata Z 535-15	FUM	SPF, trail 20 S of Hwy 210
C.pyxidata Z 545-16	FUM	SPF, Hwy 210, 1.6 km east of Marchand
C.pyxidata Z 551-10	FUM	SPF, trail 20 N of Hwy 210
C.pyxidata Z 554-4	FUM	SPF, trail 20 N of Hwy 210
C.pyxidata Z 555-16	FUM	SPF, trail 20 N of Hwy 210
C.pyxidata PN 11325-12	FUM	SWPP, Epinette trail
C.pyxidata PN 11304-20	FUM	SWPP, Epinette trail

Table 3.2. Morphological differences among four species and six morphological features. Height and diameter is measured in mm; SE is standard error; different lower case letters indicate significant differences within rows at p=0.05 using Tukey's HSD test.

	C. magyarica (n=31)		*C. humilis* (n=10)		*C. pyxidata* (n=20)		*C. pocillum* (n=10)	
Feature	Mean±SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range
Podetium height	[a]10.3±2	6-14	[b]6.3±2	1-10	[b]6.7±2	1-10	[b]6.3±2.0	2-10
Cup diameter	[a]4.8±2	3.5-6.5	[c]2.7±1	0.5-4	[b]3.7±1	1.5-6.0	[c]2.4±1.0	1.5-3.0
Squamule diameter	[a]1.8±0.2	1-4	[c]0.6±0.2	0.5-1	[ab]1.5±0.2	1-3	[bc]0.9±0.2	0.5-1.5
Apothecia diameter	[a]0.6±0.1	0.3-1.8	-	-	[a]0.6±0.08	0.3-1.5	[a]0.7±0.1	0.5-1.0
Pycnidia diameter	[a]0.2±0.01	0.2-0.3	[b]0.1±0.01	0.1-0.2	[a]0.2±0.01	0.2-0.3	[ab]0.2±0.01	0.2

Fig. 3.1 The four species examined in this study, A and B) podetia (Zraik 433-8) and squamules (Zraik 425-10) of *C. humilis*, C) podetial cup with squamules of *C. magyarica* (Zraik 434-11), D) podetium and basal rosette squamules of *C. pocillum* (Zraik 412-7), and E) podetium with brown apothecia of *C. pyxidata* (Zraik 525-13). All scale bars are 2.5 mm.

3.5 Discussion

This is the first study to report the presence of *Cladonia magyarica* and *C. humilis* in Manitoba, which represents extensions of their current distributions. The distribution of *C. magyarica* has been extended from the east and that of *C. humilis* from the west. *Cladonia magyarica* specimens contained atranorin in addition to fumarprotocetraric acid, whereas *C. pyxidata* contained only fumarprotocetraric acid, suggesting that chemistry may be an important distinguishing character. *Cladonia magyarica* also has the tallest podetium and widest cup of the four species in this study but monophyly of these characters has not been tested using molecular phylogenetic analyses. The height of podetia in *C. pyxidata* is reported to be 5-30 mm (Purvis et al. 1992; Gallé 1968) and the height in this study ranged from 6-14 mm. The cup diameter was reported to be 3-8 mm (Gallé 1968) and the measurements of the same species in this study fell within the same range. These characters may distinguish *C. magyarica* from other similar species in southern Manitoba but the squamules in the cup are not exclusive to *C. magyarica* and may occur as bullate squamules in *C. pyxidata* (Teuvo Ahti, pers. comm.). Kotelko and Piercey-Normore (2010) supported the phylogenetic placement of *C. magyarica* with *C. pyxidata* where *C. magyarica* grouped with a form of *C. pyxidata* that had upright basal squamules, but that study could not distinguish the two species using phylogenetic analysis. The presence of *C. magyarica* in southwest Manitoba is consistent with the known ecology of its distribution in dry and sandy soils. *Cladonia humilis* produces both atranorin and fumarprotocetraric acid, but the cups of *C. humilis* are covered with farinose to granular soredia while those of *C. magyarica* are esorediate. *Cladonia humilis* is distinguished from these three species but it is morphologically similar to *C. conistea* which was examined by Pino-Bodas et al. (2012). Pino-Bodas et al. (2012)

reported the podetia of *C. humilis* to range from 2.5-9.0 mm tall and the cups from 0.5-7.0 mm wide. In this study, the sizes fell within a similar range, between 1-10 mm tall and 0.5-4 mm wide, respectively. While Pino-Bodas et al. (2013) showed that *C. humilis* was not monophyletic, it was separated from *C. conista* and was distantly related to *C. pyxidata* and *C. pocillum*. Pino-Bodas et al. (2013) suggested that cortical characters may be useful in identification of these species. Squamule characters also showed differences among species (Ahti 2000; Aptroot et al. 2001) but they were not diagnostic in this study. *Cladonia pocillum* is morphologically different from *C. pyxidata* and *C. magyarica*.

Cladonia pocillum is known to contain fumarprotocetraric acid and very rarely atranorin (Kowalewska et al. 2008), but the morphological characters include large and thick squamules that form basal rosettes. The presence of both atranorin and fumarprotocetraric acid have been shown to be variable (Culberson et al. 1977a), and they have also been variable in this study. *Cladonia pyxidata* and *C. pocillum* have both been reported for Manitoba (Piercey-Normore 2003, 2007, 2008a, 2008b, 2010). These first reports of *C. magyarica* and *C. humilis* for Manitoba may be the result of insufficient collection effort in southwest Manitoba or they may have been overlooked in previous collections. The choice of morphological characters in field identification of species is difficult, as it does not always correspond with phylogenetic species. Morphological species concepts may hold true for some species within *Cladonia* (Fontaine et al. 2010, Pino-Bodas et al. 2012) but it has not been supported for other species including *C. pyxidata* and *C. pocillum* (Kotelko and Piercey-Normore 2010) and *C. humilis* (Pino-Bodas et al. 2013). Some characters may vary from one location to another because of environmental conditions (Pintado et al. 1997; Kotelko and Piercey-Normore 2010; Pino-Bodas et al. 2013).

The documentation of characters to facilitate field identification in species with high levels of variation from different geographic regions is instructive for naturalists and field biologists.

3.6 Acknowledgements

The authors thank three reviewers for their thoughtful and helpful comments; J. Bennett, C. Lewis, and T. Ahti as well as J. Bennett for the borrowed specimens from WIS. This study was supported by funding from the University of Manitoba awarded to MZ, from NSERC awarded to MPN, and collection permits from Manitoba Conservation and Parks Canada.

CHAPTER 4

The Carbon-Nutrient Balance hypothesis and phenotypic characters in six species of *Cladonia*.

***This chapter has been submitted to the journal Oecologia**

4.1 Abstract

Fruticose species of the lichen genus *Cladonia* are distributed widely in different climatic conditions and show phenotypic variability in morphology, reproductive characters, and quantity of secondary matabolites. The goal of this study was to explore the Carbon-Nutrient Balance (CNB) hypothesis by testing the relationship between the phenotypic variability of six selected lichen species from the genus *Cladonia* (*C. arbuscula, C. cariosa, C. cristatella, C. gracilis* ssp. *turbinata, C. pyxidata,* and *C. rangiferina*). Lichen specimens were collected from five locations in northern and southern Manitoba. Morphological characters measured were podetium height (mm), squamule surface area (mm^2), and number of branches on the podetium. Fecundity was measured by the apothecia diameter (mm) and number of apothecia. Two soil characteristics and canopy cover were also examined. No significant relationships were shown between morphological characters and the concentration of atranorin. Results also showed negative relationships between concentration of usnic acid and fumarprotocetraric acid with apothecia diameter. The results supported the CNB hypothesis for some species (*C. arbuscula, C. cristatella, C. gracilis* ssp. *turbinata,* and *C. pyxidata*) and their secondary metabolites (fumarprotocetraric acid and usnic acid), but not for other species (*C. cariosa, C. rangiferina*) or

for atranorin.

4.2 Introduction

Features of the biotic and abiotic environment may influence the phenotypic variability of lichens (including morphological characters, fecundity, and secondary metabolites). Phenotypic variability may represent plasticity, which refers to the condition where species that are genetically identical have phenotypic differences because of adaptation to environmental conditions (Behera 1997). Species such as *Cladonia pocillum, Ramalina capitata,* and *Pseudevernia furfuracea* showed phenotypic plasticity when they were found growing in different environmental conditions of soil pH, light and moisture (Gilbert 1977; Pintado et al. 1997; Rikkinen 1997). Phenotypic plasticity allows for differences in phenotype of individuals of the same species in different habitats which optimizes physiological processes under those conditions. Phenotypic differences have been used to compare growth of the lichen species (Muir et al. 1997; Hyvarinen et al. 1998, 2003; Gauslaa et al. 2006; Zraik et al. 2016). Lichen growth rates were correlated with the time of the year depending on the dry and moist seasons (Fisher et al.1978; Armstrong et al. 1993; Muir et al. 1997). Moisture was the most important factor influencing seasonal growth, and higher growth rates were correlated with levels of precipitation in each season (Fisher et al. 1978; Boucher et al. 1990). Lichen growth forms may also be affected by soil factors. Morphological differences of thallus squamules were observed in *C. pocillum* and *C. pyxidata,* which were thought to be related to the soil pH (Gilbert 1977; Kotelko et al. 2010; Pino-Bodas et al. 2012). Competition for space, water, nutrients, and sunlight are also major factors affecting growth especially when the resources are limited (Keddy 2001; Armstrong et al. 2007). Two highly branched fruticose species that have relatively fast growth

rates, and have a competitive advantage over space, include two reindeer lichens, *C. arbuscula* and *C. stellaris* (Ahti 1977; Ruoss et al. 1989). Since growth of thallus tissue requires large amounts of carbon in the fungal cell walls, a large amount of carbon would presumably be required to produce the biomass in these reindeer lichens compared with smaller lichens.

While there are more plant studies that focus on the Carbon-Nutrient Balance (CNB) hypothesis (Bryant et al. 1983, 1986; Waterman et al. 1989; Muzika et al. 1993; Hyvarinen et al. 2003), there are few studies on lichens (Bryant et al. 1983; Hyvarinen et al. 2003). Most vascular plants depend directly on soil to get nutrients and require larger quantities of nutrients than lichens, while most lichens receive their nutrients and moisture from atmospheric sources (and sometimes soil) and are thought to be adapted to nutrient poor conditions (Beckett et al. 2008). If sufficient nutrients are available, carbon would be used for growth of thallus tissue. However, if nutrients are unbalanced, carbon may be used for carbon-based secondary metabolites such as polyketides. A comparison between the quantity of carbon-based secondary metabolites and lichen phenotype in different environmental conditions may provide insights into the CNB hypothesis. Trade-offs are observed when an increase in one characteristic corresponds with a decrease in another characteristic. Studies on the trade-off between growth and sexual reproduction in lichens have shown inconsistent findings (Hestmark et al. 2004a, b; Gauslaa 2006). The fecundity (number of offspring from sexual reproduction) in plants and lichens is thought to happen at the expense of vegetative growth (A´lvarez-Cansino et al. 2010; Gauslaa 2006). Fecundity in lichens can be measured by number of apothecia, ascospores, and percent germination (Pringle et al. 2003; Büdel et al. 2008). The formation of lichen asexual reproductive structures such as soredia and isidia showed no reduction in thallus growth (Pringle et al. 2003). The explanation was that the asexual structures are photosynthetically active and

able to produce their own food (Pringle et al. 2003). However, a correlation was found between soredium size and amount of secondary metabolite produced (Ahti et al. 1961; Pino-Bodas et al. 2012). Studies showing the relationship between quantity of secondary metabolites and thallus growth in lichens are rare, which can provide insight into how lichens can utilize the available sources of nutrients to balance between producing more secondary metabolites and growing more thallus tissue.

The goal of this study was to examine the CNB hypothesis for six species of lichens with respect to phenotypic variability (measurements of morphological and fecundity characters) as an indication of growth compared with the quantity of secondary metabolites. The hypothesis is that when more thallus growth or reproduction has occurred there will be a smaller amount of carbon-based secondary metabolite produced because the carbon has been used in biomass production. The differences in five locations in Manitoba, Canada, were also compared to determine the extent of location as a confounding variable.

4.3. Methods and Materials

4.3.1. *Sampling and experimental design*

Lichen thallus samples for six species (*C. arbuscula, C. cariosa, C. cristatella, C. gracilis* ssp. *turbinata, C. pyxidata,* and *C. rangiferina*) were collected from five locations in Manitoba during July to September in 2013 and 2014 (Zraik et al. 2018). Briefly, five 1m x 1m quadrats were located 25m apart along each of five transects within each of five locations (Athapap, Sherridon, Sandilands, Spruce Woods, and North Star). The distance between two transects ranged from 500m to 1000m. The quadrats were placed on the same side of the transect and voucher specimens for each species were collected, placed in labelled paper bags and allowed to

air dry, and returned to the lab for further processing. These six species were chosen because they were common in the collection sites, had measureable phenotypic features, and they consistently produce three main secondary metabolites examined in this study. *C. cristatella* and *C. arbuscula* were studied for usnic acid; *C. gracilis* ssp. *turbinata* and *C. pyxidata* were studied for fumarprotocetraric acid; and *C. cariosa* and *C. rangiferina* were studied for atranorin. These six species represent three growth forms of *Cladonia* lichens where *C. pyxidata* and *C. gracilis* ssp. *turbinata* are cup lichens*; C. rangiferina* and *C. arbuscula* are highly branched reindeer lichens; and *C. cariosa,* and *C. cristatella* are club lichens (without cups). Thallus growth was examined using different features for each species (see next section). Taxonomic identification of the six species was confirmed using Ahti (2000), Brodo et al. (2001), Hinds and Hinds (2007).

4.3.2. *Measurement of Morphological, fecundity and soil characteristics*

Morphological and fecundity characteristics were examined on a total of 20 specimens collected from the five locations (4 specimens for each location). Drawings to show the features for each species are in Appendix F. The morphological characters studied were podetium height (mm) which was measured from the tallest point of the podetium to the point at which it emerged from the squamule in *C. cariosa, C. cristatella,* and *C. pyxidata*. It was measured from the tip of the podetium to the basal most part which showed discoloration or decay as it emerged from the substrate in *C. gracilis* ssp. *turbinata*. Cup diameter (mm) was measured at the widest part of the cup for *C. pyxidata* and *C. gracilis* ssp. *turbinata.* The squamule surface area (μm^2) was measured as the length multiplied by width of the squamule for each of *C. cariosa* and *C. pyxidata*. The number of branches on the podetium were counted for each of *C. gracilis* ssp. *turbinata, C. cariosa,* and *C. cristatella.* The apothecium diameter (μm) was measured at the

widest part of the apothecium. Apothecia were distinguishable from pycnidia by appearance and size where the smallest apothecium diameter was 0.5 mm, while the largest pycnidium diameter was 0.25 to 0.3 mm. The number of apothecia per podetium was counted for *C. pyxidata* only. For *C. rangiferina* and *C. arbuscula*, the only morphological character studied was podetium height (mm), which was measured from the dead basal part of the thallus to the podetial tips. Measurements were made using a dissecting microscope (Leica MZ 6) at 10× magnification with a mm ruler or an eyepiece micrometer to determine distance. All measurements were performed on dry lichens.

4.3.3. *Quantification of three main secondary metabolites*

The same specimens that were used for morphological characteristics were also prepared for quantification of the three secondary metabolites. The lichen thallus was crushed very fine with a ceramic mortar and pestle. The powder was then weighed, transferred to a glass vial and 2 mL acetone was added and left to incubate for 15 minutes on the shaker, to ensure compounds were dissolved. The extracted solvents were then transferred to new vials and another 2 mL of acetone were added to the sample and shaken again for 15 minutes. To ensure all of the metabolite was removed, a preliminary experiment was performed whereby samples were run on HPLC after each of six extractions to determine when there were no more secondary metabolites in the powder (Usnic acid, atranorin, and fumarprotocetraric acid). Since the results showed 5 extractions produced no more metabolite, a sixth extraction was done to ensure all compound was removed from the crushed specimens. The acetone extract was then filtered by a cotton filter and the acetone was evaporated off. The vial was weighed before and after the procedure to calculate the weight of the isolated compounds. A low concentration dilution (50µg/ml) was

prepared for each sample to run on High Performance Liquid Chromatography (HPLC). HPLC analysis was carried out on Waters HPLC Separations Module 2695, combined with a PDA Detector Model 2996. The column was a μBondapak® Waters C18 (3.9 X 300 mm) with a column particle diameter of 15-20 μm, and with 125 Å pores. The flow rate was 1 mL/minute. The eluent was monitored continuously at 210-600 nm, and HPLC traces were displayed at 254 nm. The gradient was held at 20% methanol to 80% water in 0.075% aqueous trifluoracetic acid for 10 minutes then the percent of methanol was linearly increased up to 80% and held at that composition for 20 minutes followed by a linear gradient back to 20% methanol for 10 minutes and held there for 10 minutes. The total run time was 60 minutes. Usnic acid, atranorin, and fumarprotocetraric acid commercial standards (ChromaDex) were used as references for this experiment. Standards were run on HPLC with 10 different concentrations from 500μg/ml to 5μg/ml, to calculate the equation for each metabolite of the three standards. Calibration curves for the standards were all calculated in Microsoft Excel. The concentration of secondary metabolites was also calculated and standardized for its content in μg for 1ml. The vials were weighed first when they were empty and again after the dried acetone extraction was added, to calculate the weight of the acetone extraction in order to determine the mass of the polyketide per mass of acetone extraction on the HPLC. The lichen thallus sample was also weighed to calculate the extraction percentage by dried tissue mass.

4.3.4. *Measurement of Soil characteristics and canopy cover*

Two soil samples (multiple representative samples and a single bulk sample) were collected from each of the 125 quadrats (25 in each of 5 locations). Multiple samples were collected from different places in the quadrat and placed within clean plastic bags (about 1kg) to

represent the surface soil in the quadrat (0 to 7 cm depth) and to determine soil characteristics, which included organic matter and sand content as described in Zraik et al. (2018). Briefly, organic matter was calculated as loss of carbon on ignition by burning the soil samples at 650˚F for 18 hours using a muffle furnace (Barnstead/Thermolyne). Sand content was determined by sieving soil samples with the Canadian standard sieves (Fisher brand U.S. Standard Brass Test Sieves; sieve number 35 (0.50 mm opening) to collect the total sand content. Percent canopy cover was estimated using 25cm by 25cm grid and held directly above the quadrat to estimate the amount of light through the canopy.

4.3.5. *Data analysis*

To determine the extent of location as a confounding variable, one-way ANOVA was performed to compare the means of 1) morphological and fecundity measurements (podetium height, cup diameter, squamule diameter, apothecium diameter, number of podetial branches and number of apothecia) for each species among the five locations; and 2) the concentration of each of the secondary metabolites (usnic acid from *C. cristatella* and *C. arbuscula*, fumarprotocetraric acid from *C. gracilis* ssp. *turbinata* and *C. pyxidata,* and atranorin from *C. cariosa* and *C. rangiferina)* among locations. All data were transformed using a log transformation (x+1) and tested for normality (Deduke et al. 2014). Regression analysis was used to examine the relationships between each of the morphological characteristics and sexual fecundity indicators as the dependent variables with the secondary metabolite quantities for each of the six species as the independent variables. Regression analysis was also used to examine the relationships between soil characteristics and the concentration of secondary metabolites for each species. All

statistical analyses were performed using JMP (Version 12, 64 bit edition, SAS Institute Inc., Cary, North Carolina, 2016).

4.4. Results

In total, there were 84 specimens: 20 specimens of each of *C. gracilis* ssp. *turbinata, C. pyxidata, C. cristatella,* and *C. arbuscula* collected from five locations (4 specimens for each location), and 12 specimens for each of *C. cariosa,* and *C. rangiferina,* which were collected from three locations (4 specimens for each location) (Athapap, Spruce Woods, and Sandilands for *C. cariosa;* and Athapap, Sherridon, and Sandilands for *C. rangiferina*). Four specimens per species were collected from each location. Each specimen consisted of a sample of 8-10 podetia and were placed in paper bags and allowed to air dry before measurements were made. Three replicates of podetia from each bag were used for morphological measurements and for HPLC (Appendix A).

4.4.1. Morphological and fecundity measurements by location

Some of the morphological features showed significant differences in size by location for each of the six species (Table 4.1; Fig. 4.1). Podetium height showed differences in four of the six species except *C. cariosa* and *C. rangiferina*, which were both found only in three of the five locations. There were significant differences in apothecium diameter with location for four species (*C. cristatella, C. pyxidata, C. gracilis* ssp. *turbinata,* and *C. cariosa*). The number of podetial branches showed significant differences by location for *C. gracilis* ssp. *turbinata* only. The cup diameter showed significant differences for *C. pyxidata* but not for *C. gracilis* ssp. *turbinata* (Fig. 4.1). *C. pyxidata* showed differences in squamule surface area by location.

Table 4.1. Measurements of morphological features (podetium height, squamule surface area, cup diameter, and number of podetial branches) from six lichen species (*C. arbuscula, C. pyxidata, C. cariosa, C. gracilis* ssp. *turbinata, C. cristatella,* and *C. rangiferina*) using one-way ANOVA. The mean of four measurements with the standard error (SE) and the range of values are shown for 20 samples from each of five locations (Sherridon, North Star, Athapap, Spruce Woods, and Sandilands). The different lower case letters indicate significant differences across locations at p=0.05 using Tukey's HSD test.

Species/Feature	Sherridon		North Star		Athapap		Spruce Woods		Sandilands	
	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range
C. arbuscula										
Podetium height (mm) P<0.0001	[a]79.4±2.1	55-85	[a]76.6±1.9	35-85	[b]65.4±3.6	30-72	[c]37±2.4	24-41	[c]28.2±1.8	18-33
C. pyxidata										
Podetium height (mm) P<0.0001	[a]12.6±1.02	10-15	[bc]7.68±0.4	5-10	[b]8.4±0.6	5.5-12	[c]5.1±0.6	4-7	[c]6±0.25	4.5-9
Cup diameter (mm) P=0.0018	[ab]4.2±0.2	4-5	[a]4.7±0.28	3-6	[a]4.8±0.35	3-7	[b]2.2±0.32	1.5-3	[a]4.09±0.28	2.5-6
Squamule area (mm^2) P=0.009	[a]4.3±1.85	2-8	[a]3.7±0.9	1-4	[b]1.84±0.27	1-4	[bc]1.01±0.01	1-1.05	[b]1.89±0.3	1-6
Apothecium diameter (mm) P=0.0071	[a]1.5±0.4	1-3	[ab]0.5±0.1	0.6-1	[b]0.73±0.1	0.5-1.5	[b]0.43±0.03	0.5-0.7	[b]0.55±0.09	0.5-1.5
No. of apothecia/cup P<0.001	[b]1.2±0.2	1-6	[b]1.6±0.4	1-8	[ab]7.2±1.3	1- 14	[a]12.4±1.2	3-17	[a]13.7±1.4	4-21
C. cariosa										
Podetium height (mm) P=0.137	–	–	–	–	[a]14.5±1.2	7-23	[a]12.2±1.3	7-20	[a]10±0.4	9-11
Squamule area (mm^2) P=0.953	–	–	–	–	[a]1.86±0.5	0.1-3.9	[a]2±0.7	1-6	[a]2.3±0.6	1-4

Apothecium diameter (mm) P=0.2164	–	–	–	–	[ab]0.7±0.1	0.5-2.5	[a]0.9±0.1	0.5-1.5	[b]0.35±0.06	0.5-0.8
No. of podetial branches P=0.1861	–	–	–	–	[ab]2.6±0.2	1-5	[a]2±0.2	1-3	[a]2.7±0.2	2-3
C. gracilis* ssp. *turbinata										
Podetium height (mm) P=0.007	[a]24.1±1.98	11-46	[ab]20.55±2.9	12-36	[ab]16.17±2.8	11-26	[b]11.25 ±1.1	9-14	[b]16.1±1.4	9-24
Cup diameter (mm) P=0.433	[a]4.47±0.3	1.5-6.5	[a]4.71±0.45	2.4-6.5	[a]3.75±0.55	2-5.5	[a]4.77±0.11	4.5-5	[a]3.95±0.23	2.5-4.8
Apothecium diameter (mm) P=0.0014	[b]1.03±0.11	0.5-2.1	[b]0.82±0.22	0.5-2.25	[b]1.56±0.74	0.5-5.2	[a]3.41±0.28	2.85-4.2	[ab]1.65±0.41	0.5-4.85
No. of podetial branches	[a]5.4±1.3	2-15	[a]5.44±0.53	3-8	[b]3.66±1.3	2-10	[b]2.05±0.4	2-3	[b]2.9±0.25	2-4
C. cristatella										
Podetium height (mm) P<0.0001	[a]16.2±1.7	10-20	[a]15.6±1.2	11-20	[a]17±1.15	15-19	[b]6.75±1.49	5-10	[b]4±1.49	3-6
Apothecium diameter (mm) P=0.0046	[a]2.3±0.54	1-3.5	[ab]1.67±0.27	0.5-2	[ab]1±0.00	0.75-1	[b]0.6±0.13	0.4-1	[b]0.4±0.04	0.5-0.7
No. of podetial branches P=0.2761	[a]3.2±0.58	2-5	[a]2.87±0.44	1-4	[a]2.66±0.33	2-3	[a]1.75±0.47	1-3	[a]2±0.4	1-3
C. rangiferina										
Podetium height (mm) P=0.354	[a]74.3±1.91	52-80	–	–	[a]71.6±2.10	48-75	–	–	[a]67.2±2.21	44-70

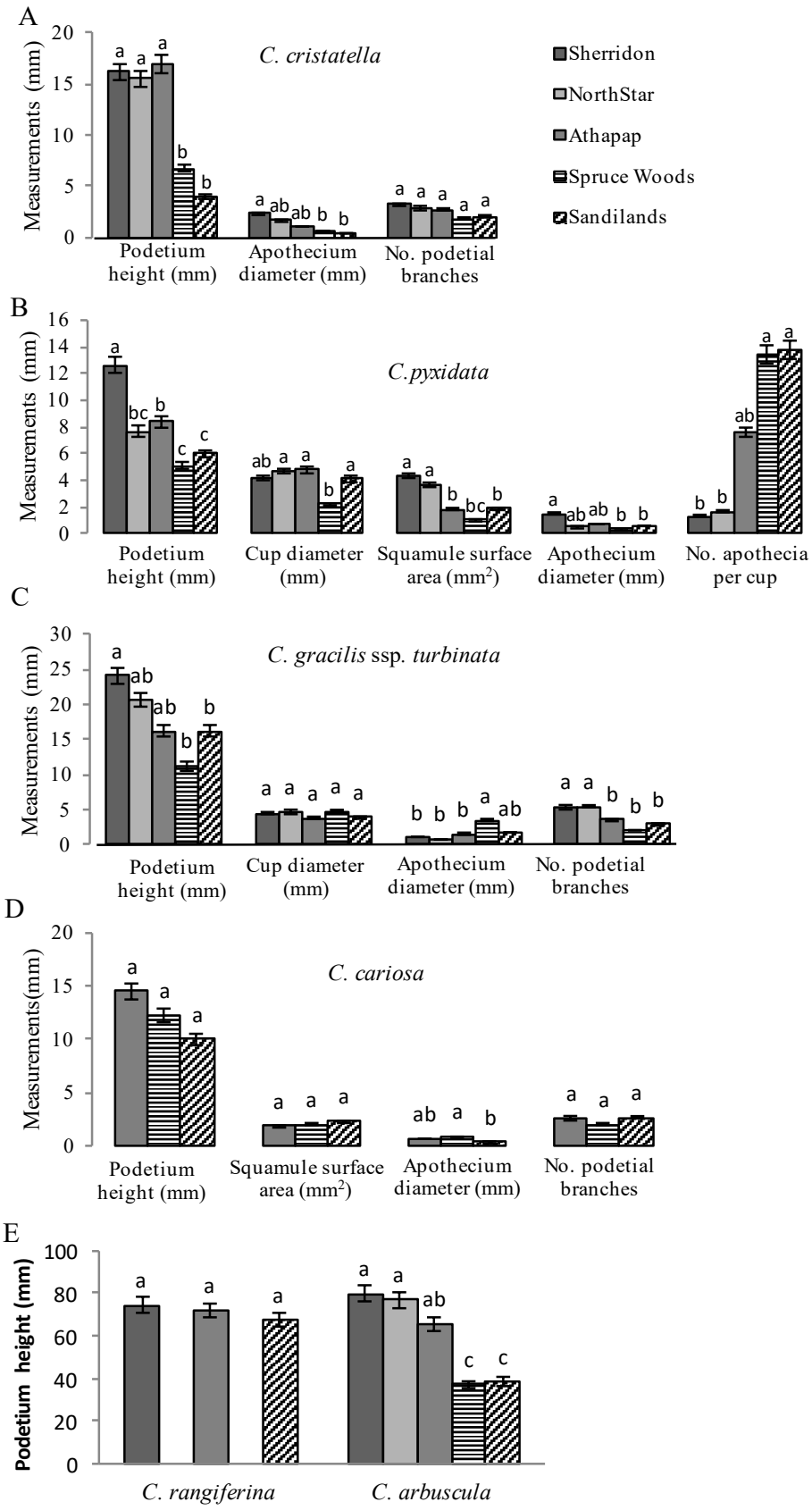

A
C. cristatella
Measurements (mm)
Sherridon
NorthStar
Athapap
Spruce Woods
Sandilands
Podetium height (mm)
Apothecium diameter (mm)
No. podetial branches
B
C.pyxidata
Podetium height (mm)
Cup diameter (mm)
Squamule surface area (mm2)
Apothecium diameter (mm)
No. apothecia per cup
C
C. gracilis ssp. turbinata
Podetium height (mm)
Cup diameter (mm)
Apothecium diameter (mm)
No. podetial branches
D
C. cariosa
Measurements(mm)
Podetium height (mm)
Squamule surface area (mm2)
Apothecium diameter (mm)
No. podetial branches
E
Podetium height (mm)
C. rangiferina
C. arbuscula

Figure 4.1. Differences in morphological and fecundity characteristics between five locations in Manitoba (Sherridon , North Star, Athapap, Spruce Woods, and Sandilands) for six species: A) *C. cristatella* for three characters (podetium height, apothecium diameter, and number of podetial branches, B) *C. pyxidata* for five characters: podetium height (mm), cup diameter (mm), squamule area (mm^2), number of apothecia per cup, and the apothecia diameter (mm), C) *C. gracilis* ssp. *turbinata* for podetium height (mm), cup diameter (mm), apothecium diameter (mm), and number of podetial branches, D) *C. cariosa* for podetium height (mm), apothecium diameter (mm), squamule area (mm^2), and number of podetial branches, and E) *C. rangiferina* and *C. arbuscula* for podetium height (mm). Note that no samples of *C. rangiferina* or *C. arbuscula* were collected from North Star or Spruce Woods. The error bars represent standard error; different lower case letters indicate significant differences within each characteristic at p=0.05 using Tukey's HSD test.

4.4.2. *Quantification of secondary metabolites by location*

Results showed significantly lower concentrations of all three secondary metabolites in Sherridon, North Star and Athapap than in Spruce Woods and Sandilands with the exception of atranorin in *C. rangiferina* where the concentration in Athapap was as high as that in Sandilands (Fig. 4.2).

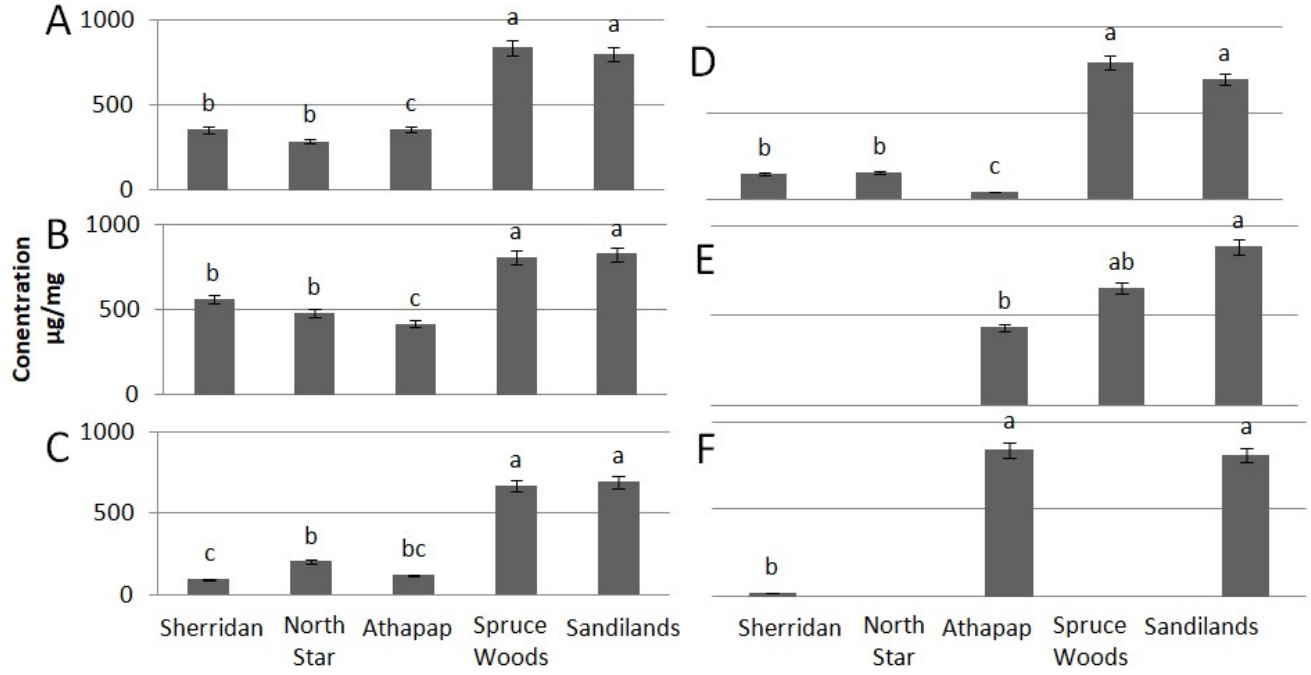


Figure 4.2. Comparison of concentration of secondary metabolites produced by six species of *Cladonia* in five locations in Manitoba, showing A) usnic acid from *C. arbuscula*, B) usnic acid from *C. cristatella*, C) fumarprotocetraric acid from *C. pyxidata*, D) fumarprotocetraric acid from *C. gracilis* ssp. *turbinata*, E) atranorin from *C. cariosa*, and F) atranorin from *C. rangiferina*. The error bars represent standard error; different lower case letters indicate significant differences across location at p=0.05 using Tukey's HSD test.

4.4.3. *Relationship between morphological and fecundity characters with secondary metabolites*

The concentration of fumarprotocetraric acid showed a negative relationship with podetium height in *C. gracilis* ssp. *turbinata* and *C. pyxidata*; with number of podetial branches in *C. gracilis* ssp. *turbinata*; and with cup diameter in *C. pyxidata* (Table 4.2). The concentration of usnic acid showed a negative relationship with the height of podetia in *C. arbuscula* and *C. cristatella* (Table 4.2). No significant relationships were found between atranorin concentration and the morphological characters in *C. cariosa* and *C. rangiferina*.

Table 4.2. A) Relationship between morphological measurements showing regression between (podetium height (mm), squamule surface area (mm^2), cup diameter (mm), and number of podetial branches) and the concentration of polyketides for six lichen species (atranorin for *C. cariosa, C. rangiferina,* fumarprotocetraric acid for *C. gracilis* ssp. *turbinata, C. pyxidata,* and usnic acid for *C. cristatella,* and *C. arbuscula)*, showing the *F* statistic, degrees of freedom (d.f.), R-squared value and the *p*-value. B) The parameter estimate (with trend direction), std error, t-ratio and p-value for morphological measurements and the concentration of polyketides. Significance was determined at $p < 0.05$.

A. Pairwise comparison	**F**	**d.f.**	***p***	**R^2**
Podetium height				
× Fumarprotocetraric acid				
C. gracilis ssp. *turbinata*	8.8088	99	0.0047*	0.155
C. pyxidata	19.5089	99	<0.0001*	0.288
× Usnic acid				
C. cristatella	6.4543	99	0.0342*	0.082
C. arbuscula	4.4705	99	0.0255*	0.098
× Atranorin				
C. cariosa	2.2512	59	0.1460	0.082
C. rangiferina	0.6654	59	0.3254	0.054
No. of podetial branches				
× Fumarprotocetraric acid				
C. gracilis ssp. *turbinata*	7.3153	99	0.0094*	0.132
× Usnic acid				
C. cristatella	2.3128	99	0.1426	0.095
× Atranorin				
C. cariosa	0.3133	59	0.5806	0.012
Cup diameter				
× Fumarprotocetraric acid				
C. gracilis ssp. *turbinata*	0.0008	99	0.9772	0.022
C. pyxidata	11.0349	99	0.0017*	0.186
Squamule surface area				
× Fumarprotocetraric acid				
C. pyxidata	1.4693	99	0.2316	0.031
× Atranorin				
C. cariosa	2.2970	59	0.1422	0.084

B. Parameter estimate	Term	Estimate	Std Error	t Ratio	Prob>|t|
Podetium height					
× Fumarprotocetraric acid					
C. gracilis ssp. *turbinata*	Intercept	551.86628	93.24322	5.92	<.0001*
	Podetia height	-12.91719	4.352221	-2.97	0.0047*
C. pyxidata	Intercept	833.89317	114.5623	7.28	<.0001*
	Podetia height	-62.57143	14.16642	-4.42	<.0001*
× Usnic acid					
C. cristatella	Intercept	1994.0753	800.2587	2.49	0.0207*
	Podetia height	-81.91163	58.13341	-1.41	0.1728
C. arbuscula	Intercept	644.5312	135.8765	5.87	0.0115
	Podetia height	-18.7462	18.3254	-2.31	0.0122
× Atranorin					
C. cariosa	Intercept	780.61224	155.4576	5.02	<.0001*
	Podetia height	-16.90833	11.2693	-1.50	0.1460
C. rangiferina	Intercept	744.6894	211.3654	4.89	0.0012
	Podetia height	54.6879	14.5781	3.54	0.1568
No. of podetial branches					
× Fumarprotocetraric acid					
C. gracilis ssp. *turbinata*	Intercept	449.602	67.39195	6.67	<.0001*
	No. branch on tip	-34.80884	12.86987	-2.70	0.0094*
× Usnic acid					
C. cristatella	Intercept	2143.3945	840.2405	2.55	0.0182*
	No. branch on tip	-454.1476	298.6293	-1.52	0.1426
× Atranorin					
C. cariosa	Intercept	480.49794	148.8217	3.23	0.0035*
	branch on tip	32.083878	57.32083	0.56	0.5806
Cup diameter					
× Fumarprotocetraric acid					
C. gracilis ssp. *turbinata*	Intercept	290.46393	152.8787	1.90	0.0639
	Cup diam.	-0.974423	33.91461	-0.03	0.9772
C. pyxidata	Intercept	812.8622	143.401	5.67	<.0001*
	cup diameter	-106.2392	31.9816	-3.32	0.0017*
Squamule surface area					
× Fumarprotocetraric acid					
C. pyxidata	Intercept	427.34962	67.77103	6.31	<.0001*
	squamulus area mm^2	-26.1789	21.59724	-1.21	0.2316
× Atranorin					
C. cariosa	Intercept	509.06335	58.43442	8.71	<.0001*
	squamulus area mm^2	24.656956	16.26902	1.52	0.1422

* Significance was determined at $p < 0.05$

Table 4.3 A). Relationship (regression) between fecundity indicators (apothecium diameter (mm), and number of apothecia on cup) and the concentration of polyketides for six lichen species (*C. cariosa, C. rangiferina, C. gracilis* ssp. *turbinata, C. pyxidata, C. cristatella,* and C. arbuscula), showing F statistic, degrees of freedom (d.f.), R-squared value and the p-value. Significance was determined at $p < 0.05$. B). The parameter estimate (with trend direction), std error, t-ratio and p-value for fecundity indicators and the concentration of polyketides.

A. Pairwise comparison	***F***	**d.f.**	***p***	**R^2**
Apothecium diameter × Fumarprotocetraric acid				
C. gracilis ssp. *turbinata*	8.0268	99	0.0069*	0.151
C. pyxidata	5.8230	99	0.0320*	0.0708
× Usnic acid				
C. cristatella	12.7854	99	0.0001*	0.043
× Atranorin				
C. cariosa	0.9249	59	0.3454	0.035
No. of apothecia on cup × Fumarprotocetraric acid				
C. pyxidata	1.4319	99	0.2393	0.038

B. Parameter estimate					
Apothecium diameter	**Term**	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
× Fumarprotocetraric acid					
	Intercept	180.5325	59.45972	3.04	0.0040*
C. gracilis ssp. *turbinata*	apothecia diam.	91.493004	32.29358	2.83	0.0069*
	Intercept	506.66034	90.5669	5.59	<.0001*
C. pyxidata	apothecia diam.	-201.392	119.8645	-1.68	0.1014
× Usnic acid	Intercept	1445.0311	587.3124	2.46	0.0222*
C. cristatella	apothecia diam.	-357.2548	359.0094	-1.00	0.3305
× Atranorin	Intercept	627.23402	86.60636	7.24	<.0001*
C. cariosa	apothecia diam.	-93.62123	97.34853	-0.96	0.3454
No. of apothecia on cup					
× Fumarprotocetraric acid					
	Intercept	332.96362	57.10564	5.83	<.0001*
C. pyxidata	no. apothecia on cup	0.6944579	0.580346	1.20	0.2393

* Significance was determined at $p < 0.05$

The concentration of fumarprotocetraric acid showed significant relationship with apothecium diameter in *C. gracilis* ssp. *turbinata* and *C. pyxidata* (Table 4.3). The concentration of usnic acid showed significant relationship with apothecium diameter in *C. cristatella*. No significant results for atranorin in *C. cariosa.*

4.4.4. *Relationship between soil features and canopy cover with secondary metabolites*

Usnic acid was negatively correlated with percent organic matter in the soil and canopy cover and it showed a positive relationship with the sand content for *C. cristatella* and *C. arbuscula* (Table 4.4). Fumarprotocetraric acid showed a negative relationship with percent organic matter and canopy cover but it showed a positive relationship with the sand content for *C. gracilis* ssp. *turbinata* and *C. pyxidata* (Table 4.4). However, atranorin did not show any significant relationships for *C. cariosa* and *C. rangiferina* (Table 4.4).

Table 4.4. A). Relationship between environmental characteristics (organic matter, canopy cover, sand) and each of the three polyketide concentrations (usnic acid, fumarprotocetraric acid, and atranorin) for six lichen species (*C. arbuscula, C. cristatella, C. pyxidata, C. gracilis* ssp. *turbinata, C. cariosa,* and *C. rangiferina*), using regression analysis showing the *F* statistic, degrees of freedom (d.f.), the R-squared value, and the *p*-value. B). The parameter estimate (with trend direction), std error, t-ratio and p-value between environmental characteristics (organic matter, canopy cover, sand) and each of the three polyketide concentrations Significance was determined at $p < 0.05$.

A. Pairwise comparison	***C. arbuscula***				***C. cristatella***			
Usnic acid	***F***	**d. f.**	***p***	**R^2**	***F***	**d. f.**	***p***	**R^2**
× Organic matter	12.6995	99	0.0377*	0.809	10.6053	99	0.0473*	0.779
× Canopy cover	13.5414	99	0.0167*	0.887	12.8022	99	0.0373*	0.810
× Sand	16.9157	99	0.0260*	0.849	20.7105	99	0.0199*	0.873
	C. pyxidata				***C. gracilis* ssp. *turbinata***			
Fumarprotocetraric acid	***F***	**d. f.**	***p***	**R^2**	***F***	**d. f.**	***p***	**R^2**
× Organic matter	24.8820	99	0.0068*	0.937	30.9751	99	0.0114*	0.911
× Canopy cover	31.9940	99	0.0052*	0.947	28.9194	99	0.0046*	0.951
× Sand	11.2131	99	0.0441*	0.789	33.9611	99	0.0101*	0.918
	C. cariosa				***C. rangiferina***			
Atranorin	*F*	d. f.	*p*	**R^2**	***F***	**d. f.**	***P***	**R^2**
× Organic matter	1.5887	59	0.4270	0.614	0.3233	59	0.6709	0.244
× Canopy cover	0.9910	59	0.5014	0.497	0.3745	59	0.6504	0.272
× Sand	0.5996	59	0.5805	0.375	0.0002	59	0.9919	0.0001

* Significance was determined at $p < 0.05$

B. Parameter estimate		*C. arbuscula*				*C. cristatella*			
Usnic acid	Term	Estimate	Std Error	t Ratio	Prob>|t|	Estimate	Std Error	t Ratio	Prob>|t|
Organic matter	Intercept	839.18543	106.5805	7.87	0.0043*	836.43037	81.10514	10.31	0.0019*
	OM	-19.2383	5.398532	-3.56	0.0377*	-13.37851	4.108151	-3.26	0.0473*
Canopy cover	Intercept	923.85903	94.21491	9.81	0.0023*	887.99632	86.49777	10.27	0.0020*
	canopy cover	-17.4982	3.606428	-4.85	0.0167*	-11.84691	3.311025	-3.58	0.0373*
Sand	Intercept	-608.0563	280.7596	-2.17	0.1189	-196.0462	182.2823	-1.08	0.3609
	sand	16.098499	3.914177	4.11	0.0260*	11.564994	2.541267	4.55	0.0199*

Parameter estimate		**C. pyxidata**				**C. gracilis ssp. turbinata**			
Fumarprotocetraric acid	Term	Estimate	Std Error	t Ratio	Prob>|t|	Estimate	Std Error	t Ratio	Prob>|t|
Organic matter	Intercept	734.66471	68.44724	10.73	0.0017*	806.96087	95.63148	8.44	0.0035*
	OM	-23.22684	3.467001	-6.70	0.0068*	-26.95911	4.843942	-5.57	0.0114*
Canopy cover	Intercept	817.6948	72.10898	11.34	0.0015*	912.01588	81.41976	11.20	0.0015*
	canopy cover	-20.28241	2.76024	-7.35	0.0052*	-23.92304	3.116645	-7.68	0.0046*
Sand	Intercept	-869.3239	372.7441	-2.33	0.1019	-1188.847	272.0329	-4.37	0.0222*
	sand	17.401179	5.196568	3.35	0.0441*	22.101314	3.792514	5.83	0.0101*

Parameter estimate		**C. cariosa**				**C. rangiferina**			
Atranorin	Term	Estimate	Std Error	t Ratio	Prob>|t|	Estimate	Std Error	t Ratio	Prob>|t|
Organic matter	Intercept	805.27928	162.844	4.95	0.1270	916.58167	711.3472	1.29	0.4202
	OM	-12.48775	9.907555	-1.26	0.4270	-17.37671	30.55939	-0.57	0.6709
Canopy cover	Intercept	826.06916	212.3043	3.89	0.1601	1042.9841	855.8097	1.22	0.4374
	canopy cover	-10.02893	10.07416	-1.00	0.5014	-17.74178	28.9929	-0.61	0.6504
Sand	Intercept	114.06204	719.4363	0.16	0.8999	525.1417	2603.915	0.20	0.8733
	sand	7.2383037	9.347405	0.77	0.5805	0.4952402	38.77902	0.01	0.9919

4.5. Discussion

4.5.1. *Relationship between secondary metabolites and morphological characters*

The negative relationship between each of fumarprotocetraric acid and usnic acid concentration with podetium height may suggest that the production of new thallus occurs at the expense of the production of these polyketides. Negative correlation is a result of trade-offs of resources for demand in expense on another (Rhoades 1979). The podetium height represents thallus growth in many *Cladonia* spp. and available carbon would be expected to produce new thallus tissue. An increased podetium height may be an important adaptation to ensure better wind dispersal of ascospores, which would make it an important adaptation. The morphological interpretation of the podetium is described by Ahti (1982) where it may be considered to be part of the thallus development in some species and the stipe of the ascoma in other species depending on the location of ascogonial development. If increased height of the podetium provides an advantage to the species, then a trade-off between secondary metabolite production and podetium growth would be expected. This relationship was present in *C. arbuscula* and *C. cristatella*, which supports the CNB hypothesis (Bryant et al. 1983; Hyvarinen et al. 2003) whereby an imbalance in the available nutrients needed for thallus growth allows the excess carbon to be used for secondary metabolites. However, if the lichen is in an environment with excess light, which is thought to trigger production of usnic acid (McEvoy et al. 2006; Armaleo et al. 2008), it would be expected that thallus growth, i.e. podetial height, would be reduced. The high concentration of usnic acid in southern locations (Spruce Woods and Sandilands) where the canopy cover was less than the other three locations (Table 2.3), showed the lowest podetial height suggesting a trade-off between quantity of usnic acid and production of new thallus.

The cup diameter in *C. pyxidata* also showed a negative relationship with fumarprotocetraric acid but not in *C. gracilis* ssp. *turbinata* suggesting that the cup growth may be more biologically relevant for *C. pyxidata* than for *C. gracilis* ssp. *turbinata.* For example, the aeroles in the *C. pyxidata* cup are thought to be dispersed by splash cup mechanism with rainwater (Bailey 1966; Bailey et al. 1968). The splash cup mechanism of dispersal is also known throughout the bryophytes and vascular plants (Brodie 1951; Brodie and Gregory 1953; Amador et al. 2013). If the mechanism is important for maintenance of the species it would be selectively retained in an optimal cup size and shape for those species in which it is important such as *C. pyxidata*. However, if they do not provide an important mechanism for the species, selection may be relaxed in terms of the size and shape as in *C. gracilis* ssp. *turbinata* which do not produce soredia or aeroles. On the other hand, the negative relationship with number of podetial branches in *C. gracilis* ssp. *turbinata* may indicate that the branches have an important role in its evolution. A function of fumarprotocetraric acid is thought to increase tolerance of lichens to acidic substrata (Hauck et al. 2009b) and it may play a role in herbivory (Hesbacher et al. 1995). In contrast, atranorin showed no relationship with the morphological characters examined and did not support the CNB hypothesis. A function of atranorin is thought to be related to protection of the algae from light and it has been shown to screen light by reflectance (BeGora et al. 2001; Solhaug et al. 2009; 2010), but a trade-off was not supported by the species examined in this study.

4.5.2. *Relationship between secondary metabolites and sexual fecundity characters*

The strong relationship between usnic acid and fumarprotocetraric acid concentrations with apothecium diameter in *C. cristatella, C. pyxidata* and *C. gracilis* ssp. *turbinata* suggested

that sexual reproduction may also occur at the expense of secondary metabolite production. Sexual reproduction provides an evolutionary advantage by increasing genetic variation among populations (Burger 1999). The size of the apothecia in *C. cristatella* and *C. pyxidata* from Sherridon (a northern location) was three to five times larger than those in southern locations (Spruce Woods and Sandilands). The larger size of the apothecia and number of apothecia per cup are indicators for high levels of fecundity. The low concentration of usnic and fumarprotocetraric acids in Sherridon suggests that high fecundity may occur at the expense of producing secondary metabolites. The northern locations in this study (Sherridon, North Star, and Athapap) appeared to have used more carbon (and balanced nutrient levels) for producing tissues in growth and reproduction than for secondary metabolite production in support of the CNB hypothesis. Since usnic acid was reported to occur near the algal cells and in the apothecia and pycnidia, supporting a UV protective role for usnic acid around the algae as well as the developing spores (Culberson et al. 1993; Liao et al. 2010), it might be expected that the relationship would have been a strong positive relationship. However, it is not known if the usnic acid present was mainly concentrated around the ascospores or elsewhere in the thallus or where the reduction in synthesis occurred.

Both *Cladonia gracilis* ssp. *turbinata* and *C. pyxidata* produce abundant apothecia. The negative relationship between apothecial diameter in *C. gracilis* ssp. *turbinata* and *C. pyxidata* with fumarprotocetraric acid concentration may suggest that the species producing fumarprotocetraric acid use carbon to produce bigger apothecia rather than produce more fumarprotocetraric acid. While the apothecia of *Cladonia* spp. may actually represent a conglomerate of many hymenial discs of tissue (Ahti 1982), a reasonable assumption is that larger apothecia result in larger number of ascospores produced. Fumarprotocetraric acid is

thought to play a role in the tolerance of *L. conizaeoides* to acidic soil (Hauck et al. 2009a, b) but there is no known light regulating role. Our results could not support the tolerance to soil pH because even though the soil in Sandilands and Spruce Woods was close to neutral, the fumarprotocetraric acid concentration was significantly higher than in Sherridon and North Star which had a lower pH (Zraik et al. 2018).

4.5.3. *Relationship between soil features, canopy cover and secondary metabolites*

This study provides support for the CNB hypothesis for usnic and fumarprotocetraric acid for the respective species, *Cladonia cristatella, C. pyxidata,* and *C. gracilis* ssp. *turbinata*. However, the CNB hypothesis was not supported for a relationship of atranorin with growth or reproduction in *C. cariosa*. The CNB hypothesis suggests that when a plant or lichen is growing in soils with an imbalance in nutrients, the available carbon may be converted to carbon-based secondary metabolites such as polyketides. When they are growing in soils with balanced nutrient levels, the carbon may be used in thallus growth rather than production of secondary metabolites. The positive relationship of percent sand content with each of usnic acid and fumarprotocetraric acid concentrations suggests that physical soil characteristics may also affect secondary metabolite production, which may be more relevantly explained by a negative correlation between percent sand content and organic matter.

The negative relationship between percent canopy cover and usnic and fumarprotocetraric acid concentrations suggests that light exposure has an effect on production of secondary metabolites. The role of usnic acid in UV light protection has been shown in a number of other studies (Culberson et al. 1993; McEvoy et al. 2006; Armaleo et al. 2008; Liao et al. 2010). While there has been no hypothesis proposed for a role for fumarprotocetraric acid in

light protection, the negative relationships shown for *C. pyxidata* and *C. gracilis* ssp. *turbinata* may represent an indirect effect if light affects humidity or other parameters. Previous studies on atranorin showed a role for atranorin in light protection by reflecting light and preventing photo inhibition (BeGora et al. 2001; Solhaug et al. 2009, 2010) and as an antioxidant (Gaikwad et al. 2012), but these relationships were not supported by this study.

In conclusion, this study provides evidence for the CNB hypothesis under certain conditions for *C. gracilis* ssp. *turbinata, C. pyxidata, C. cristatella,* and *C. arbuscula* by comparing the morphological, fecundity, and soil characteristics with secondary metabolite concentrations. Two secondary metabolites, usnic acid and fumarprotocetraric acid, supported the hypothesis by producing a negative relationship with the morphological and fecundity characteristics. Comparisons with the atranorin concentrations did not support the hypothesis. However, more studies are needed to explore causation of these relationships. This study also provided a mechanism to measure growth in *Cladonia* species and evidence that the podetial height in these species as well as cup diameter as in *C. pyxidata* have biologically and evolutionarily significant roles in the maintenance of the species. Neither the squamule size nor cup diameter in *C. gracilis* ssp. *turbinata* had significant implications. Similarly, the biological significance of larger apothecia also requires further study with respect to the capacity to produce more spores and viability of the spores. Overall, this study provides baseline measurements for future studies in addition to providing important insights into the biologically significant features in these species with respect to the CNB hypothesis.

4.6 Acknowledgements

The authors thank T. Lightly and M. Abdel-Hameed for assistance with HPLC analysis. This project was supported by a Faculty of Science award to MZ, GETS to MPN for MZ, an NSERC Discovery Grant to MPN, and collection permits from Manitoba Conservation and Parks Canada.

CHAPTER 5

Effects of environmental factors (temperature and humidity) on the production of secondary metabolites in lichens.

***This chapter will be submitted to an appropriate journal.**

5.1 Abstract

Secondary metabolites produced by lichens are found in different quantities within the same lichen species growing in similar habitats. The goal of this chapter was to explore whether humidity and temperature affects the stability of three secondary metabolites (atranorin produced by *C. cariosa,* fumarprotocetraric acid produced by *C. chlorophaea,* and usnic acid produced by *C. stellaris*) and to compare the results with a source of commercially produced secondary metabolite. Specimens were incubated in humid and dry conditions and in three temperatures (4°C, 18°C, and 32°C). High Performance Liquid Chromatography (HPLC) was performed at the beginning of the experiment to detect the concentration at time zero and four month intervals for one year. Results showed that high temperature and dry conditions had the greatest effect on degradation of secondary metabolites. A comparison of the HPLC peaks showed new peaks in both the experiments (commercial and lichen thallus sources) suggesting that a partial degradation of atranorin, usnic acid and fumarprotocetraric acid had occurred over time. Degradation may account for some of the variation reported in natural habitats. Turnover time of the secondary products in a lichen thallus would have implications for the amount of energy, nutrients, and carbon required for secondary metabolite production.

5.2 Introduction

Lichen-forming fungi produce 1,050 known secondary metabolites (Stocker-Wörgötter 2008) and many of these are polyketides. Polyketides are variable in structure and are produced by the successive condensation of small carboxylic acids, by a mechanism similar to that of fatty acid biosynthesis (Hopwood and Sherman 1990). The biosynthesis of polyketides takes place by consecutive reactions catalyzed by multidomain polyketide synthases (PKS) enzymes, which act on acetyl-CoA, propionyl-CoA, or butyryl CoA as the carbon building blocks to form the phenolic derivatives that make up these small molecules through the acetyl-polymalonyl pathway (Elix et al. 1996). Large numbers of variable polyketide structures are produced through this pathway, with each having different biological activities (Stocker-Wörgötter 2008; Nielsen et al. 2009). The PKS enzymes are encoded by PKS genes that sometimes occur as large numbers of paralogs in fungal genomes including *Cladonia grayi* (Armaleo et al. 2011) and *C. uncialis* (Abdel-Hameed et al. 2016). Fewer polyketides are produced than there are PKS genes in the genome. Non-lichenized fungi, such as *Aspergillus nidulans*, are also known to produce fewer numbers of polyketides than there are PKS genes (Sanchez et al. 2010) suggesting that the genes are silent and that plasticity may be present in PKS gene expression and biosynthesis of the polyketide. The many PKS paralogs present in fungal genomes are thought to be influenced by environmental conditions, but the function of the genes and the cues that trigger their expression are still unknown (Keller et al. 2008). While lichens inhabit almost every ecosystem, few studies have examined the ecological distribution of lichen secondary metabolites (Piercey-Normore 2007; Toni and Piercey-Normore 2013). If specific environmental conditions can trigger expression of some of the silent PKS genes (Gerke et al. 2012) novel polyketides may be

discovered that will improve our understanding of the ecology of lichens and they may be useful in biotechnology or pharmaceutical industries.

The functions of secondary metabolites in lichens are diverse and they are hypothesized to play roles in humidity control (Rundel 1978; Lawrey 1986; Culberson et al. 1992; Stocker Wörgötter 2001), light screening or herbivory (Gauslaa 2005; Nimis et al. 2006; Stocker-Wörgötter 2008). The production of grayanic acid in cultures of *Cladonia grayi* corresponded with the production of aerial hyphae of the lichen culture (Culberson et al. 1992) suggesting that lower humidity may play a role in triggering its biosynthesis. Some of the secondary metabolites produced by lichen-forming fungi are thought to serve as protection against excess sunlight by acting as a sun-screen in the cortical layer for UV light, which damages DNA and interferes with photosynthesis by lichen algae (McEvoy et al. 2006, 2007; Armaleo et al. 2008). The atranorin concentration in the cortex of *Parmotrema hypotropum* was positively correlated with the number of light hours per year, while norstictic acid showed a negative correlation with number of light hours and was thought to be influenced by humidity changes within the thallus (Armaleo et al. 2008). Fumarprotocetraric acid levels varied within an ecological transect that extended from higher elevations in the Appalachian Mountains to lower elevations along the North Carolina coast. It was surmised that fumarprotocetraric acid provided a protective role for the lichen against environmental conditions such as temperature, drought and UV level (Culberson et al. 1977a). Higher usnic acid concentration was correlated with high humidity sites in the lichen *Flavocetraria nivalis* (Bjerke et al. 2004). These and other studies have shown correlations between an environmental parameter and changes in polyketide concentration, (Fox et al.2008; Armaleo et al., 2008; McEvoy et al., 2006, 2007; Bjerke et al., 2002; Rundel, 1969) but little is known about the causative effect of the environment in lichens. More information is available for

non-lichenized fungi (Sanchez et al. 2008; Hoffmeister et al. 2007; Worgotter et al. 2004). Secondary metabolites are not limited to lichens living in extreme conditions, but are also produced by lichens in temperate boreal regions. High levels of polyketides produced by temperate boreal lichens is explained by the slow growing nature of lichen thalli in general and exposure to changing conditions over prolonged periods of time (e.g., Hamada et al. 1982). Other conditions that influence secondary metabolite production may include nutrient availability and pH of the substrate (Fox and Howlett 2008; Timsina et al. 2013). The pH level of the soil was hypothesized to lead to morphological differences between two morphotypes of *C. pyxidata* and *C. pocillium* (Gilbert 1977; Kotelko et al. 2010). If the role of secondary metabolites in the natural lichen thallus is related to environmental conditions as suggested by these studies, then the secondary metabolites would either be expected to be stable over time and different conditions or they would be expected to have short turnover times while the thallus maintains a sufficient quantity for protection.

While evidence supports the concept that environmental factors trigger the biosynthesis of polyketides, the chemical stability of polyketides may also be affected by environmental conditions such as temperature (Culberson et al. 1977c; Hamada et al. 1982), light (Quilhot et al. 1992), or ozon gas level (Quilhot et al. 1996). Atranorin is considered a thermally unstable depside (Culberson et al. 1977b) and its concentration was correlated with temperature changes (James et al. 1991). A depside is a polyphenol composed of two or more monocyclic aromatic units linked by an ester bond. Studies on the decomposition of secondary metabolites have focused on the thermopyrolosis (the pyrolosis of compounds under very high temperatures to break large molecules into smaller molecules) (Irwin 1982; Mauzelaar et al. 1982; Saiz-Jimenez et al. 1991). Secondary metabolites produced by *C. rangiferina* were degraded completely after

drying the lichen thalli at 85°C, and for *C. mitis* and *U. muhlenbergii* at 80°C (Culberson et al. 1977b). Therefore, it is expected that higher temperatures would have a greater effect on the degradation of polyketides.

The objectives of this study were 1) to test the effects of three temperatures and two humidity levels on the production of three secondary metabolites produced by lichen thalli under controlled lab conditions (*in vivo*) over different periods of time, and 2) to test the stability of pure commercial products of three secondary metabolites (*in vitro*) under the same experimental conditions. The hypothesis is that high temperature and humidity will decrease the quantity of secondary metabolites over time, more than low temperature and less humidity.

5.3 Materials and Methods

5.3.1 *Experimental design and sample collection*

For the *in vivo* experiment, three lichen species (*C. cariosa, C. chlorophaea* and *C. stellaris*) were selected because they are common in Manitoba and many thalli grow within close vicinity of one another to reduce effects from possible genetic variation. They were collected from Sherridon Road (N54° 77’ 72.9” W101° 47’ 37.5”), which is composed of exposed bedrock outcrops with Jack pine, aspen, birch, white spruce, willow and alder. The three species that were chosen produce atranorin (*C. cariosa*), fumarprotocetraric acid (*C. chlorophaea*) and usnic acid (*C. stellaris*). A total of 18 thalli for each lichen species consisted of three replicates for each of the moisture (with and without water) and temperature treatments (4°C, 18°C and 32°C) resulting in a total of 54 thalli that were tested.

5.3.2 *Testing the stability of in vivo secondary metabolites*

Each lichen thallus was placed into a glass jar containing filter paper on the bottom (Whatman; WHA10010155). Glass jars were completely covered with aluminum foil to prevent any light from entering the jar. The thalli in nine jars were watered with distilled autoclaved water and thalli in another nine jars were left dry. The 9 jars were divided into 3 groups: the first group was incubated at 4°C and watered with 5 mL of water every 45 days; the second group was incubated at 18°C and watered with 7.5 mL water every 30 days; and the third group was incubated at 32°C and watered with 10 mL water every 15 days. Since evaporation was high at 32°C, the volume and frequency of adding water was increased. A sample of the thallus stalk (i.e. podetium) from each jar was taken at each of four time periods of zero days, 120 days, 240 days, and 360 days ensuring that the weight of each sample was the same each time.

For HPLC analysis, the lichen thallus was finely crushed with a ceramic mortar and pestle, weighed, and the secondary metabolites extracted with acetone. The extraction process was repeated six times to ensure all secondary metabolites were removed from the thallus sample. The acetone extract was then filtered by cotton filters, the filtrate was dried, and the vial was weighed again to calculate the weight of the filtered compound. A low concentration from diluting the sample (50 µg/ml) was prepared for each sample to run on HPLC. HPLC analysis was carried out on Waters HPLC Separations Module 2695, combined with a PDA Detector Model 2996. The column was a µBondapak® Waters C18 (3.9 X 300 mm) with a column particle diameter of 15-20 µm, and with 125 Å pores. The flow rate was 1 mL/minute. The eluent was monitored continuously at 210-600 nm, and HPLC traces were displayed at 254 nm. The gradient was held at 20 % methanol to 80 % water in 0.075 % aqueous trifluoracetic acid for 10 minutes then the percent of methanol was linearly increased up to 80 % and held at that

composition for 20 minutes followed by a linear gradient back to 20 % methanol for 10 minutes and held there for 10 minutes. The total run time was 60 minutes. Standards of usnic acid, atranorin, and fumarprotocetraric acid (ChromaDex, USA) were used as references for this experiment. Samples for running on HPLC were collected four times as described above. The final dry weight of the extract was calculated in micrograms per milligram of dried chemical (See methods 4.3.3). In order to prepare the sample for HPLC, it was dissolved in a known volume of acetone, mixed and macerated using a sonicator (BRANSON 3510). Dilutions of known concentrations were prepared which were run on HPLC to produce a calibration curve.

5.3.3 *Testing the stability of in vitro secondary metabolite standards*

For the *in vitro* experiment, three standards of secondary metabolites (atranorin, fumarprotocetraric acid and usnic acid) (ChromaDex, USA) were prepared by dissolving 1 mg of each compound in 1 mL of acetone and subjecting them to the same procedure as above. These commercial products were used to prepare the calibration curve for the three secondary metabolites in order to calculate the concentrations. The commercial standards were added to HPLC vials for HPLC analysis as indicated above. The acetone was allowed to evaporate and the compounds were placed in glass jars and subjected to the same experimental conditions as in the *in vivo* experiment with moist and dry treatments, and three temperatures (4°C, 18°C, and 32°C) using three replicates for each treatment. Results were recorded at each of four time periods of zero days, 45 days, 90 days, and 135 days and changes in concentration were calculated depending on the calibration curve equation for each of atranorin, fumarprotocetraric acid and usnic acid.

5.3.4 *Data analyses*

In order to test whether the secondary metabolite degrades over time, a one-way ANOVA compared the means of polyketide concentrations for each of the four time periods of the *in vivo* and *in vitro* experiments. To test the effect of temperature and humidity, and whether there was any statistical interaction on the production of secondary metabolites, a two-way ANOVA compared means of percent loss after the end of each of the *in vivo* and *in vitro* experiments. A one-way ANOVA compared the means of percent loss between the three polyketides for each treatment. To determine whether secondary metabolite degradation was affected more as a metabolite within thallus or as a pure metabolite outside the thallus, a one-way ANOVA compared the means of loss between the two experiments. The percent loss refers to the difference between the concentration of the compound at the beginning of the experiment (zero days) and the concentration at the end of experiment (after 360 days or 135 days for the *in vivo* or *in vitro* experiment, respectively) divided by the concentration of the compound at zero days. It is calculated using the equation where percent loss = Concentration (zero days) - Concentration (last day of experiment) / Concentration (zero days) * 100. Tukey's Honestly Significant difference (HSD) test was used to test significant differences at $p = 0.05$.

5.4 Results

5.4.1 *Change in concentration of polyketides in vivo*

The *in vivo* experiment showed the trends in secondary metabolite concentration loss over time (Fig. 5.1). The differences between temperatures within each condition showed similar downward trends over time. The in vivo experiment showed similar trends in secondary metabolites concentration loss over time (Fig. 5.1). The differences between temperatures within

each condition showed similar downward trends over time. Variation in the concentration at day 0 may have resulted from the natural variation in the thallus. The concentration of usnic acid from *C. stellaris*, varied from 25 to 95 ug/mg at day 0 (Fig. 5.1 A and B); of fumarprotocetraric acid from C. chlorophaea, varied from <5 to 120 ug/mg (Fig.5.1 C and D); and for atranorin from C. cariosa, it varied from 55 to 95 ug/mg at day 0 (Fig. 5.1 E and F). The one-way ANOVA test results for Figures 5.1 and 5.2 are in appendix G., where we try to test whether there are significant differences in concentration of polyketide between the four times of the experiment. Dry conditions with high temperature of 32°c showed significant loss in the concentration of the three polyketides (Fig. A, C, and E). Moist conditions showed more stability for the polyketides between the time 0 (0 day) and time 4 (360 days) (Fig. B, D, F).

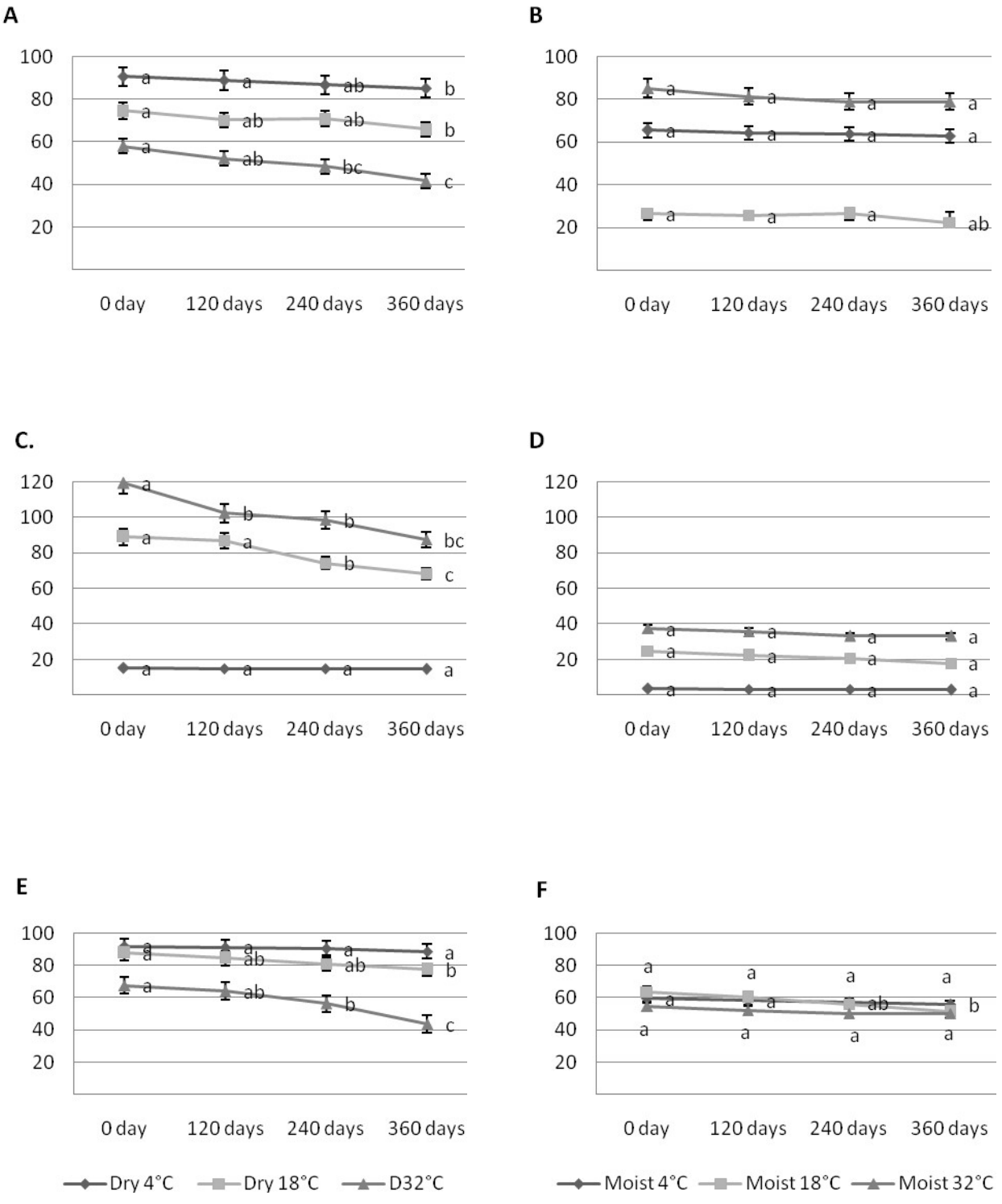


Figure 5.1. Changes in quantity of polyketide Y axis (µg/mg) in the lichen thallus (*in vivo* experiment) over time for each temperature (4°C, 18°C and 32°C), and dry and moist conditions

showing A) usnic acid from *C. stellaris* in the dry treatment; B) usnic acid from *C. stellaris* in the moist treatment; C) fumarprotocetraric acid from *C. chlorophaea* in the dry treatment; D) fumarprotocetraric acid from *C. chlorophaea* in the moist treatment, E) atranorin from *C. cariosa* in the dry treatment, and F) atranorin from *C. cariosa* in the moist treatment. Day 0 is the concentration of polyketide at the beginning of the experiment and is different for each treatment because of natural variation. The vertical bars are standard error and the different lower case letters indicate significant differences over time within each treatment at $p = 0.05$ using Tukey's HSD test.

5.4.2 *Change in concentration of polyketides in vitro*

In general, the percent loss of all polyketides seemed to be similar for both dry and moist conditions at the same temperature conditions. 0 day is the concentration of polyketide at the beginning of the experiment and is different for each polyketide because of different polyketide standard preparation for each experiment. More usnic acid was present at 4°C in dry and moist conditions (except day 0 in 4°C dry) and the smallest amount was at 32°C in the moist condition but the lines were overlapping in the dry condition (Fig. 5.2 A and B). The amount of fumarprotocetraric acid overlapped in the dry conditions but the smallest amount was in the moist condition at 18°C (Fig.5.2 C and D). The amount of atranorin was greatest at 18°C in dry conditions and the smallest amounts at 32°C in moist conditions (Fig. 5.2 E and F).

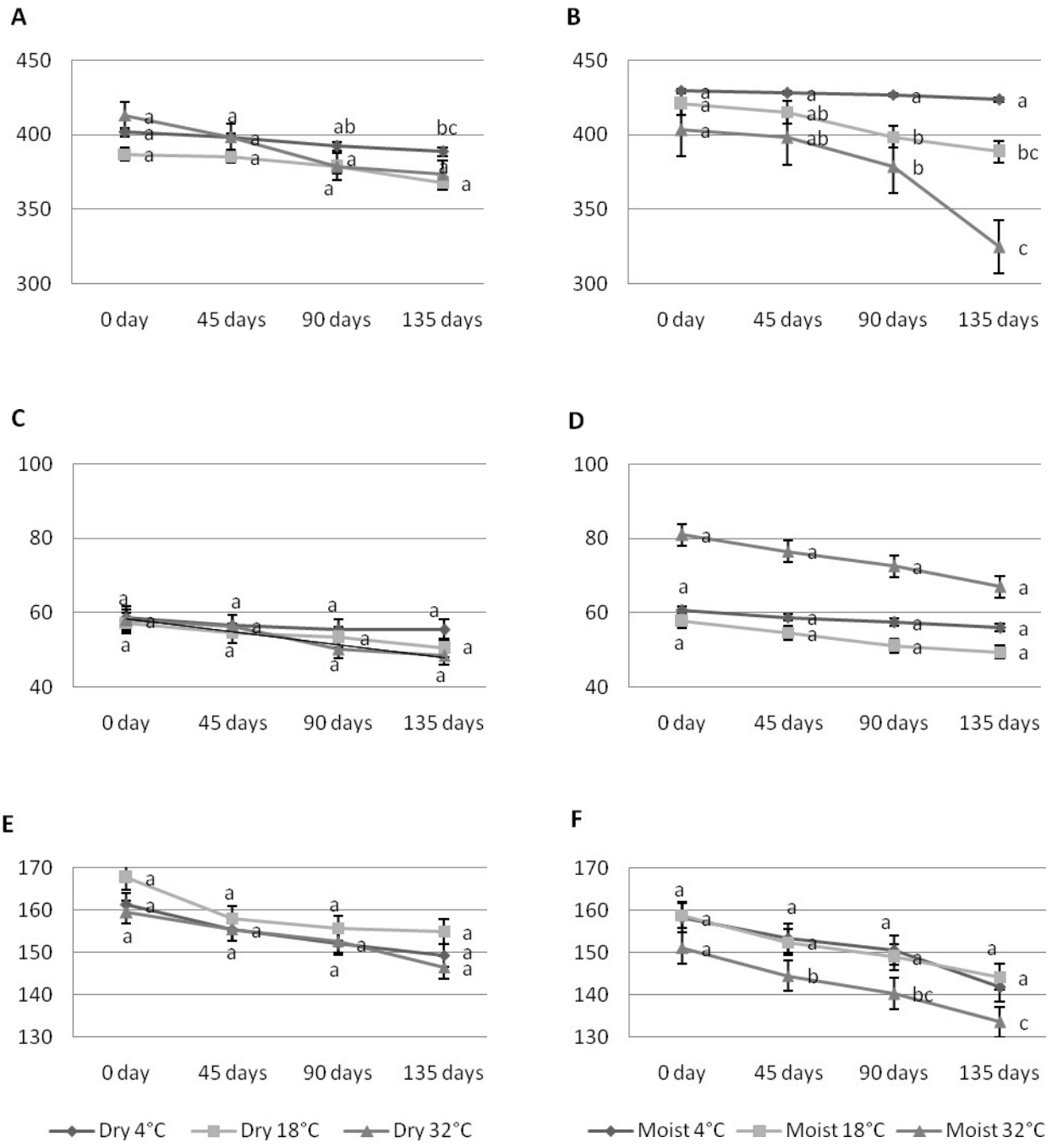


Figure 5. 2. Change in concentration of polyketide Y axis (ug/mg) as the pure commercial standards solvent (*in vitro* experiment) over time for each temperature (4°C, 18°C and 32°C), under dry and moist conditions, showing A) usnic acid in the dry condition, B) usnic acid in the moist condition, C) fumarprotocetraric acid in the dry treatment, D) fumarprotocetraric acid in the moist condition, E) atranorin in the dry condition, and F) atranorin in the moist condition. The vertical bars are standard error

and the different lower case letters indicate significant differences over time within each treatment at p = 0.05 using Tukey's HSD test.

5.4.3 *Loss of secondary metabolites*

For the *in vivo* experiment, fumarprotocetraric acid was the least stable of the three polyketides, which is recorded to have four most significant losses out of six treatments (Fig. 5. 3A). Atranorin had a significant loss in one treatment (dry at 32°C). Usnic acid had the lowest percent loss in only one of the six treatments (moist at 4°C; Fig. 5.3A). For the *in vitro* experiment, fumarprotocetraric acid had the greatest loss in three of the six treatments (Fig. 5.3B) but neither atranorin nor usnic acid had significant losses (Fig. 5.3B).

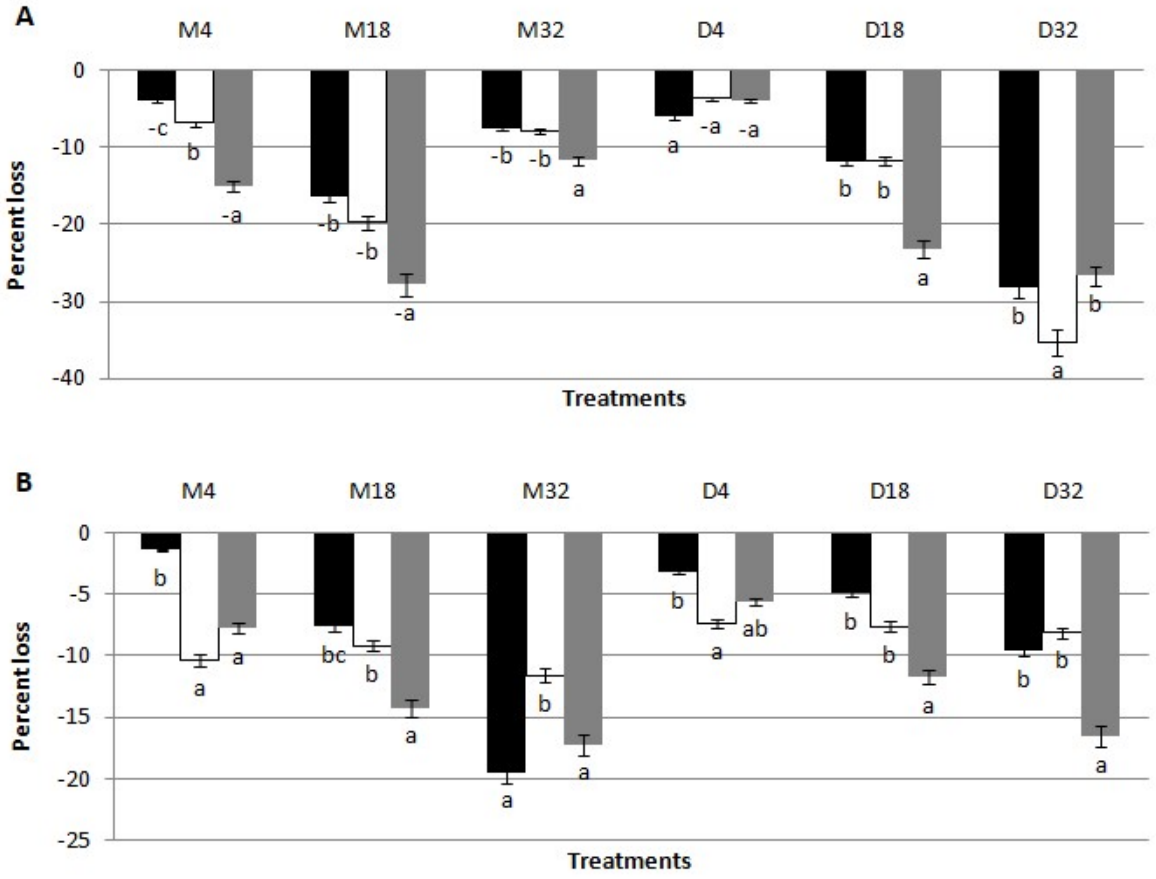


Figure 5.3. Comparison of percent loss of secondary metabolites for A) the *in vivo*, and B) the *in vitro* experiment showing (usnic acid as the black bar, atranorin as the white bar, and fumarprotocetraric acid as the gray bar) within each of the treatements (D is dry; M is moist) and three temperatures (4°C, 18°C and 32°C) using one-way ANOVA. The error bars represent the standard error (SE) from three replicates; different lower case letters indicate significant differences between polyketides within clustered treatments at p=0.05 using the Tukey's HSD test.

5.4.4 *Loss of secondary metabolites between in vivo and in vitro and under same treatment condition*

The percent loss of each secondary metabolite from the lichen thallus (*in vivo*) (after 120 days of incubation) and the commercial standard (*in vitro*) (after 135 days of experiment incubation) was calculated to allow for a similar period of time between the two experiments (*in vivo* and *in vitro*). In general, the percent lost from lichen thallus was less than the loss of the standard with two exceptions (fumarprotocetraric acid at 4°C in moist conditions and usnic acid at 4°C in dry conditions) (Fig 5.4). A second trend is that the percent loss was generally greater in the high temperature treatments than the low temperature treatments (Fig. 5.4). The one-way ANOVA (Appendix H) compared the percent loss of each polyketide in the dry/moist condition with the source (*in vitro* vs. *in vivo*).

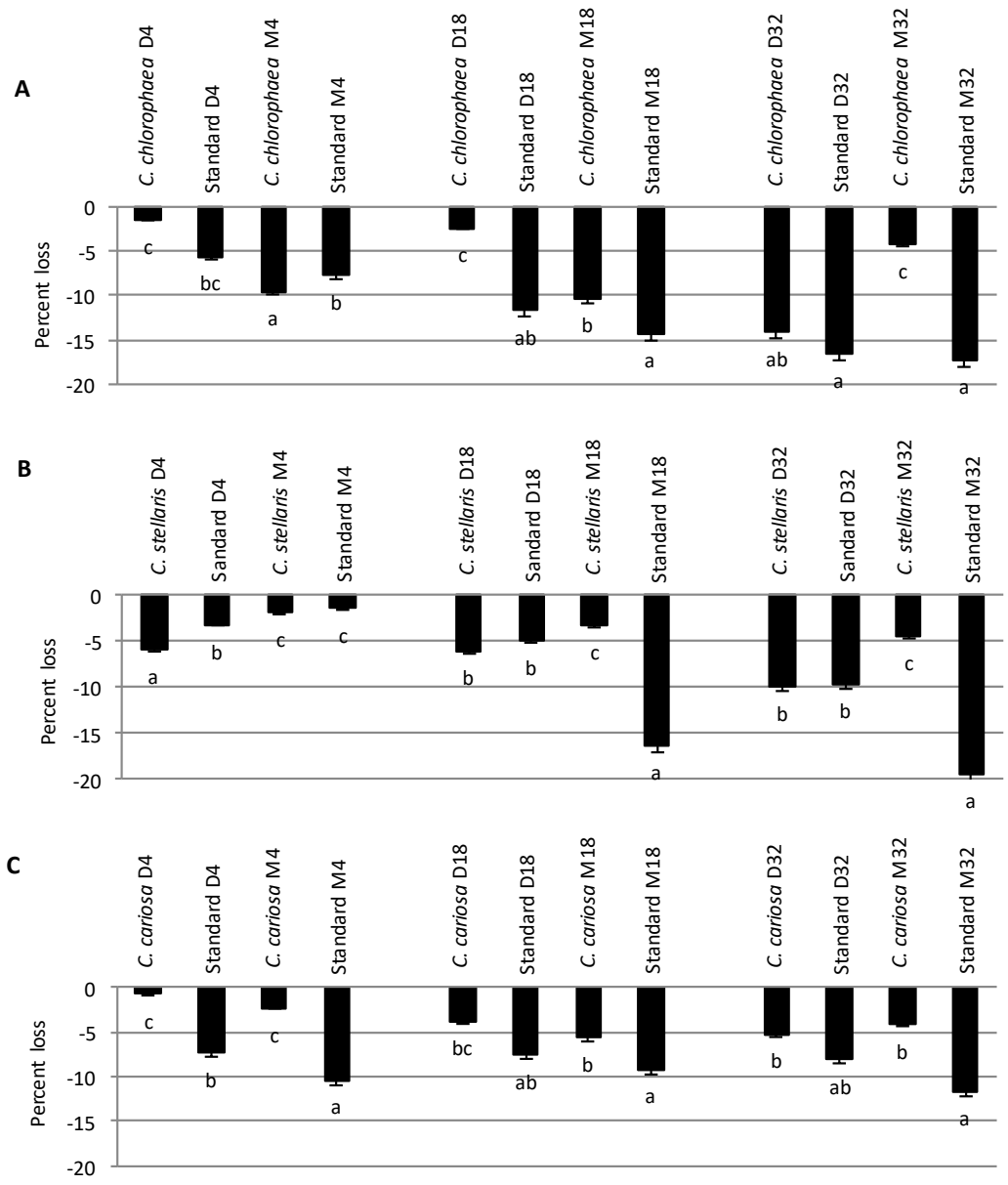


Figure 5.4. Comparison of the percent loss of polyketides from lichen thallus (*in vivo*) and from the commercial standards (*in vitro*) for each of the three temperatures (4°C, 18°C and 32°C) under dry (D) and moist (M) conditions. The percent loss was calculated over 120 days (*in vivo*) and 135 days (*in vitro*) showing A) fumarprotocetraric acid, B) usnic acid and C) atranorin. The error bars represent the standard error (SE) from three replicates; different lower case letters indicate significant differences within clustered temperature treatments at p=0.05 using the Tukey's HSD test.

5.4.5. *Synergistic effect of temperature and humidity on percent polyketide loss*

The effect of temperature and humidity on the percent loss for lichen secondary metabolites was examined by two-way ANOVA. The results showed a significant interaction between temperature and humidity for the percent loss of usnic acid for both *in vivo* and *in vitro* experiments (Table 5.1). The temperature x humidity interaction was significant for the *in vivo* conditions for atranorin, but not for the *in vitro* experiment, which showed only humidity to be significant. The temperature x humidity interaction was significant for fumarprotocetraric acid in the *in vivo* experiment but there was no significant interaction in the *in vitro* experiment. Humidity and temperature had individual effects in the *in vitro* experiment (Table 5.1).

Table 5.1. Effect of temperature and humidity on percent loss of secondary metabolites (usnic acid, atranorin, and fumarprotocetraric acid) over time for each of the *in vivo* (within lichen thallus) and *in vitro* (commercial standard) experiments based on two-way ANOVA, showing *F* statistic, degrees of freedom (d.f.), and *p*-value with significance at $p \leq 0.05$.

Usnic acid	**d.f.**	***F****	***p***
In vivo			
Temperature	2	70.42	**<0.0001***
Humidity	1	44.28	**<0.0001***
Temperature*humidity	2	68.90	**<0.0001***
In vitro			
Temperature	2	94.30	**<0.0001***
Humidity	1	47.90	**<0.0001***
Temperature*humidity	2	43.26	**<0.0001***
Atranorin	**d.f.**	***F***	***p***
In vivo			
Temperature	2	75.21	**<0.0001***
Humidity	1	23.92	**0.0004***
Temperature*humidity	2	101.33	**<0.0001***
In vitro			
Temperature	2	2.16	0.1574
Humidity	1	19.15	**0.0009***
Temperature*humidity	2	0.67	0.5294
Fumarprotocetraric acid	**d.f.**	***F***	***p***
In vivo			
Temperature	2	30.41	**<0.0001***
Humidity	1	0.02	0.8891
Temperature*humidity	2	21.39	**0.0001***
In vitro			
Temperature	2	94.26	**<0.0001***
Humidity	1	10.31	0.0075*
Temperature*humidity	2	1.02	0.3895

* The means between the two factors (secondary metabolites with temperature, humidity and interaction between them) are significant

5.4.6 *Degradation of secondary metabolites over time*

5.4.6.1. *Usnic Acid*

The amount of usnic acid produced by *Cladonia stellaris* at 32°C in dry conditions showed a retention time of 24.1 minutes with six additional peaks representing unknown compounds (Fig. 5.5 A). Usnic acid had a retention time (24.1 min) and UV spectra (λmax 232.6 and 280.9 nm) identical to that of a standard sample of usnic acid (ChromaDex). After 120 days two additional peaks were detected, which were not detected at time 0 (Fig. 5.5 B). After each of 240 and 360 days there were only six additional peaks (Fig. 5.5 C and D).

The amount of commercial usnic acid standard at 32°C in dry conditions showed a retention time of 21.8 minutes at zero days and UV spectra (λmax 232.6 and 280.9 nm) (Fig. 5.5 E). At 45 days, there were two new peaks present at 18.7 minutes and at 19.5 minutes with the usnic acid peak at 21.0 minutes (Fig. 5.5 F). Two peaks could also be detected, in addition to usnic acid, in each of the 90 and 135 day time periods (Fig. 5.5 G and H).

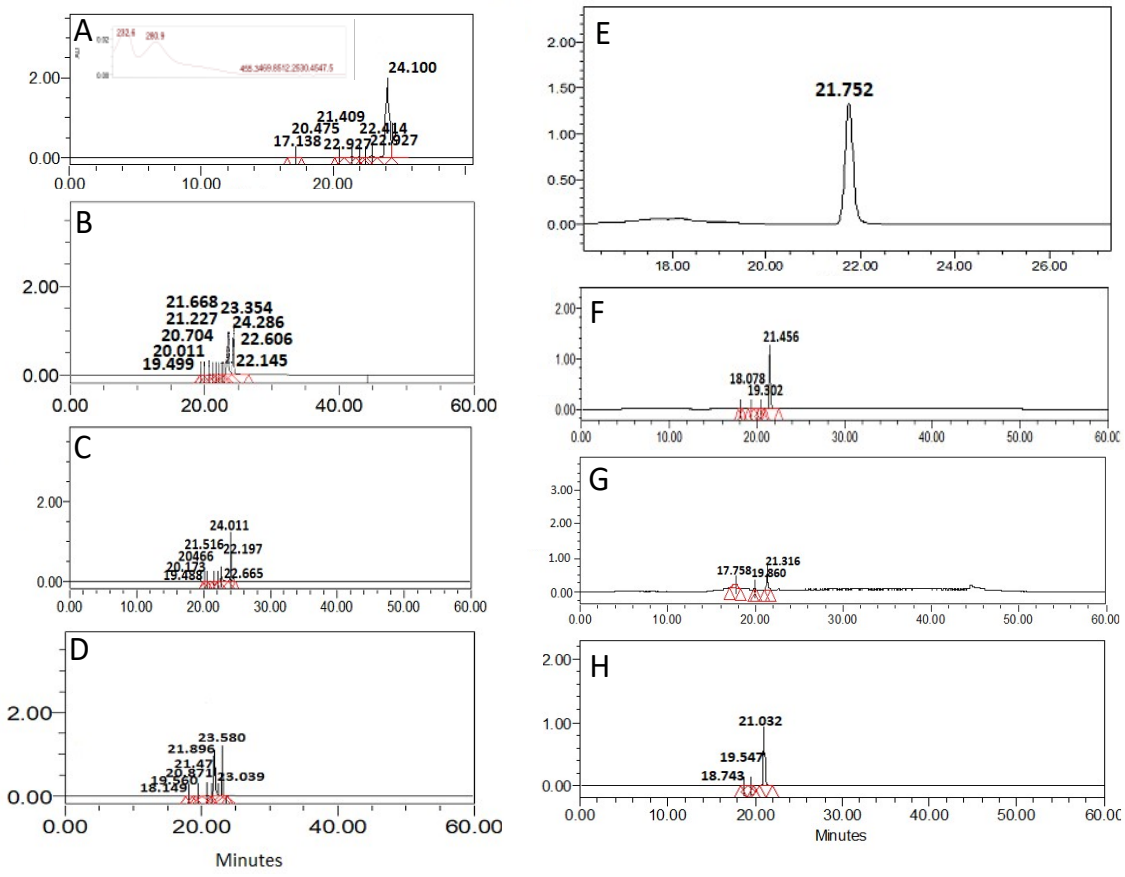


Figure 5.5. HPLC chromatograms of usnic acid from A-D) *Cladonia stellaris* (*in vivo* experiment) and E-H) the commercial standard (*in vitro* experiment) at 32°C and dry conditions showing four time periods at A) zero days showing one peak at 24.1 minutes for usnic acid and 6 other unknown peaks, and the insert with the UV absorbance at 254 nm (λmax 232.6 and 282 nm), B) 120 days with the usnic acid peak at 24.3 minutes and eight other unknown peaks, C) 240 days with an usnic acid peak at 24.0 minutes and six other unknown peaks, D) at 360 days with an usnic acid peak at 23.6 minutes and six other unknown peaks, E) zero days showing one peak at 21.7 minutes for usnic acid, and the insert with the UV absorbance at 254 nm (λmax 232.6 and 280.9 nm), F) 45 days with the usnic acid peak at 21.0 minutes and two other unknown peaks, G) 90 days with an usnic acid peak at 21.3 minutes and two other unknown peaks, and H) at 135 days with an usnic acid peak at 21.0 minutes and two other unknown peaks.

5.4.6.2. *Fumarprotocetraric acid*

The fumarprotocetraric acid produced by *Cladonia chlorophaea* at 32°C in dry conditions showed a retention time of 24.3 minutes at time zero (Fig. 5.6A), 21.5 minutes at 120 days (Fig. 5.6B), 21.4 minutes at 240 days (Fig. 5.6C), and 20.8 at 360 days (Fig. 5.6D). Two new peaks appeared in addition to the peak for fumarprotocetraric acid at 120 and 240 days, and a third new peak appeared at 360 days.

The fumarprotocetraric acid commercial standard at 32°C in dry conditions showed a retention time of 20.7 and UV spectra (λmax 212.6, 239.6, and 317.7 nm) at time zero (Fig. 5.6E), 20.4 minutes at 45 days (Fig. 5.6F), 20.2 minutes at 90 days (Fig. 5.6G), and 20.2 minutes at 135 days (Fig. 5.6H). Two new peaks appeared in addition to the peak for fumarprotocetraric acid at each of 45, 90 and 135 days.

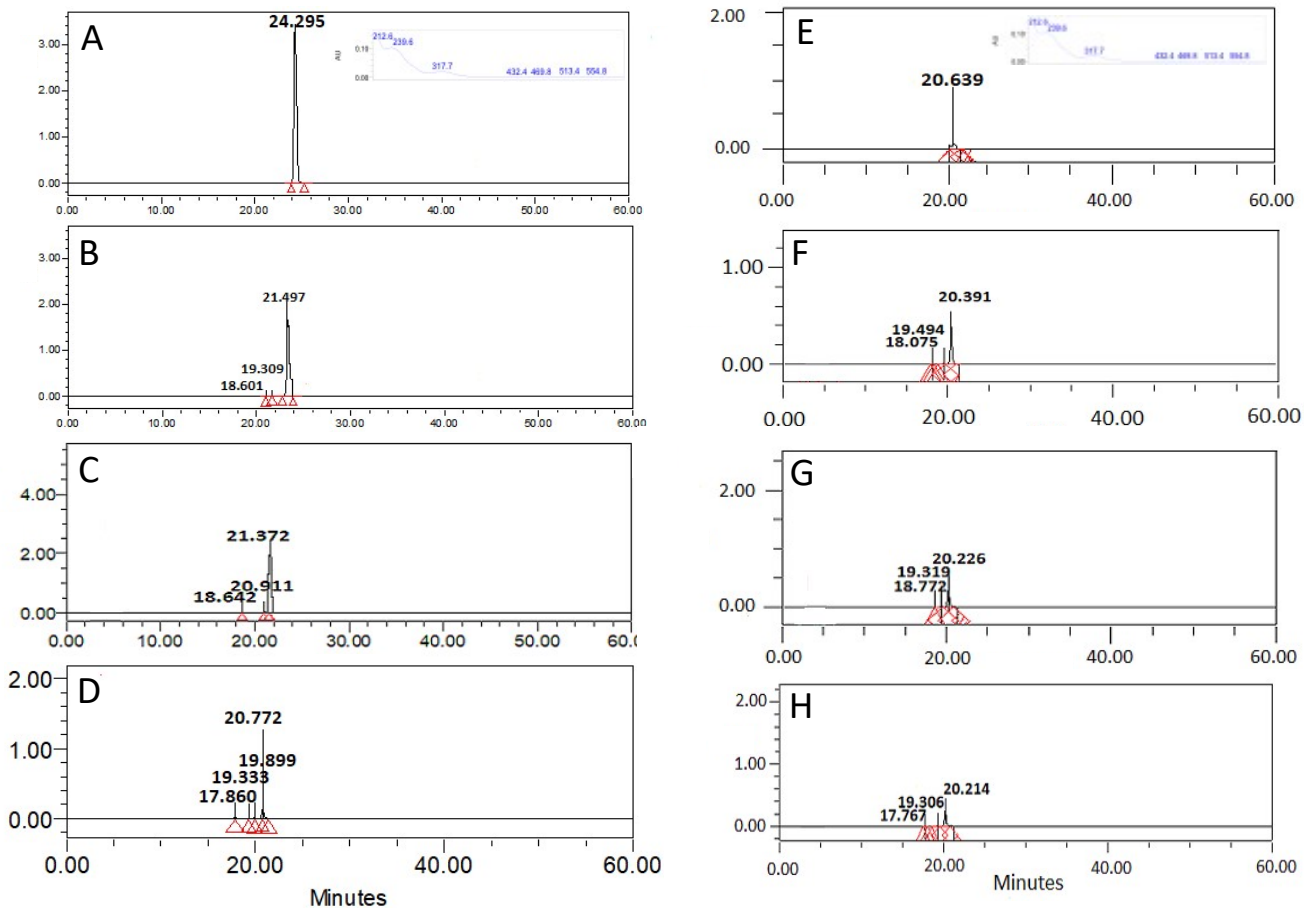


Figure 5.6. HPLC chromatograms of fumarprotocetraric acid from A-D) *Cladonia chlorophaea* (*in vivo* experiment) and E-H) the commercial standard (*in vitro* experiment) at 32°C and in dry conditions showing four time periods at A) zero days showing one peak at 24.3 minutes for fumarprotocetraric acid, and the insert with the UV absorbance at 254 nm (λmax 212.6 and 317.7 nm), B) 120 days showing two additional peaks, C) 240 days showing two additional peaks, D) 360 days showing three additional peaks, E) zero days showing one peak at 20.6 minutes for fumarprotocetraric acid, and the insert with the UV absorbance at 254 nm (λmax 212.6 and 317.7 nm), F) 45 days with the fumarprotocetraric acid peak at 20.4 minutes and two other unknown peaks, G) 90 days with an fumarprotocetraric acid peak at 20.2 minutes and two other unknown peaks, and H) at 135 days with an fumarprotocetraric acid peak at 20.2 minutes and two other unknown peaks.

5.4.6.3. *Atranorin*

The atranorin produced by *Cladonia cariosa* at 32°C in dry conditions showed a retention time of 24.1 minutes at time zero (Fig. 5.7A), 21.0 minutes at 120 days (Fig. 5.7B), 22.8 minutes at 240 days (Fig. 5.7C), and 21.3 at 360 days (Fig. 5.7D). Nine additional peaks appeared in addition to the peak for atranorin at 120 days and six new peaks appeared at each of 240 days and 360 days.

The amount of atranorin commercial standard at 32°C in dry conditions showed a retention time of 21.9 minutes and UV spectra (λmax 251.4 and 320.1 nm) at time zero (Fig. 5.7E), 21.7 minutes at 45 days (Fig. 5.7F), 21.505 minutes at 90 days (Fig. 5.7G), and 21.5 minutes at 135 days (Fig. 5.7H). Two new peaks appeared in addition to the peak for atranorin at each of 45, 90 and 135 days.

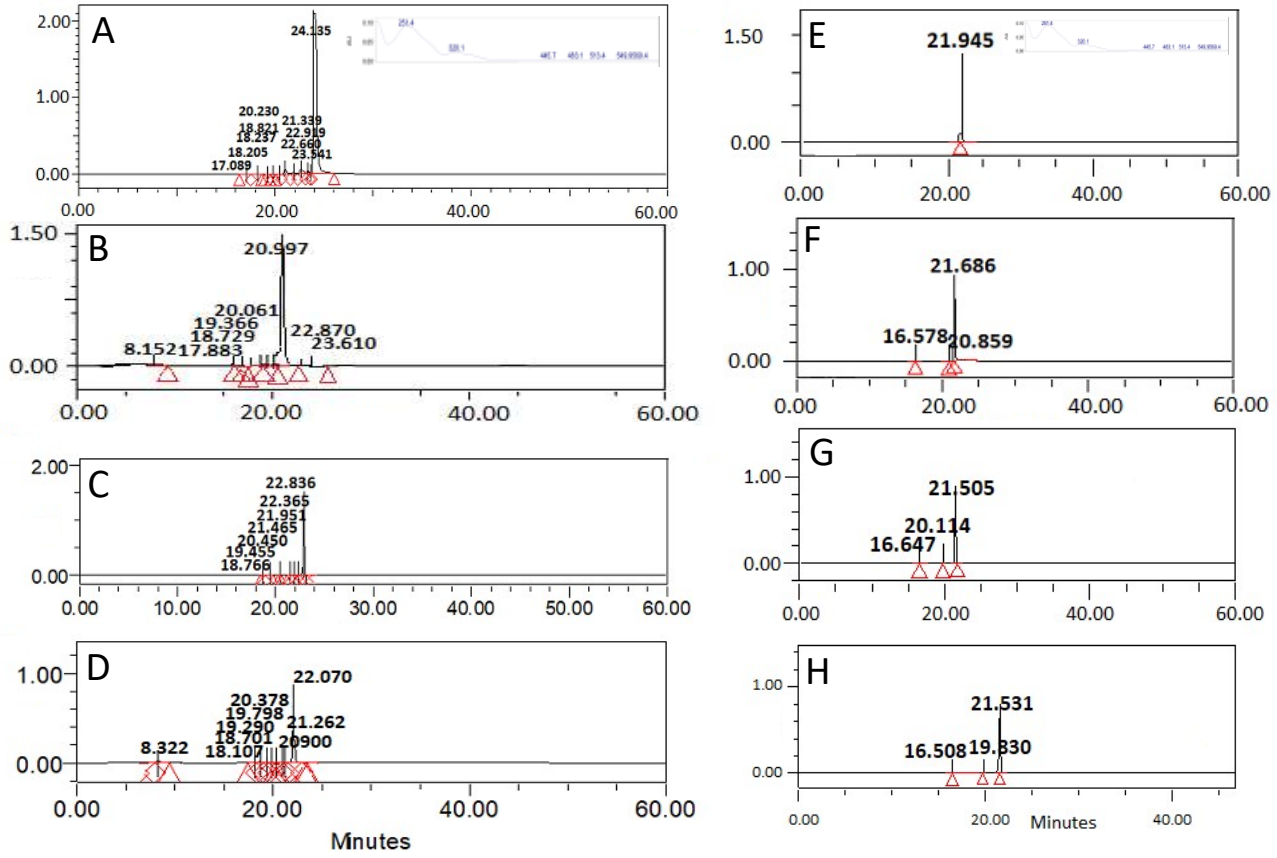


Figure 5.7. HPLC chromatogram of atranorin from A-D) *Cladonia cariosa* (*in vivo* experiment) and E-H) the commercial standard at 32°C and in dry conditions showing four time periods at A) zero days showing one peak with retention time of 24.1 minutes for atranorin, nine additional peaks for unknown compounds, and the insert with the UV absorbance at 254 nm (λmax 251.4 and 320.1 nm), B) 120 days showing a peak for atranorin with retention time of 21.0 minutes, and six additional peaks, C) 240 days showing a peak for atranorin with retention time of 22.8 minutes, and six additional peaks, and D) 360 days showing a peak for atranorin with retention time of 21.3 minutes, and six additional peaks, E) zero days showing one peak for atranorin with a retention time of 21.9 minutes, and the insert with the UV absorbance at 254 nm (λmax 251.4 and 320.1 nm), F) 45 days with the atranorin peak at 21.7 minutes retention time and two other unknown peaks, G) 90 days with an atranorin peak at 21.5 minutes retention time and two other unknown peaks, and H) at 135 days with an atranorin peak at 21.5 minutes retention time and two other unknown peaks.

5.5 Discussion

The stability of three secondary metabolites in this study decreased with higher temperatures supporting the hypothesis that high temperature will decrease the quantity of secondary metabolites over time. However, moisture seemed to affect fumarprotocetraric acid and atranorin more than usnic acid. This suggests that higher temperatures (ie. 18°C and 32°C) would be sufficient to degrade the molecular stucture of these compounds even within a 45 day time period. The rates of degradation varied but was greatest at 32°C and most stable at 4°C. While the loss of secondary metabolites increased with higher temperatures, higher temperatures may result from high levels of light in nature. Since light was eliminated in this experiment, it did not influence the temperature but in nature it may have an effect on temperature or a direct effect on secondary metabolite production (Rundel 1969; Quilhot et al. 1991; BeGora et al. 2001; McEvoy et al. 2006; Nybakken et al. 2006; Armaleo et al. 2008). Other studies have demonstrated the degradation of secondary metabolites under harsher conditions than were used in this study. For example, the thermal decomposition of atranorin was shown to occur at 230°C when exposed for 15 minutes (Huneck et al. 1989), at 85°C for 22 hours, or at 105°C for 15 minutes for atranorin and anziaic acid (Culberson et al. 1977b). The results from this study suggest that the practice of high temperature drying of samples at 40°C in preparation for herbarium storage (Mirando et al. 1977) may result in degradation of some polyketides. It is thought that depsides such as atranorin may split at the ester bond in high temperatures (250°C; Huneck et al. 1968), and the thermal decomposition of atranorin produced orcinol, 5-orcinol, and 4-O-demethylbarbatol (Huneck et al. 1989). These phenolic compounds could combine with some derivatives such as *p*-methan to produce new active cannabionl derivatives. The new peaks

in this study that were detected after the incubation period of the samples and the standards revealed that some of usnic acid, fumarprotocetraric acid and atranorin may have degraded producing new compounds that could be detected by HPLC analysis. Alternatively, the extra peaks from the thallus samples may be additional metabolites that were present in small quantities. Further studies would determine the identity of the compounds and their chemical structures.

The percentage of secondary metabolite lost differed depending on whether they were exposed as pure commercial products or present within a lichen thallus. Higher temperatures of 18°C and 32°C resulted in more degradation of fumarprotocetraric acid as a commercial standard than in the lichen thallus. Usnic acid and atranorin showed a similar trend but only in the moist conditions. If more loss occurs when the polyketide is alone than in the lichen thallus (Fig. 4), the thallus may help to protect the molecule against degradation. Thallus tissues have layers of organized cells and the existence of secondary metabolites within these tissue layers may give the secondary metabolites protection against temperature and drought conditions (Büdel et al. 2008). The presence of some pigments like melanin which have protective functions (Solhaug et al. 2003) may protect the cells from exposure to excess sunlight and the pigments may also protect the polyketides. The HPLC traces showed that other molecules were present at 32°C in dry conditions over the time period of the study suggesting that degradation of the polyketides may have occurred. The conditions that result in greater loss of the compound, might suggest that the lichen must undergo a higher turnover rate of the compound in order to maintain a constant amount in the thallus, which would result in the lichen putting more energy into production of the metabolite. But if the temperature is lower (i.e. 4°C) the turnover rate would be shorter because of less degradation of usnic acid.

The three secondary metabolites differed in their stability where fumarprotocetraric acid was the most unstable of the three secondary metabolites and usnic acid was more stable than atranorin. The molecular structure of fumarprotocetraric acid contains more hydroxyl groups and is prone to more hydrolysis than the other two molecules with more thermochemolysis products for fumarprotocetraric acid (PubChem Compound Database; CID=5317419; MacGillivary 1999). The molecular structure of different polyketides results in different rates of degradation which may be affected differently by different environmental parameters. The difference in structure and rate of degradation might suggest that the lichen expends more energy for biosynthesis of some polyketides than others. In general, usnic acid and atranorin are more stable under different sunlight intensities and light and shaded sites (Stephenson et al. 1979). Degradation of some polyketides may be useful as a source of carbon for bioactivity. Usnic acid content in the thallus of *C. mitis* was lower in summer and spring, and the highest content was in autumn and winter (BeGora et al. 2001). Summer and spring seasons may be more appropriate for carbon as it is needed for growth and respiration, which could be explain the excessive use of polyketides in their study. The poor nutrient conditions may have shunted carbon to produce more polyketides than thallus tissues but the environmental and seasonal timing could also play a role to increase or decrease the production of polyketides.

In conclusion, the loss of secondary metabolites that was recorded in these experiments showed that temperature and moisture affect the stability of these compounds, but the extent of their effects depend on the type of metabolite. The variation in quantity reported in other studies have focused on the biosynthesis of the polyketides but the degradation of the polyketides may also play an important role in the natural variation. The results also have implications for processing and herbarium storage of lichen specimens. Heating may not be the best approach if

secondary metabolites are the focus of the research in the herbarium. The results also raise interesting questions about the turnover time of the secondary products in a lichen thallus. Turnover time would have implications for the amount of energy, nutrients, and carbon required for secondary metabolite production and therefore explain the variable results reported for the carbon-nutrient balance hypothesis in many studies. It might also suggest that individuals of the same species that are present at different latitudes with temperature optima would be expected to produce, or maintain, different amounts of the same secondary metabolite. Overall, this study showed a significant decrease in stability of atranorin, fumarprotocetraric acid and usnic acid with increasing temperature which is well within the range of typical air temperatures in the boreal regions and it raised questions for further consideration around the expense of secondary products by lichen fungi. Finally, further work needs to be done to identify the new compounds that were formed from the hypothesized partial decomposition of the secondary metabolites. This research may also lead to a better understanding of their biosynthesis.

5.6 Acknowledgements

The authors thank T. Lightly (University of Manitoba) for assistance in HPLC analysis. This project was supported by a Faculty of Science award to MZ, GETS to MPN for MZ, and an NSERC Discovery Grant to MPN, and collection permits from Manitoba Conservation and Parks Canada.

CHAPTER 6

Final Discussion and Conclusion

The study of chemical ecology in lichens is best represented by a model that shows the close connection between biotic and abiotic factors that affect secondary metabolite production in lichens. The model in Fig. 6.1 shows some of the trade-offs through the carbon-nutrient balance hypothesis (CNBH; Bryant et al. 1983). The model shows a complex connection of networks with inputs (biotic and abiotic factors) and biological activity (growth, fecundity and secondary metabolite production) of the system representing the lichen thallus, which illustrates the linkages between lichens and the surrounding ecosystem. The biotic factors include the presence of other lichen species that influence the degree of humidity maintained in the microhabitat or herbivores that feed on the lichens. The abiotic factors include the environmental and terrestrial factors such as temperature, sunlight, water, atmospheric gases, and soil properties. The trade-off between an essential component of the lichen (thallus growth, secondary metabolite production, and fecundity) and growth or secondary metabolite production depends on the balance between nutrient levels and the availability of carbon. This model was designed using the genus *Cladonia*: however, it may be applied to a wider range of lichen species. This thesis examined questions relevant to this model.

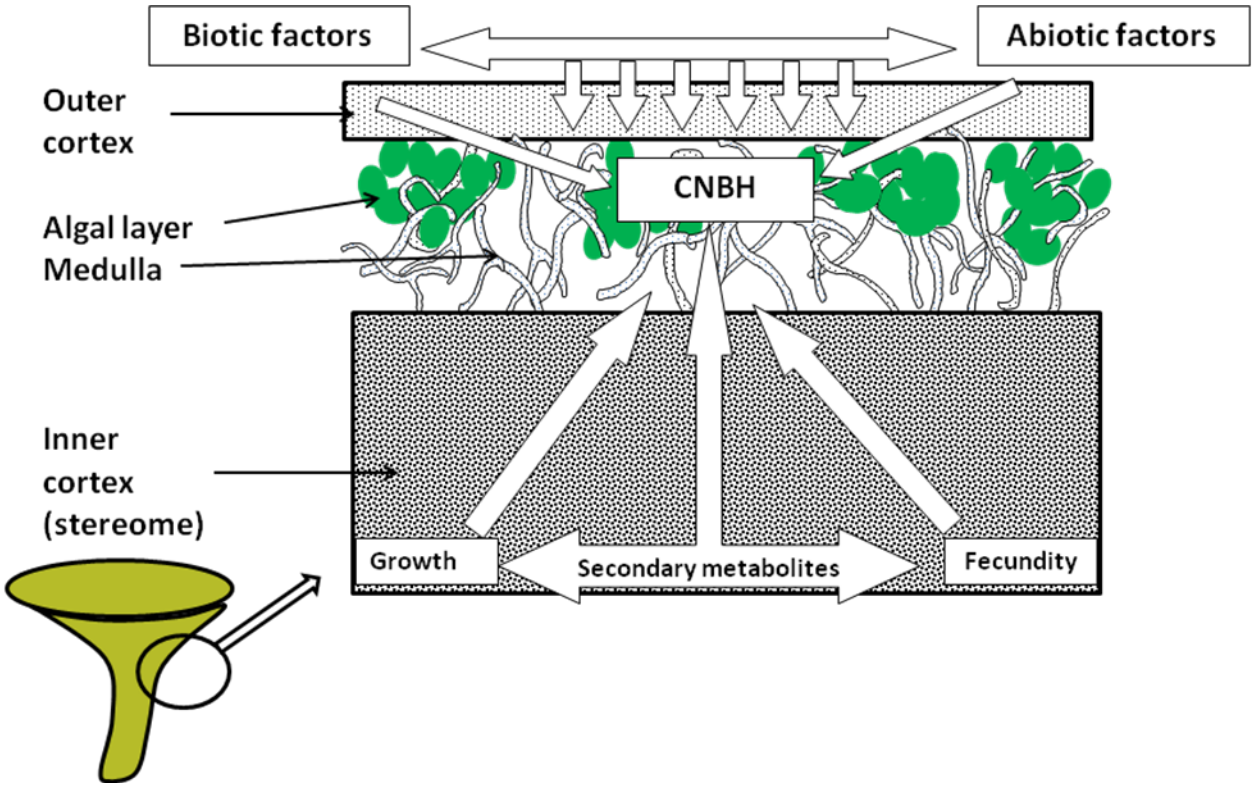


Fig. 6.1. A model representing the relationship between the biotic and abiotic factors that influence the carbon-nutrient balance hypothesis (CNBH). The thallus growth, fecundity and secondary metabolites are the main carbon consumers that affect the CNBH in lichens. When an outer cortex is present, it forms a partial barrier to external conditions. The medullary hyphae maintain moisture and air spaces for photosynthesis by the algal layer. The stereome forms support for the podetium to stand erect from a basal substratum. See text for more detail.

The general goal of this thesis was to examine the CNB hypothesis in lichens by exploring the chemical ecological and macromorphological variability in lichens that colonize sandy soils in five locations in southern and northern Manitoba. The CNBH in lichens was addressed by examining the distribution of secondary metabolites and species assemblages with respect to soil characteristics (Chapter 2), which resulted in a description of new species reports for Manitoba and the description of diagnostic features for a group of species that are directly attached to soil (Chapter 3). The results from Chapter 2 led to questions around plasticity in morphological features with respect to environmental parameters (Chapter 4) and the direct effect of temperature and moisture on polyketide production and degradation (Chapter 5).

If lichens are directly attached to soil, any soil characteristics that affect nutrient transfer and moisture retention would be expected to influence thallus growth, secondary metabolite production and fecundity. In Chapter 2, the significant relationship between species assemblages and rounded sand particles may indicate that the angular shape of the sand particles may minimize the spaces between sand particles but the round shape may allow for more water and air movement between particles (Lipiec et al. 2016). In sandy soil with a higher content of angular particles, the smaller spaces between particles would make the soil heavier and lose water more slowly (Lipiec et al. 2016). The association of *Cladonia cariosa, C. humilis, C. magyarica* and *Peltigera rufescens*, with round sand grains; and the association of *Cladonia amaurocraea, C. deformis, C. stellaris, C. uncialis* and *Stereocaulon tomentosum*, with angular sand grains may suggest functional relationships related to their morphology. The mat of rhizines in *P. rufescens* and the persistent basal squamules in *C. cariosa, C. humilis,* and *C. magyarica* would be in more direct contact with the soil and be more influenced by soil conditions than tall podetia with disintegrating basal squamules such as in *C. amaurocraea, C. deformis, C. stellaris,*

C. uncialis, and *Stereocaulon tomentosum*. The finding that *C. magyarica* and *C. humilis* were relatively common on the sandy soils in Spruce Woods Provincial Park was somewhat unexpected. Neither species had previously been reported in Manitoba even though they occurred in neighbouring states (http://lichenportal.org/portal/taxa/index.php?taxon=53432) and provinces (Brodo et al. 2013). This finding led to reporting their presence and a more detailed description of the morphology in Chapter 3.

Chapter 2 also showed that some secondary metabolites corresponded with soil conditions. The presence of usnic acid was significantly higher in the more acidic sites of Sherridon and North Star (Table 2.6), where usnic acid may help the lichen tolerate acidic substrata (Hauck et al. 2009**a**). The presence of atranorin in lichen species in the less acidic sites, such as Spruce Woods Provincial Park and to a lesser extent Athapap (Table 2.5), may reflect the higher pH levels in the habitat. However, the pH of the soil alone does not seem to influence the type of secondary metabolite produced by lichens in this study. A combination of soil pH, water retention properties, light availability, genetics of the species, and other factors may have a synergistic effect on secondary metabolite production. A more accurate approach to examining polyketide production is to measure the effect of environmental conditions on the quantity, rather than presence and absence, of the metabolite. This led to hypotheses that soil conditions may influence quantity of secondary metabolite production in Chapter 4.

The soil conditions, which seem to influence some lichen species and not other species, can be represented by the geological history of Manitoba. The extent of weathering may be determined by the shape of the sand grains where rounded sand grains may reflect the glacial activity in the region (Cho et al. 2006). The highest percent of rounded sand grains in the Sandilands Provincial Forest revealed that this location may have had the highest degree of

weathering conditions compared to other locations. Geologically, the Sandilands Provincial Forest was buried under deep water of the glacial Lake Aggasiz before the water disappeared over thousands of years, and formed the layer of sandy till covering lake clay currently in the area (Lee 1968). Since angular and large sand grains have had less erosion on the grain than round and fine sand grains (Cho et al. 2006), the percentage of rounded sand grains in the Spruce Woods Provincial Park was expected to be higher than reported because the sand was thought to be washed heavily from the Rocky Mountains for long periods of time (Huck and Whiteway 2013). However, Spruce Woods Provincial Park had the largest amount of fine sand grains, which may also represent erosion of the sand grains. Rounded sand grains were the second highest in Athapap of the five locations, which was located under shallow water seas and remnants of Lake Agassiz (Johnston 1946). The weathering caused by this ancient lake explains the relatively high content of eroded sand grains in Athapap compared to Sherridon and North Star. Sherridon and North Star are dominated by exposed ridges of volcanic lava flows and intrusive rock (Brownell and Kinkel 1935), which was subjected to less weathering because of the absence of a long-term aquatic environment resulting in angular sand grains as the dominant shape.

The results of Chapter 2 showed that soil features, including the weathering of sand grains, pH, and organic matter levels, influenced the species assemblage of lichens that rely on soil for their nutrients. Variability in soil features would presumably also affect growth of the lichen and therefore the consumption of carbon by thallus tissue. Since polyketides are carbon-based and are thought to have protective functions in nature (Huneck et al. 1999; Liao et al. 2010), there may be a trade-off between production of polyketides and growth of thallus tissue. This led to the hypothesis that when more thallus growth or reproduction has occurred there will

be a smaller amount of carbon-based secondary metabolite produced because the carbon has been used in biomass production (Chapter 4). The northern locations in this study (Sherridon, North Star, and Athapap) appeared to have used more carbon for producing tissues in growth and reproduction than for secondary metabolite production in support of the CNB hypothesis. The polyketides, usnic acid and fumarprotocetraric acid, supported the CNB hypothesis by producing a negative relationship with the morphological and fecundity characteristics, while atranorin did not support the hypothesis. There are very few studies that show the relationship between secondary metabolites and vegetative growth in lichens (Bidussi et al. 2013). Studies on this topic are more common for vascular plants, which show that plants in poor nutrient conditions tend to produce more carbon-based secondary metabolites and fewer nitrogen-based compounds (Shigo et al. 1973; Mckey et al. 1979; Gartlan et al. 1980). With low growth rates, the high content of secondary metabolites played a deterrent role against herbivores.

Chapter 4 focused on environmental conditions that influenced the production of polyketides, but the degradation of polyketides was not taken into account. This led to a hypothesis that changes in environmental conditions such as temperature or humidity may lead to the loss of polyketides. Chapter 5 examined how temperature and humidity could have an effect on stability and the loss or the increase in concentration of polyketides in the lichen thallus. Chapter 5 showed a significant decrease in the stability of atranorin, fumarprotocetraric acid and usnic acid with increasing temperature. It also showed the presence of unknown compounds in HPLC analysis, which may have resulted from partial degradation of the polyketides. Lichen secondary metabolites are thought to be stable compounds but may be completely degraded under very high temperatures (Culberson et al. 1977b; Huneck et al. 1989) of 230°C. However, slightly higher temperatures, such as 40°C, could partially degrade them

(Mirando et al. 1977). These results were consistent with those of Chapter 5 where atranorin, fumarprotocetraric acid, and usnic acid showed significant degradation with a high temperature of 37°C and dry conditions. If degradation occurs at these temperatures, which are relatively common temperatures in nature, the lichen must have a turnover rate to produce more polyketide in order to maintain the level found within the thallus.

In conclusion, the results of this thesis show relationships between secondary metabolites (concentration and presence/absence) and the morphological, fecundity and soil characteristics, which contributed to an understanding of the role of the CNB hypothesis in lichens. Two out of three compounds supported the CNB hypothesis. The findings provided important insights into the biologically and evolutionarily significant features in these species with respect to the CNB hypothesis. It highlights the relationship between the lichen thallus and the soil characteristics for certain species and it showed that a combination of factors (biotic and abiotic) that affected morphology and polyketide production. The thesis provided new reports of two species for Manitoba (*Cladonia magyarica* and *C. humilis*) and distinguished among their close allies. Further study is needed to understand degradation of polyketides, and the new products produced from degradation. The algal selectivity is the productive partner in lichens with respect to carbon fixation and may also affect the CNB hypothesis.

Future directions

The model describing the effects of ecosystem factors (biotic and abiotic) on the CNB hypothesis shows effects on the biological activities (growth, fecundity) and these activities could increase or decrease the production of secondary metabolites by the presence of available carbon that is necessary in all bioactivity. In addition, since only three polyketides are tested

under the CNB hypothesis in this thesis, questions are raised about other polyketides such as zeorin and barbatic acid which showed a strong relationship with acidic soil. Further study is needed to address these questions. An experiment is needed to identify the unknown HPLC products that were produced from the hypothesized degradation of secondary metabolites by using NMR and other analytical techniques. The effect of light, as a factor in addition to temperature and moisture, on the degradation of secondary metabolites is unknown and potentially would play a large role in polyketide degradation. The elimination of light from an experiment might stop the production of new carbon-based molecules because of the prevention of photosynthesis, which is thought to be the main source of carbon-based molecules for the synthesis of polyketides. If light was added to the experiment, photosynthesis would result in more carbon-based molecules and polyketide synthesis would be expected to increase or at least become stabilized, compensating for the partial degradation observed during this experiment. An experiment is also needed to test the fecundity levels of lichens with secondary metabolite production by comparing the germination percent (or other fecundity measures) with the concentration of polyketides produced.

LITERATURE CITED

Abdel-Hameed, M., Bertrand, R.L., Piercey-Normore, M.D., and Sorensen, J.L. 2016. Putative identification of the usnic acid biosynthetic gene cluster by de novo whole-genome sequencing of a lichen-forming fungus. Fungal Biology 120(3): 306–316.

Ahti, T. 1961. Taxonomic studies on reindeer lichens (*Cladonia*, subgenus *Cladina*). Annales Botanici Fennici Vanamo 32(1): 1–160.

Ahti, T. 1966. Correlation of the chemical and morphological characters in *Cladonia chlorophaea* and allied lichens. Annales Botanicci Fennici 3: 380–389.

Ahti, T. 1977. Lichens of the boreal coniferous zone. *In* M. R. D. Seaward (ed.): Lichen Ecology. Academic Press. London, pp. 145-181.

Ahti, T. 1980. Nomenclatural notes on *Cladonia* species. Lichenologist 12: 125–133.

Ahti, T. 1982. The morphological interpretation of cladoniform thalli in lichens. Lichenologist 14(2): 105-113.

Ahti, T. 2000. *Cladoniaceae*, Flora Neotropica Monograph 78. New York Botanical Garden. New York.pp. 1-362.

Ahti, T., and DePriest, P.T. 2001. New combinations of *Cladina* epithets in *Cladonia* (Ascomycotina: Cladoniaceae) Mycotaxon 78: 499–502.

Ahti, T., and Stenroos, S. 2013. Cladoniaceae. *In*: Ahti, T., Stenroos, S., and Moberg, R. (eds.), Nordic Lichen Flora, Volume 5. pp. 7-87.

Álvarez-Cansino, L., Zunzunegui, M., Barradas, M.C.D., and Esquivias, M.P. 2010. Gender-specific costs of reproduction on vegetative growth and physiological performance in the

dioecious shrub *Corema album*. Annals of Botany 106: 989–998.

Amador, G.J., Yamada, Y., McCurley, M., and Hu, D.L. 2013. Splash cup plants accelerate raindrops to disperse seeds. Journal of the Royal Society Interface. 10: 20120880. http://dx.doi.org/10.1098/rsif.2012.0880

Andrews, J.T., Barry, R.G. 1978. Glacial inception and disintegration during the last glaciation. Annual Review of Earth and Planetary Sciences 6: 205–228.

Aptroot, A., Sipman, H.M., and van Herck, C.M. 2001. *Cladonia monomorpha*, a neglected cup lichen from Europe. Lichenologist 33: 271–283.

Arculus, R.J., Ishizuka, O., Bogus, K., and the Expedition 351 Scientists. 2015. Izu-Bonin-Mariana arc origins: continental crust formation at an intra-oceanic arc: foundation, inception, and early evolution. International Ocean Discovery Program Preliminary Report, 351.

Armaleo, D., Zhang, Y., and Cheung, S. 2008. Light might regulate divergently depside and depsidone accumulation in the lichen *Parmotrema hypotropum* by affecting thallus temperature and water potential. Mycologia 100: 565–576.

Armaleo, D., Sun, X., and Culberson, C.F. 2011. Insights from the first biosynthetic gene cluster for a lichen depside and depsidone. Mycologia 103(4): 741–754.

Armstrong, R.A. 1993. Factors determining lobe growth in foliose lichen thalli. New Phytologist 124(4): 675–679.

Armstrong, R.A., and Welch, A.R. 2007. Competition in lichen communities. Symbiosis 43: 1–12.

Asplund, J., Solhaug, K., and Gauslaa, Y. 2010. Optimal defense: snails avoid reproductive parts of the lichen *Lobaria scrobiculata* due to internal defense allocation. Ecology 91: 3100–

3105.

Athukorala, S.N.P., Pino-Bodas, R., Stenroos, S., Ahti, T., and Piercey-Normore, M.D. 2016. Phylogenetic relationships among reindeer lichens of North America. Lichenologist 48(3): 209–227.

Bačkor, M., Ivanova, V., Laatsch, H., Lokajová, V., and Bačkorová, M. 2013. Allelopathic effects of lichen secondary metabolites on photobiont *Trebouxia erici.* Allelopathy Journal. 31: 189–198.

Bailey, R.H. 1966. Studies on the dispersal of lichen soredia. Jour. Linn. Soc. Bot. 59: 479–490.

Bailey, R.H., and Garrett, R.M. 1968. Studies of the discharge of ascospores from lichen apothecia. Lichenologist 4: 57–65.

Bailey, K.A., and Syme, E.C. 1989. Geology of the Flin Flon-White Lake area. Geological Report GR87-1, Manitoba Energy and Mines, Government of Manitoba, Winnipeg, Manitoba.p. 313.

Baloch, E., Lücking, R., Lumbsch, H.T., and Wedin, M. 2010. Major clades and phylogenetic relationships between lichenized and non-lichenized lineages in Ostropales (Ascomycota: Lecanoromycetes). Taxon 59(5): 1483–1494.

Bannatyne, B.B. 1975. High-calcium limestone deposits of Manitoba. Mineral Resources Division Publication 75-1, Province of Manitoba Department of Mines, Resources, and Environmental Management, Winnipeg, Manitoba.p.103.

Baquerizo, M.D., Maestre, F.T., Rodríguez, J.G.P., and Gallardo, A. 2013. Biological soil crusts promote N accumulation in response to dew events in dryland soils. Soil Biology and Biochemistry 62: 22–27.

Barger, N.N., Castle, S.C., and Dean, G.N. 2013. Denitrification from nitrogen-fixing biologically crusted soils in a cool desert environment, southeast Utah, USA. Ecol Process 2: 16. Online. https://doi.org/10.1186/2192-1709-2-16.

Benton, J., and Jones, J.R. 1999. Soil Analysis Handbook of Reference Methods. CRC Press, NY.pp. 27-37, 231.

Beckett, R.P., Kranner, I., and Minibayeva, F.V. 2008. Stress physiology and the symbiosis. In Lichen Biology, 2nd edition, ed. T. H. Nash, pp. 40-68. Cambridge: Cambridge University Press.

Behera, N. 1997. Effect of phenotypic plasticity on adaptation and evolution: a genetic algorithm analysis. Current Science 73: 968–976.

BeGora, M.D., Fahselt, D. 2001. Usnic acid and atranorin concentrations in lichens in relation to bands of UV irradiance. Bryologist 104: 134–140.

Belnap, J. 1996. Soil surface disturbances in cold deserts: effects on nitrogenase activity in cyanobacterial-lichen soil crusts. Biology and Fertility of Soils 23: 362–367.

Belnap, J., Rosentreter, R., Leonard, S., Kaltenecker, J.H., Williams, J., and Eldridge, D. 2001. Biological Soil Crusts: Ecology and Management. Technical reference 1730-2. U.S. Department of the interior.

Belnap, J., and Eldridge, D.J. 2003. Disturbance and recovery of biological soil crusts. Pages 364–383 in J. Belnap, and O. L. Lange, editors. Biological soil crusts: structure, function, and management. Springer-, Berlin, Germany.

Belnap, J., and Lange, O.L. 2003. Biological soil crusts: structure, function, and management. Ecological studies 150. Springer, Berlin.

Bidussi, M., Goward, T., Gauslaa, Y. 2013. Growth and secondary compound investments in the epiphytic lichens *Lobaria pulminaria* and *Hypogymnia occidentalis* transplanted along an altitudinal gradient in British Columbia. Botany 91: 621–630.

Bignell, E., Negrete-Urtasun, S., Calcagno, A.M., Haynes, K., Arst, H.N., and Rogers, T. 2005. The *Aspergillus* pH-responsive transcription factor PacC regulates virulence. Mol. Microbiol. 55(4): 1072–84.

Bjerke, J.W., Elvebakk, A. 2004. Distribution of the lichen genus *Flavocetraria* (Parmeliaceae, Ascomycota) in the southern hemisphere. New Zealand Journal of Botany 42(4): 647–656.

Bjerke, J. W., Lerfall, K., and Elvebakk, A. (2002) Effects of ultraviolet radiation and PAR on the content of usnic and divaricatic acids in two arctic-alpine lichens. Photochem Photobiol Sci 1:678–685.

Botting, R.S., and Fredeen, A.L. 2006. Contrasting terrestrial lichen, liverwort, and moss diversity between old-growth and young second-growth forest on two soil textures in central British Columbia. Canadian Journal of Botany 84: 120–132.

Boucher, V.L., and Nash, T.H. 1990. The Role of the Fruticose Lichen *Ramalina menziesii* in the Annual Turnover of Biomass and Macronutrients in a Blue Oak Woodland. Botanical Gazette 151(1): 114–118.

Brodie, H.J. 1951. The splash-cup dispersal mechanism in plants. Canadian Journal of Botany 29: 224–234.

Brodie, H. J., and Gregory P.H. 1953. The action of wind in the dispersal of spores from cup-shaped plants structures. Can. Jour. Bot. 31(4): 402–410.

Brodo, I. M.1973. Substrate ecology. *In* The Lichens. *Edited by* V. Ahmadjian, and M.E. Hale M.E. Academic Press, New York and London. pp. 401–441.

Brodo, I. 1981. Lichens of the Ottawa region. Syllogeus 29: 1–137.

Brodo, I., Sharnoff, S.D., and Sharnoff, S. 2001. Lichens of North America. New Haven: Yale University Press. 795 pp.

Brodo, I.M., Harris, R.C., Buck, W., Lendemer, J.C., and Lewis C. 2013. Lichens of the Bruce Peninsula, Ontario: Results from the 17th Tuckerman Workshop, 18-22 Sept. 2008. Opuscula Philolichenum 12: 198–232.

Brownell, G.M., and Kinkel, A.R. Jr. 1935. The Flin Flon Mine: Geology and paragnesis of the ore deposit. *In* The Flin Flon Mine. *Edited by* The Mines Branch, Department of Mines and Natural Resources. Industrial Development Board of Manitoba. pp. 261-286.

Bryant, J.P., Chapin, F.S., and Klein, D.R. 1983. Carbon-nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40: 357–368.

Bryant, J.P., Chapin, F.S. 1986. Browsing-Woody Plant Interactions During Boreal Forest Plant Succession. In Forest Ecosystems in the Alaskan Taiga, ed. Cleve, K.V., ChapinIII, F.S., Flanagan, P.W., Viereck, L.A., and Dyrness, C.T. Springer International Publishing AG.

Buckow, R., Kastell, A., Terefe, N.S., and Versteeg, C. 2010. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. Journal of Agricultural and Food Chemistry. 58: 10076-10084.

Büdel, B., and Scheidegger, C. 2008. Thallus morphology and anatomy. In Lichen Biology, 2nd edition, ed. T. H. Nash, pp. 40-68. Cambridge: Cambridge University Press.

Bu'Lock, J.D. 1961. Intermediary metabolism and antibiotic synthesis. Advances in Applied Microbiology 3: 293–342.

Burger, R. 1999. Evolution of genetic variability and the advantage of sex and recombination in changing environments. Genetics 153(2): 1055-1069.

Bu, C., Wu, S., Han, F., Yang, Y., and Meng., J. 2015. The combined effects of moss-dominated biocrusts and vegetation on erosion and soil moisture and implications for disturbance on the Loess Plateau, China. PLoS One 10:e0127394.

Burkin, A., Kononenko, G. 2014. Metabolites of toxigenic fungi in lichens of genera *Nephroma, Peltigera, Umbilicaria*, and *Xanthoria.* Biol. Bulletin 41(3): 216–222.

Calcott, M.J., Ackerley, D.F., Knight, A., Keyzers R.A., and Owen, J.G. 2018. Secondary metabolism in the lichen symbiosis. Chem. Soc. Rev. 47: 173–176.

Calvo, A.M., Wilson, R.A., and Keller, N.A. 2001. Relationship between secondary metabolism and fungal development. Microbiology and Molecular Biology Reviews 66: 447–459.

Campbell, C.D., and Vereras, J.C. 2010. Biosynthesis of lovastatin and related metabolites formed by fungal iterative PKS enzymes. Bioploymers 93: 755–763.

Canadian climate data and information archives. 2013. Canadian Climate Normals 1971-2000. Retrieved from: http://climate.weatheroffice.gc.ca. Canadian weather. 2013. Caracuel, Z., Casanova, C., Roncero, M.I., Di Pietro, A., and Ramos, J. 2003. pH response transcription factor PacC controls salt stress tolerance and expression of the P-Type Na+ -ATPase Ena1 in *Fusarium oxysporum*. Eukaryote Cell 2(6): 1246–52.

Chamizo, S., E. Rodrguez-Caballero, J. R. Roman, and Y. Canton. 2017. Effects of biocrust on soil erosion and organic carbon losses under natural rainfall. Catena 148: 117–125.

Chen, G., Wang, H., and Pei, Y.J. 2014. Secondary metabolites from marine-derived microorganisms. Asian Nat. Prod. Res. 16: 105–122.

Cheng, S.C., Huangb, M.Z., and Shieab J. 2011.Thin layer chromatography/mass spectrometry. Planar Chromatography 1218(19): 2700–2711.

Chesworth, W. 2008. Encyclopaedia of Soil Science. Springer. pp.605.

Chiang, Y.M., Szewczyk, E., Davidson, A.D., Keller, N., Oakley, B.R., and Wang, C.C.C. 2009. A gene cluster containing two fungal polyketide synthases encodes the biosynthetic pathway for a polyketide, Asperfuranone, in *Aspergillus*. Journal of the American Chemical Society 131: 2965–2970.

Chiang, Y.M., Oakley, B.R., Keller, N.P., and Wang, C.C.C. 2010. Unraveling polyketide synthesis in members of the genus *Aspergillus*. Appl. Microbiol. Biotechnology 86: 1719–1736.

Cho, G.C., Dodds, J., and Santamarina, J.C., 2006. Particle shape effects on packing density, stiffness, and strength: natural and crushed sands. J. Geotech. Geoenviron. Eng. 132: 591–602.

Consortium of North America Lichen Herbaria (CNALH). 2016. Available at http://lichenportal.org/portal/index.php. Accessed September 09, 2016.

Cook, K.H., Vizy, and E. K. 2015. Detection and analysis of an amplified warming of the Sahara Desert. Journal of Climate 28: 6560.

Cornejo, C., and Scheidegger, C. 2013. New morphological aspects of cephalodium formation in the lichen *Lobaria pulmonaria* (Lecanorales, Ascomycota). Lichenologist 45(1): 77–87.

Crittenden, P.D. 2000. Aspects of the ecology of mat-forming lichens. Rangifer 20(3): 127–139.

Culberson, W.L. 1969. The chemistry and systematics of some species of the *Cladonia cariosa* group in North America. Bryologist 72: 377–386.

Culberson, C. 1972. Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. Journal of Chromatography A 72: 113–125.

Culberson, C.F., Culberson, W.L. and Arwood, D.A. 1977a. Physiography and fumarprotocetraric acid production in the *Cladonia chlorophaea* group in North Carolina. Bryologist. 80: 71–75.

Culberson, C.F., Culberson, W.L., and Johnson A. 1977b. Thermally induced chemical artifacts in lichens. Phytochemistry. 16: 127–130.

Culberson, W. L., Culberson, C. F. and Johnson A. 1977c. Correlations between secondary-product chemistry and ecogeography in the *Ramalina siliquosa* lichens. Plant Systematics and Evolution. 127: 191–200.

Culberson, C. F., Culberson, W. L., and Johnson, A. 1983. Genetic and environmental effects on growth and production of secondary compounds in *Cladonia cristatellea*. Biochemical Systematics and Ecology. 11: 77–84.

Culberson, W. L. 1986a. Chemistry and sibling speciation in the lichen-forming fungi: ecological and biological considerations. Bryologist 89: 123–131.

Culberson, C. F. 1986. Biogenetic relationships of the lichen substances in the framework of systematics. Bryologist. 89: 91–98.

Culberson, C. F., Culberson, W. L., and Johnson, A. 1988. Gene flow in lichens. American Journal of Botany. 75: 1135–1139.

Culberson, C.F., and Armaleo, D. 1992. Induction of a complete secondary-product pathway in a cultured lichen fungus. Experimental Mycology 16: 53–63.

Culberson, C. F., Culberson, W. L., and Johnson, A. 1993. Occurrence and histological

distribution of usnic acid in the *Ramalina siliquosa* species complex. Bryologist 96: 181–184.

De Bono, J.P., and McDowell, G.R. 2015. An insight into the yielding and normal compression of sand with irregularly-shaped grains using DEM. Powder Technology 271: 270–277.

Deduke, C., Timsina, B., and Piercey-Normore, M. D. 2012. Effect of environmental change on secondary metabolite production in lichen-forming fungi. In International perspectives on global environmental change, Ed. S. Young, pp. 197-230. Croatia: InTech.

Deduke, C. and Piercey-Normore, M.D. 2014. A potential trade-off with stictic acid improves ascospore viability in *Xanthoparmelia cumberlandia*. Bryologist. DOI:bryo-117-03-10.3d.

DePriest, P. T. 2004. Early molecular investigations of lichen-forming symbionts: 1986-2001. Annual Review of Microbiology 58: 273–301.

Doering, J., Deduke, C., and Piercey-Normore, M.D. 2016. Variation in lichen species assemblages and secondary metabolites surrounding *Stereocaulon* species in boreal forest of Northwestern Manitoba. Evansia 33(2): 70–79.

Dornelas, M. 2010. Disturbance and change in biodiversity. Philosophical Transactions of the Royal Society B. 365(1558): 3719–3727.

Egan, R.S., Morgan, S., Wetmore, C.M., and Ladd, D. 2002. Additions to the lichen flora of Nebraska. Transactions of the Nebraska Academy of Sciences 28: 1–13.

Eldridge, D.J., and Koen, T.B. 1998. Cover and floristics of microphytic soil crusts in relation to indices of landscape health. Plant Ecology 137: 101–114.

Elix, J. A., Johnston, J. and Parker, J. L. 1988. A computer program for the rapid identification of lichen substances. Mycotaxon. 31: 89–99.

Elix, J. A. 1996. Biochemistry and secondary metabolites. In Lichen Biology, ed. T. H. Nash III, pp. 154-180. United Kingdom: Cambridge University Press.

Elix, J. A., and Stocker-Wörgötter, E. 2008. Biochemistry and secondary metabolites. *In* Lichen Biology, 2nd edition. *Edited by* T.H. Nash III. Cambridge University Press, Cambridge, pp. 104–133.

Esslinger, T. L. 2008. A cumulative checklist for the lichen-forming, lichenicolous and allied fungi of the continental United States and Canada. North Dakota State University: http://www.ndsu.nodak.edu/instruct/esslinge/chcklst/chcklst7.htm

Evans, A.W. 1943. Michrochemical studies on the genus *Cladonia*. Rhodora 45: 417–438.

Fahselt, D. 1981. Lichen products of *Cladonia stellaris* and *C. rangiferina* maintained under artificial conditions. Lichenologist. 13: 87–91.

Fahselt, D. 1994. Carbon metabolism in lichens. Symbiosis 16: 117–165.

Fair, J. D. and Kormos, C. M. 2008. Flash column chromatograms estimated from thin-layer chromatography data. J. chromatogram 1211(1-2): 49–54.

Farkas, E.E., and Lökös, L.S. 1994. Distribution of the lichens *Cladonia magyarica* Vain. and *Solorinella asteriscus* Anzi in Europe. Acta Botanica Fennica 150: 21–30.

Feige, G.B., Lumbsch, H.T., Huneck, S., and Elix, A. 1993. Identification of lichen substances by a standardized high-performance liquid chromatographic method. Journal of Chromatography. 646(2): 417–427.

Fisher, P.J., and Proctor, M.C. 1978. Observations on a season's growth in *Parmelia caperata* and *P. sulcata* in South Devon. Lichenologist 10: 81–88.

Fischer, T., Gypser, S., Subbotina, M., and Veste, M. 2014. Synergic hydraulic and nutritional feedback mechanisms control surface patchiness of biological soil crusts on tertiary sands at a post-mining site 62(4): 293–302.

Fontaine, K.M., Ahti, T., and Piercey-Normore, M.D. 2010. Convergent evolution in *Cladonia gracilis* and allies. Lichenologist 42: 323–338.

Fox, E.M., and Howlett, B.J. 2008. Secondary metabolism: regulation and role in fungal biology. Current Opinion in Microbiology 11: 481–487.

Friedmann, E. I. 1982. Endolithic microorganisms in the Antarctic cold desert. Science, 215, 1045-1053.

Friedl, T. and Budel, B. 1996. Photobionts. In: Lichen Biology. (ed Nash III. T. H.). Cambridge University Press. Cambridge.

Gaikwad, S., Verma, N., Sharma, B. O., and Behera, B. C. 2012. Growth promoting effects of some lichen metabolites on probiotic bacteria. Journal of Food Sciences and Technology 2012: 1–8.

Gallé, L. 1968. The xerothermic lichen species, *Cladonia magyarica* Vain. A Móra Ferenc Múzeum Évkönyve (MFMÉ). 237–268.

Galun, M. 1988. Handbook of Lichenology. Chapter VII. B. 2. CRC press. Florida. USA.

Gartlan, J. S.1981. Food selection by black colobus monkeys (*Colobus satanus*) in relation to plant chemistry. Biol. J. Linn. Soc. 16: 115–146.

Gauslaa, Y., Ohlson, M., and Rolstad, J. 1998. Fine-scale distribution of the epiphytic lichen *Usnea longissima* on two even-aged neighbouring *Picea abies* trees. J. Veg. Sci 9: 95–102.

Gauslaa, Y. 2005. Lichen palatability depends on investments in herbivore defense. Oecologia

143: 94–105.

Gauslaa, Y., Marit L., Solhaug, K., Mikael, O. 2006. Growth and ecophysiological acclimation of the foliose lichen *Lobaria pulmonaria* in forests with contrasting light climates. Oecologia 147(3):406-416.

Gehring, J.L., and Delph, L.F. 2006. Effects of reduced source-sink ratio on the cost of reproduction in females of *Silene latifolia.* International Journal of Plant Science 167:843–854.

Gerke, J., Bayram, O., Feussner, K., Landesfeind, M., Shelest, E., Feussner, I., and Braus, G.H. 2012. Breaking the Silence: Protein Stabilization Uncovers Silenced Biosynthetic Gene Clusters in the Fungus *Aspergillus nidulans.* Appl environ microb 78: 8234–8244.

Gilbert, O. L. 1977. Fungal plasticity in *Cladonia pocillum*. Lichenologist 9:172–173.

Goffinet, B., and Bayer, R.J. 1997. Characterization of mycobionts of photomorph pairs in the Peltigeraceae (lichenized Ascomycetes) based on internal transcribed spacer sequences of the nuclear ribosomal DNA. Fungal Genetics and Biology 21: 228–237.

Hartman, T. 1996. Diversity and variability of plant secondary metabolism: a mechanistic view. Entomologia Experimentalis et Applicata 80: 177–188.

Hamada, N. 1982. The effect of temperature on the content of the medullary depsidone salazinic acid in *Ramalina siliquosa* (lichen). Canadian Journal of Botany 60: 383–385.

Hammer, S. 1995. A synopsis of the genus *Cladonia* in the Northwestern United States. Bryologist 98: 1–28.

Hartmann, T. 2007. From waste products to ecochemicals: Fifty years research of plant secondary metabolism. Phytochemistry 68: 2831–2846.

Hauck, M., and Huneck, S. 2007. Lichen substances affect metal adsorption in *Hypogymia physodes*. Journal of Chemical Ecology 33: 219–233.

Hauck, M. 2008. Susceptibility to acidic precipitation contributes to the decline of the terricolous lichens *Cetraria aculeata* and *Cetraria islandica* in central Europe. Environmental Pollution 152: 731–735.

Hauck, M., Jürgens, S.R., Willenbruch, S., Huneck, S., and Leuschner, C. 2009*a*. Dissociation and metal-binding characteristics of yellow lichen substances suggest a relationship with site preferences of lichens. Annals of Botany 103: 13–22.

Hauck, M., Jürgens, S.R., Huneck, S., and Leuschner, C. 2009*b*. High acidity tolerance in lichens with fumarprotocetraric, perlatolic or thamnolic acids is correlated with low pKa1 values of these lichen substances. Environmental Pollution 157: 2776–2780.

Hawksworth, D. L. 1976. Lichen chemotaxonomy. *In:* D. H. Brown, D. L. Hawksworth and R. H. Bailey (eds.): Lichenology: Progress and Problems. Academic Press, London, pp. 139-184.

Hawksworth, D. L. and Honegger, R. 1994. The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. *In:* M. A. J. Willams (eds.) Plant Galls: Organisms, Interactions, Populations. Claredon Press, Oxford, pp. 77–98.

Heiđmarsson, S. 2003. Molecular study of *Dermatocarpon miniatum* (Verrucariales) and allied taxa. Mycol. Res. 107(4): 459–468. Doi:10.1017/S0953756203007652.

Helms, G., Friedl, T., Rambold, G. and Mayrhofer, H. 2001. Identification of photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. Lichenologist. 33: 73–86.

Hesbacher, S., Giez, I., Embacher, G., Fiedler, K., Max, W., Trawoger, A., Turk, R., Lange, O. L., and Proksch, P.1995. Sequestration of lichen compounds by lichen-feeding members of the Arctiidae (Lepidoptera). Journal of Chemical Ecology 21: 2079–2089.

Hestmark, G., Skogesal, O., and Skullerud, Q. 2004a. Growth, reproduction, and population structure in four alpine lichens during 240 years of primary colonization. Canadian Journal of Botany 82: 1356–1362.

Hestmark, G., Skogesal, O., and Skullerud, O. 2004b. Growth in the alpine saxicolous lichens *Allantoparmelia alpicola* and *Melanelia stygia*. Nova Hedwigia 78: 301–309.

Hinds, J.W., and Hinds, P.L. 2007. Machrolichens of New England. Bronx, NY: The New York Botanical Garden Press. 584 pp.

Hoffmeister, D. and Keller, N. P. 2007. Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat. Prod. Rep. 24:393-416.

Honegger, R. 1992. Lichens: mycobiont-photobiont relationships. *In*: Algae and symbioses. Plants, Animals, Fungi, Viruses, Interactions Explored. W. Reisser (ed.), Bristol: Biopress. pp. 255–275.

Honegger, R. 1998. The lichen symbiosis – what is so spectacular about it? Lichenologist. 30: 193–212.

Hopwood, D. A. and Sherman, D. H. 1990. Molecular Genetics of polyketides and its comparison to fatty acid biosynthesis. Annual Review of Genetics. 24: 37–62.

Horn, R. 2011. Friction phenomena in soils. *In:* Gliński, J., Horabik, J., and Lipiec, J. (eds.): Encyclopedia of Agrophysics. Springer, Dordrecht, Heidelberg, London, New York, pp. 316–318.

Huneck, S. 1973. Nature of lichen substances. *In*: V. Ahmadjian (ed.): The Lichens. Academic Press, London. pp. 495–522.

Huneck, S., and Schmidt, J. 1989. Thermal decomposition of lichen depsides. A Journal of Chemical Sciences. 44(10): 1283–1289.

Huneck, S. 1999. The significance of lichens and their metabolites. Naturwissenschaften 86: 559–570.

Huck, B., and Whiteway, D. 2013. In Search of Canada's Ancient Heartland. Heartland Associates.

Hyvarinen, M., and Crittenden, P.D. 1998. Growth of the cushion-forming lichen, *Cladonia portentosa*, at nitrogen-polluted and unpolluted heathland sites. Environmental and Experimental Botany. 40: 67–76.

Hyvarinen, M., Walter, B., and Koopmann, R. 2003. Impact of fertilization on phenol content and growth rate of *Cladina stellaris*: a test of the carbon-nutrient balance hypothesis. Oecologia 134: 176–181.

Inderjit, I., and Mallik, A.U. 2002. Chemical Ecology of Plants: Allelopathy in Aquatic and Terrestrial Ecosystems. Birkhauser. Germany. pp. 1-56.

Irwin, W.J. 1982. Analytical pyrolysis: a comprehensive guide. Marcel Dekker Inc. New York, 1982.

James, P.W., Hawksworth, D.L., and Rose, F. 1977. Lichen communities in the British Isles: a preliminary conspectus. pp. 296–409.

James, P.D., Edwaeds, C.D., and Dawson, M. 1991. The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat. Journal of General Microbiology. 137(7): 1715–1720.

Johnston, W.A. 1946. Glacial Lake Agassiz, with special reference to the mode of deformation of the beaches. Geological Survey Bulletin No. 7, Mines and Geology Branch, Department of Mines and Resources, Ottawa, Ontario. pp. 1-27.

Jun, R., and Rozé, F. 2005. Monitoring bryophyte and lichen dynamics in sand dunes: example on the French Atlantic coast. *In:* Herrier, J.L., Mees, J., Salman, A., Seys, J., Van Nieuwenhuyse, H., and Dobbelaere. (eds.): Dunes and Esturaries, 2005. Proceedings of the International Conference on Nature Restoration Practices in European Coastal Habitats, Koksijde, Belgium. 19-23 September, 2005. VLIZ Special Publication 19. pp. 291–313.

Junttila, S., Laiho, A., Gyenesei, A., and Rudd, S. 2013. Whole transcriptome characterization of the effects of dehydration and rehydration on *Cladonia rangiferina*, the grey reindeer lichen. BMC Genomics 14: 870–883.

Kalliokoski, J. 1953. Interpretations of the structural geology of the Sherridon-Flin Flon region, Manitoba. Geological Survey Bulletin No. 25, Mines and Geology Branch, Department of Mines and Resources, Ottawa, Ontario.pp. 1-18

Karunaratne, V., Bombuwela, K., Kathirgamanathar, S., and Thadani, V.M. 2005. Lichens: a chemically important biota. Journal of the National Science Foundation of Sri Lanka 33(3): 169–186.

Kappen, L., and Rogers, R.W. 1977. Lichens of arid regions. Journal of Hattori Bot. 53: 305.

Kappen, L. 1988. Ecophysiological relationships in different climatic regions. In*:* Galun, M. (ed.): Handbook of lichenology, 2. CRC Press, Boca Raton, pp. 37–100.

Kappen, L., and Valladares, F. 1999. Opportunistic growth and desiccation tolerance: the ecological success of poikilohydrous autotrophs. *In*: Pugnaire, F.I., and Valldares, F. (eds.): Handbook of functional plant ecology. Marcel Dekker, New York. pp. 121–194.

Kebarle, P. A. 2000. Brief overview of the present status of the mechanisms involved in electrospray mass spectrometry. J. Mass Spectrom. 35: 804–817.

Keddy, P.A. 2001. Competition. Population and community Biology series. Kluwer Academic Publishers, pp. 61, 97.

Keller, N.P., and Hohn, T.M. 1997. Metabolic pathway gene clusters in filamentous fungi. Fungal Genetics and Biology 21: 17–29.

Keller, N.P., Turner, G., and Bennett, J.W. 2005. Fungal secondary metabolism - from biochemistry to genomics. Nature Reviews Microbiology 3: 937–947.

Keller, N.P., and Shwab, E.K. 2008. Regulation of secondary metabolite production in filamentous ascomycetes. Mycological Research. 112: 225–230.

Kirk, P.M., Cannon, P.F., David, J.C., and Stalpers, J.A. 2001. Ainsworth and Bisby's Dictionary of the Fungi. 9th edition. CAB International, Wallingford, Oxon.pp. 161

Kirk, P.M., Cannon, P.F., Minter, D.W., and Stalpers, J.A. 2008. Dictionary of the Fungi, 10th Edition. United Kingdom: CABI Europe. pp. 202

Koricheva, J. 2002. The carbon-nutrient balance hypothesis is dead: long live the carbon-nutrient balance hypothesis? Oikos 98: 3.

Kotelko, R., and Piercey-Normore, M.D. 2010. *Cladonia pyxidata* and *C. pocillum*: genetic evidence to regard them as conspecific. Mycologia 102: 534–545.

Kowalewska, A., Kukwa, M., Ostrowska, I., Jabłońska, A., Oset, M., and Szok, J. 2008. The lichens of the *Cladonia pyxidata-chlorophaea* group and allied species in Poland. Herzogia 21: 61–78.

Kowalski, M., Hausner, G., and Piercey-Normore, M.D. 2011. Bioactivity of secondary metabolites and thallus extracts from lichen fungi. Mycoscience 52: 413–418.

Kupiec, T. 2004. Quality-Control Analytical Methods: High-Performance Liquid Chromatography. International Journal of Pharmaceutical Compounding 8(3): 223–227.

Kuzyk, R. 2013. Terrestrial lichen abundance in relation to stand structure and silvicultural history. M.SC.F. thesis. Faculty of natural resources management. Lakehead University.pp.12. Kytoviita, M.M. 2009. No allelopathic effect of the dominant forest-floor lichen *Cladonia stellaris* on pine seedling 23(2): 435–441.

Lalley, J.S., Viles, H.A., Henschel, J.R., and Lalley, V. 2006. Lichen-dominated soil crusts as arthropod habitat in warm deserts. Journal of Arid Environments. 67: 579–593.

Lan, S., Wu, L., Zhang, D., Hu, C. 2012. Successional stages of biological soil crusts and their microstructure variability in Shapotou region (China). Environmental Earth Sciences 65(1): 77–88.

Langhans, T., Schwabe, A., and Storm, C. 2009. Community Assembly of Biological Soil Crusts of Different Successional Stages in a Temperate Sand Ecosystem, as Assessed by Direct Determination and Enrichment Techniques. Microb. Ecol. 58: 394–407. DOI 10.1007/s00248-009-9532-x

Larson, D.W. 1987. The absorption and telease of water by lichens. Bibliotheca Lichenologica.25: 351-360.

Lawrey, J.D. 1984. Lichen secondary compounds influence herbivore choice. Bioscience 34: 109.

Lawrey, J.D. 1986. Biological role of lichen substances. Bryologist. 89: 111–122.

Lechowski, Z., Mej, E., and Bialczyk, J. 2006. Accumulation of biomass and some macroelements in tomato plants grown inmedia with (+) usnic acid. Environmental and Experimental Botany 56: 239–244.

Lee, H.A. 1968. Quaternary Geology (part 1 of Ch. 9). *In*: Beals, C.S. (ed.): Science, History, and Hudson Bay. Vol 2, pp. 503–542.

Legendre, P., and Legendre, L. 1998. Numerical Ecology, 2nd English edition. Developments in Environmental Modelling No. 20. Elsevier Science, Philadelphia, PA.

Lewry, J.F., and Collerson, K.D. 1990. The Trans-Hudson Orogen: extent, subdivision, and problems. *In:* Lewry, J.F., and Stauffer, M.R. The Early Proteozoic Trans-Hudson Orogen of North America. Geological Association of Canada Special Paper 37: 1-14.

Liao, C., Piercey-Normore, M. D., Sorensen J. L., and Gough, K. 2010. *In situ* imaging of usnic acid in selected *Cladonia* spp. by vibrational spectroscopy. Analyst 135: 3242–3248.

Lipiec, J., Siczek, A., Sochan, A., and Bieganowski, A. 2016. Effect of sand grain shape on root and shoot growth of wheat seedlings. Geoderma 265: 1–5.

Litterski, B., and Ahti, T. 2004. World distribution of selected European *Cladonia* species. Symbolae Botanicae Upsalienses 34: 205–236.

Lokajová, V., Bačkorová, M., and Bačkor, M. 2014. Allelopathic effects of lichen secondary metabolites and their naturally occurring mixtures on cultures of aposymbiotically grown lichen photobiont *Trebouxia erici* (Chlorophyta). South African Journal of Botany 93: 86–91.

Lumbsch, H. T. 1998a. The taxonomic use of metabolic data in lichen-forming fungi. In Chemical Fungal Taxonomy (J. C. Frisvad, P. D. Bridge &D. K. Arora, eds). New York: M. Dekker.

Lumbsch, H. T. 1998b. The use of metabolic data in lichenology at the species and subspecies levels. Lichenologist. 30, 357-367.

MacGillivary, T.F. 1999. Analysis of lichens under environmental stress using pyrolysis GS-MS and pyrolysis GC-FID. Master thesis. Department of Chemistry. Memorial University of Newfoundland.

Manitoba Geology. 2017. Mineral Resources. Online. http://gov.mb.ca/iem/geo/exp-sup/mbgeology.html#sbz. Accessed May 15, 2017.

Manojlovic, N., Rankovic, B., Kosanic, M., Vasiljevic, P., and Stanojkovic, T. 2012. Chemical composition of three *Parmelia* lichens and antioxidant, antimicrobial and cytotoxic activities of some their major metabolites. Phytomedicine.19: 1166 –1172.

Marie, V., Gross, N., Hill D., Martin, R., Wirth, D., Wright, I. J., and Soussana, F. 2013. Disentangling coordination among functional traits using an individual-centred model: impact on plant performance at intra- and inter-specific levels. PLoS One 8: 1–16.

Matos, P., Pinho, P., Aragon, G., Martinez, I., Nunes, A., Soares A.M., and Branquinho, C. 2014. Lichen traits responding to aridity. Journal of Ecology 103(2): 451–458.

Mauzelaar, H.L.C., Haverkamp, J., and Hileman, F.D.P. 1982. Pyrolysis mass spectrometry of recent and fossil biomaterials. Compendium and atlas; Elsevier Scientific Publishing Co. Amsterdam. pp. 290.

McEvoy, M., Nybakken, L., Solhaug, K.A., and Gauslaa, Y. 2006. UV triggers the synthesis of the widely distributed secondary lichen compound usnic acid. Mycological Progress 5: 221–229.

McEvoy, M., Solhaug, K. A., Gauslaa, Y. 2007. Solar radiation screening in usnic acid containing cortices of the lichen *Nephroma arcticum*. Symbiosis 43: 143–150.

McKey, D. 1974. Adaptive patterns in alkaloid physiology. American Naturalist 108: 305–320.

McKey, D.1979. The distribution of secondary compounds within plants. *In*: Rosenthal, G.A., and Janzen, D.H. (eds.): Herbivores: Their interaction with secondary plant metabolites. Academic Press, New York, pp. 55–133.

Melo, M.G.D., Araújo, A.A.S., Serafini, M.R., Carvalho, L.F., Bezerra, M.S., Ramos, C.S., Bonjardim, L.R., Albuquerque-Júnior, R.L.C., Lima, J.T., Siqueira, R.S., Fortes, V.S., Fonseca, M.J.V., Quintans-Júnior, L.J. 2011. Anti-inflammatory and toxicity studies of atranorin extracted from *Cladina kalbii* Ahti in rodents. Brazilian Journal of Pharmaceutical Sciences 47(4): 861–872.

Miadlikowska, J. and Lutzoni, F. 2006. Phylogenetic revision of the genus *Peltigera* (lichen-forming ascomycetes) based on morphological, chemical and large subunit nuclear ribosomal DNA data. International Journal of Plant Science. 161: 925–958.

Mietzsch, E., Lumbsch, H. T. and Elix, J. A. 1993. Notice: a new computer program for the identification of lichen substances. Mycotaxon 47: 475–479.

Millot, M., Di Meo, F., Tomasi, S., Boustie, J., and Trouillas, P. 2012. Photoprotective capacities of lichen metabolites: A joint theoretical and experimental study. J. Photochem. Photobiol. B Biol. 111: 17–26.

Mirando, M., and Fahselt, D. 1977. The effect of thallus age and drying procedure on extractable lichen substances. Can. J. Bot. 56: 1499–1504.

Mishler, B.D. 2001. The biology of bryophytes. American Journal of Botany 88(11): 2129–2131.

Moiseeva, E.N. 1961. Biochemical properties of lichens and their practical importance. Akademia Nauk SSSR. Botanischeski Institut im. Moscow. 82 pp.

Muggia, L.S., Pérez-Ortega, A., Fryday, T., and Spribille, M.G. 2014. Global assessment of genetic variation and phenotypic plasticity in the lichen-forming species *Tephromela atra*. Fungal Diversity 64(1): 233–251.

Muir, P. S., Shirazi, A. M., and Patrie, J. 1997. Seasonal growth dynamics in the lichen *Lobaria pulmonaria.* Bryologist 100: 458–464.

Muzika, R. 1993. Terpenes and phenolics in response to nitrogen fertilization: a test of the carbon/nutrient balance hypothesis. Chemoecology 4: 3–7.

Nash, T.H. III, Ryan, B.D., Gries, C., and Bungartz, F. 2002. Lichenflora of the greater Sonoran Desert region. Vol. 1. The pyrenolichens and most of the squamulose and marolichens. Lichen Unlimited; Tempe. Arizona, USA.p. 532

Nash, T.H. III, Ryan, B.D., Diederich, P., Gries, C., and Bungartz, F. 2004. Lichen flora of the greater Sonoran Desert region, Vol. 2. Most of the microlichens, balance of the macrolichens, and the lichenicolous fungi. Lichen Unlimited; Tempe. Arizona, USA.p.742.

Nash, T.H. 2008. Lichen biology. Cambridge University Press. UK. p.104-152.

Nash, T.H. III, Gries, C., and Bungartz, F. 2011. Lichen flora of the greater Sonoran Desert region, Vol. 3. Lichen Unlimited; Temp. Arizona, USA.p. 567.

National Center for Biotechnology Information. PubChem Compound Database; CID=5317419, https://pubchem.ncbi.nlm.nih.gov/compound/5317419 (accessed May 17, 2018).

Nimis, P.L., and Skert, N. 2006. Lichen chemistry and selective grazing by the coleopteran *Lasioderma serricorne*. Environ. Exp. Bot. 55: 175–182.

Nicholson, S.E. 2011. Dryland climatology. Cambridge University Press. pp. 158.

Nielsen, K. F., Mogensen, J. M., Johansen, M., Larsen, T. O., and Frisvad, J. C. 2009. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Anal Bioanal Chem. 395: 1225–1242.

Nguyen, K.H., Chollet-Krugler, M., Gouault, N., and Tomasi, S. 2013. UV-protectant metabolites from lichens and their symbiotic partners. Nat. Prod. Rep. 30(12): 1490-1508.

Nourish, R., and Oliver, R.W.A. 1976. Chemotaxonomic studies on the *Cladonia chlorophaea-pyxidata* complex and some allied species in Britain. *In:* Brown, D.H., Hawksworth, D.L., and Beiley, R.H. (eds.): Lichenology: progress and problems. Academic Press, London. Pp. 185–214.

Nybakken, L., and Julkunen-Tiito, R. 2006. UV-B induces usnic acid in reindeer lichens. Lichenologist. 38(5): 477–485.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M., Henry, H., and Wagner, H. 2015. Vegan: Community Ecology Package. Available from: http://cran.rproject.org/web/packages/vegan/vegan.pdf.Accessed 7 August, 2015.

Orange, A. 1992. A key to the *Cladonia chlorophaea* group in Europe, using microcrystal tests. British Lichen Society Bulletin 70: 36–42.

Orange, A., James, P.W., and White, F.J. 2001. Microchemical methods for the identification of lichens. London: British Lichen Society. 101 pp.

Osyczka, P., and Rola, K. 2013. Phenotypic plasticity of primary thallus in selected *Cladonia* species (lichenized Ascomycota: Cladoniaceae). Biologia (Poland) 68(3): 365–372.

Pérez-Ortega S., Ortiz-Álvarez S., Allan Green T.G., and de los Ríos, A. 2012. Lichen myco- and photobiont diversity and their relationships at the edge of life (McMurdo Dry Valleys, Antarctica). FEMS Microbiology Ecology 82: 429–448.

Piercey-Normore, M.D. 2003. A field survey of the genus *Cladonia* (Ascomycotina) in Manitoba, Canada. Mycotaxon 86: 233–248.

Piercey-Normore, M.D. 2005. Lichens from the Hudson Bay Lowlands: Northeastern coastal regions of Wapusk National Park in Manitoba. Canadian Journal of Botany 83: 1029–1038.

Piercey-Normore, M.D. 2006. Lichens from the Hudson Bay Lowlands: Diversity in the southeastern peatlands of Wapusk National Park, Manitoba. Canadian Journal of Botany 84: 1781–1793.

Piercey-Normore, M.D. 2007. The genus *Cladonia* in Manitoba: Exploring taxonomic trends with secondary metabolites. Mycotaxon 101: 189–199.

Piercey-Normore, M.D. 2008a. A Survey of Lichens and Bryophytes in White Spruce, *Picea glauca.* The Canadian Field-Naturalist 122(3): 199–204.

Piercey-Normore, M.D. 2008b. Lichens from the Hudson Bay Lowlands: A survey of white spruce tree islands on a calcareous beach ridge in northeastern Manitoba. Canadian Field-Naturalist 122: 199–204.

Piercey-Normore, M.D. 2010. Lichens from the Hudson Bay Lowlands: northwestern interior

treeline peatlands of Wapusk National Park, Manitoba. Botany 88: 923–929.

Pietrasiak, N. 2012. Effects of land surface characteristics on pedogenesis, biological soil crust community diversity, and ecosystem functions in a Mojave Desert piedmont landscape. Ph.D. Dissertation. University of California, Riverside., U.S.A. 208 pp.

Pietrasiak, N., Regus, J.U., Johansen, J.R., Lam, D., Sachs, J.L. and Santiago, L.S. 2013. Biological soil crust community types differ in key ecological functions. Soil Biology and Biochemistry 65: 168–171.

Pino-Bodas, R., Ahti, T., Stenroos, S., Martin, M.P., and Burgaz, A.R. 2012. *Cladonia conista* and *C. humilis* (Cladoniaceae) are different species. Bibliotheca Lichenologica 108: 161–176.

Pino-Bodas, R., Burgaz, A.R., Martín, M.P., and Lumbsch, H.T. 2012. Species delimitations in the *Cladonia cariosa* group (*Cladoniaceae*, Ascomycota). Lichenologist 44: 121-135.

Pino-Bodas, R., Ahti, T., Stenroos, S., Martín, M.P., and Burgaz, A.R. 2013. Multilocus approach to species recognition in the *Cladonia humilis* complex (Cladoniaceae, Ascomycota). American Journal of Botany 100: 664–678.

Pintado, A., Valladares, F., and Sancho, L.G. 1997. Exploring phenotypic plasticity in the lichen *Ramalina capitata*: Morphology, water relations, and chlorophyll content on north- and south-facing populations. Annals of Botany 80: 345–353.

Poole, C.F. 2003. Thin-layer chromatography: challenges and opportunities (Review). Journal of Chromatography A. 1000: 963–984.

Pringle, A., Chen, D., and Taylor, J. W. 2003. Sexual fecundity is correlated to size in the lichenized fungus *Xanthoparmelia cumberlandia*. Bryologist 106: 221–225.

Pugh, G. J. F. 1980. Strategies in fungal ecology. Transactions of the British Mycological Society 75: 1–15.

Purvis, O.W., Coppins, B.J., Hawksworth, D.L., James, P.W., and Moore, D.M. 1992. The Lichen Flora of Great Britain and Ireland. London: British Lichen Society and Natural History Museum. 710 pp.

Quilhot, W., Pera, W., Flores, E., Hidalgo, M. E., Fernandez, E., and Leighton, G. 1991. Temporal variation in usnic acid concentration in *Usnea aurantiaco-atra* in the Antarctica (Jaq.) Bory. Instituto Antartico Chileno, Ser. Cientifica. 41: 99–106.

Quilhot, W., Hidalgo, M.E., Pera, W., and Flores, E.1992. Possible biological role of secondary metabolites on Antarctic lichens. Instituto Antartico Chileno, Ser. Cientifica. 42: 53–59.

Quilhot, W., Fernandez, C., Rubio, M. E., Cavieres, M. E., Hidalgo, M.E., and Galloway, D. 1996. Preliminary data on the accumulation of usnic acid related to ozone depletion in two Antarctic lichens. Instituto Antartico Chileno, Ser. Cientifica. 46: 105–111.

Rajeev, L., Rocha, U., Klitgord, N., Luning, E.G., Fortney, J., Axen, S.D., Shih, P.M., Bouskill, N.J., Bowen, B.P., et al. 2013. Dynamic cyanobacterial response to hydration and dehydration in a desert biological soil crust. ISME Journal 7: 2178–2191.

Rambold, G., Friedl, T. and Beck, A. 1998. Photobionts in lichens: possible indicators of phylogenetic relationship? Bryologist 101: 392–397.

R Core Team. 2013. R: A language and environment for statistical computing [Computer program] R Foundation for Statistical Computing. Vienna

Richardson, D.H.S., and Smith, D.C. 1996. The physiology of the symbiosis in *Xanthoria aureola* (Ach.) Erichs. Lichenologist 3: 202–206.

Richardson, D.H.S. 1999. War in the world of lichens: parasitism and symbiosis as

exemplified by lichens and lichenicolous fungi. Mycol. Res. 103: 641–650.

Rikkinen, J. 1997. Habitat shifts and morphological variation of *Pseudevemia furfuracea* along a topographical gradient. Symb. Bot. Ups 32: 223–245.

Rhoades, D.F. 1979. Evolution of plant chemical defense against herbivores. *In:* Rosenthal, G.A., and Janzen, D.H. (eds.): Herbivores: their interaction with secondary plant metabolites. Academic Press, New York, NY. pp. 3–54.

Robinson, E.D., Ponzetti, J.M, and Bakker, J.D. 2013. Long-term changes in biological soil crust cover and composition. Ecological Processes 2: 5–142.

Rogers, R.W. 1977. Lichens of hot arid and semi-arid lands. In: Seaward, M.R.D. (ed.): Lichen Ecology. Academic Press. London. UK.pp. 211-252.

Rosentreter, R., Rai, H., and Upreti, D.K. 2014. Distribution Ecology of Soil Crust Lichens in India: A Comparative Assessment with Global Patterns. In: Rai, H, Upreti, D.K(eds.): Terricolous Lichens in India, Springer Science New York.pp. 21-31.

Rundel, P.W. 1969. Clinical variation in the production of usnic acid in *Cladonia subtenuis*. Bryologist 72: 40–44.

Rundel, P.W. 1978. The ecological role of secondary lichen substances. Biochemical Systematics and Ecology 6: 157–170.

Ruoss, E., and Ahti, T. 1989. Systematics of some reindeer lichens (*Cladonia* subg. *Cladina*) in the southern hemisphere. Lichenologist 21: 29–44.

Saiz-Jimenez, C., Grimalt, J., Garcia-Rowe, J., and Ortega-Clavo, J.J. 1991. Analytical pyrolysis of lichen thalli. Symbiosis 11: 313–326.

Sanchez, J.F., Chiang, Y.M., Szewczyk, E., Davidson, A.D., Ahuja, M., Oakley, C.E., Bok, J.W., Keller, N., Oakley, B.R., and Wang, C.C. 2010. Molecular genetic analysis of the

orsellinic acid/F9775 gene cluster of *Aspergillus nidulans*. Mol. Biosyst. 6: 587–593.

Sanchez, J. F., Chiang, Y.M., and Wang, C. C. C. 2008. Diversity of polyketide synthases found in the *Aspergillus* and *Streptomyces* genomes. Molecular Pharmaceutics. 5:226-233.

Schlech-Pietsch, S., Wagner, U., and Anderson, T.H. 1994. Changes in composition of polysaccharides and aggregate stability after carbon amendments to different textured soils. Applied Soil Ecology 1: 145–154.

Schmitt, I., Kautz, S., and Lumbsch, H.T. 2008. 6-MSAS-like polyketide synthase genes occur in lichenized ascomycetes. Mycological Research 112: 289–296. doi: 10.1016/j-mycres.2007.08.023.

Schroeckh, V., Scherlach, K., Nutzmann, H.W., Shelest, E., Schmidt-Heck, W., Schuemann, J., Martin, K., Hertweck, C., and Brakhage, A.A. 2009. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. Proc. Natl. Acad. Sci. USA 106: 14558–14563.

Shields, L.M., Mitchell, C., and Drovet, F. 1957. Alga- and lichen-stabilized surface crusts as soil nitrogen sources. Amer. Jour. Bot. 44: 489–498.

Shigo, A. L.1973. Insect and disease control: forest fertiliza- tion relations. - In: Forest fertilization. U.S. For. Serv. Gen. Tech. Rept., NE-3: 117-121.

Solhaug, K. A.; Gauslaa, Y.; Nybakken, L., and Bilger, W. (2003). UV-induction of sun-screening pigments in lichens. New Phytologist. 158:91-100.

Solhaug, K. A., Lind, M., Nybakken, L., and Gauslaa, Y. 2009. Possible functional roles of cortical depsides and medullary depsidones in the foliose lichen *Hypogymnia physodes*. Flora 204: 40-48.

Solhaug, K.A., Larsson, P., and Gauslaa, Y. 2010. Light screening in lichen cortices can be quantified by chlorophyll fluorescence techniques for both reflecting and absorbing pigment. Planta 231: 1003-1011.

Sparks, D. 2003. Environmental Soil Chemistry. Academic Press. London. UK.

Stenroos, S., Hyvönen, J., Myllys, L., Thell, A., and Ahti, T. 2002. Phylogeny of the genus *Cladonia*s. lat. (Cladoniaceae, Ascomycetes) inferred from molecular, morphological, and chemical data. Cladistics 18: 237–278.

Stephenson, N. L., and Rundel, P. W. 1979. Quantitative variation and the ecological role of vulpinic acid and atranorin in the thallus of *Letharia vulpina*. Biochememical Systematics and Ecology. 7: 263–267.

Stocker-Wörgötter, E., and Turk, R.1987. The synthesis of lichen *Verrucaria macrostoma* under laboratory conditions. Nova Hedwigia 44: 55–68.

Stocker-Wörgötter, E. 2001. Experimental studies of the lichen symbiosis: DNAanalyses, differentiation and secondary chemistry of selected mycobionts, artificial resynthesis of two- and tripartite symbioses. Symbiosis 30: 207–227.

Stocker-Worgotter E., Elix, J. A., and Grube, M. 2004. Secondary chemistry of lichen-forming fungi: Chemosyndromic variation and DNA-analyses of cultures and chemotypes in the *Ramalina farinacea* complex. Bryologist 107:152-162.

Stocker-Wörgötter, E. 2008. Metabolic diversity of lichen-forming ascomycetous fungi: culturing, polyketide and shikimate metabolite production, and PKS genes. Natural Products Reports 25: 188–200.

Su, Y.G., Zhao, X., Li, A.X., Li, X.R. and Huang, G. 2011. Nitrogen fixation in biological soil crusts from the Tengger desert, northern China. European Journal of Soil Biology 47: 182–187.

Tehler, A., Wedin, M. 2008. Systematics of lichenized fungi. *In:* Nash III, T.H. (e.d): Lichen Biology, 2nd edition. Cambridge: Cambridge University Press, pp. 336–352.

Teller, J. T., Leverington, D. W. 2004. Glacial Lake Agassiz: a 5000 year history of change and its relationship to the δ180 record of Greenland. Geological Society of America Bulletin 116: 729–742.

Thomson, J.W. 1967. The lichen genus *Cladonia* in North America. University of Toronto Press. pp. 1-184.

Timsina, B.A., Sorensen, J.L., and Weihrauch, D. 2013. Effect of aposymbiotic conditions on colony growth and secondary metabolite production in the lichen-forming fungus *Ramalina dilacerata*. Fungal Biology 117(11-12): 731–43.

Toldi, O., Tuba, Z., and Scott, P. 2009. Vegetative desiccation tolerance: is it a goldmine for bioengineering crops? Plant Sci 176:187–199.

Toni, S. A., and Piercey-Normore, M. D. 2013. Chemical ecology of lichens and species composition of cryptogams among three boreal habitats in eastern Manitoba. Botany. 91: 53–61.

Wang, B.C., He, R., and , Li, Z.H. 2010. The stability and antioxidant activity of anthocyanins from blueberry. Food Technology and Biotechnology, 48: 42-49.

Waterman, P.G., and Mole, S. 1989. Soil nutrients and plant secondary compounds. Phytochemistry Research. University of Strathclyde, Glasgow. pp. 1-181.

Whiton J.C., and Lawrey J.D. 1982. Inhibition of *Cladonia cristatella* and *Sordaria fimicola* Ascospore Germination by Lichen Acids. The Bryologist. 85(2): 222–226.

Wilkes, K., Howard, L.R., Brownmiller, c., and Prior, R.L. 2014. Changes in chokeberry (*Aronia melanocarpa* L.) polyphenols during juice processing and storage. Journal of Agricultural and Food Chemistry, 62: 4018-4025.

Wong, P.Y., and Brodo, I.M. 1992. The lichens of southern Ontario, Canada. Syllogeus 69: 1–79.

Zraik, M., Booth, T., and Piercey-Normore, M.D. 2016. Reports of *Cladonia magyarica* and *C. humilis* in Manitoba. Evansia 33(3): 136–144.

Zraik, M., Booth, T., and Piercey-Normore, M.D. 2018. Relationship between lichen species assemblages, secondary metabolites and soil characteristics in Manitoba. Botany 96: 267–279.

Appendix A. Specimens examined in this study showing collection numbers, secondary metabolites detected by TLC and HPLC (AT-atranorin, FUM-fumarprotocetraric acid), and location of collection which were all within Manitoba, Canada. Specimen collection numbers are those of Zraik (Z). All collections are from Manitoba in Sherridon(S), North Star(NS), Athapap(A), Spruce Woods(SW), and Sandilands(SA).

Cladonia species (collection number)	Secondary metabolite	Location of collection/transect
C.arbuscula Z111-4	US	S/1
C.arbuscula Z121-12	US	S/2
C.arbuscula Z132-2	US	S/3
C.arbuscula Z143-4	US	S/4
C.arbuscula Z221-11	US	NS/2
C.arbuscula Z225-13	US	NS/2
C.arbuscula Z235-12	US	NS/3
C.arbuscula Z245-11	US	NS/4
C.arbuscula Z311-2	US	A/1
C.arbuscula Z322-10	US	A/2
C.arbuscula Z323-12	US	A/2
C.arbuscula Z332-12	US	A/3
C.arbuscula Z422-3	US	SW/2
C.arbuscula Z433-3	US	SW/3
C.arbuscula Z434-9	US	SW/3
C.arbuscula Z455-1	US	SW/5
C.arbuscula Z523-6	US	SA/2
C.arbuscula Z531-1	US	SA/3
C.arbuscula Z541-13	US	SA/4
C.arbuscula Z551-12	US	SA/5
C.gracilis spp. *turbinata* Z121-11	FUM	S/2
C.gracilis spp. *turbinata* Z142-17	FUM	S/4
C.gracilis spp. *turbinata* Z143-8	FUM	S/4
C.gracilis spp. *turbinata* Z155-4	FUM	S/5
C.gracilis spp. *turbinata* Z212/11	FUM	NS/1
C.gracilis spp. *turbinata* Z232-16	FUM	NS/3
C.gracilis spp. *turbinata* Z241-1	FUM	NS/4
C.gracilis spp. *turbinata* Z251-14	FUM	NS/5
C.gracilis spp. *turbinata* Z323-12	FUM	A/2
C.gracilis spp. *turbinata* Z331-5	FUM	A/3
C.gracilis spp. *turbinata* Z334-7	FUM	A/3
C.gracilis spp. *turbinata* Z352-8	FUM	A/5
C.gracilis spp. *turbinata* Z425-9	FUM	SW/2
C.gracilis spp. *turbinata* Z442-5	FUM	SW/4
C.gracilis spp. *turbinata* Z455-3	FUM	SW/5
C.gracilis spp. *turbinata* Z455-4	FUM	SW/5
C.gracilis spp. *turbinata* Z514-1	FUM	SA/1
C.gracilis spp. *turbinata* Z541-17	FUM	SA/4
C.gracilis spp. *turbinata* Z543-18	FUM	SA/4

C.gracilis spp. *turbinata* Z553-14	FUM	SA/5
C. cristatella Z114-10	US-BAR	S/1
C. cristatella Z121-15	US-BAR	S/2
C. cristatella Z131-7	US-BAR	S/3
C. cristatella Z154-16	US-BAR	S/5
C. cristatella Z212-13	US-BAR	NS/1
C. cristatella Z214-3	US-BAR	NS/1
C. cristatella Z234-9	US-BAR	NS/3
C. cristatella Z252-7	US-BAR	NS/5
C. cristatella Z332-14	US-BAR	A/3
C. cristatella Z333-5	US-BAR	A/3
C. cristatella Z334-9	US-BAR	A/3
C. cristatella Z335-11	US-BAR	A/3
C. cristatella Z412-10	US-BAR	SW/1
C. cristatella Z413-4	US-BAR	SW/1
C. cristatella Z415-7	US-BAR	SW/1
C. cristatella Z415-14	US-BAR	SW/1
C. cristatella Z545-14	US-BAR	SA/4
C. cristatella Z551-5	US-BAR	SA/5
C. cristatella Z552-1	US-BAR	SA/5
C. cristatella Z554-1	US-BAR	SA/5
C. pyxidata Z111-1	FUM	S/1
C. pyxidata Z131-8	FUM	S/3
C. pyxidata Z135-6	FUM	S/3
C. pyxidata Z153-9	FUM	S/5
C. pyxidata Z211-9	FUM	NS/1
C. pyxidata Z221-13	FUM	NS/2
C. pyxidata Z232-18	FUM	NS/3
C. pyxidata Z255-16	FUM	NS/5
C. pyxidata Z311-4	FUM	A/1
C. pyxidata Z322-11	FUM	A/2
C. pyxidata Z333-1	FUM	A/3
C. pyxidata Z345-3	FUM	A/4
C. pyxidata Z411-3	FUM	SW/1
C. pyxidata Z421-1	FUM	SW/2
C. pyxidata Z423-6	FUM	SW/2
C. pyxidata Z425-8	FUM	SW/2
C. pyxidata Z512-18	FUM	SA/1
C. pyxidata Z525-13	FUM	SA/2
C. pyxidata Z534-12	FUM	SA/3
C. pyxidata Z554-4	FUM	SA/5
*C.cariosa Z*313-11	AT	A/1
*C.cariosa Z*333-2	AT	A/3
*C.cariosa Z*3342-13	AT	A/4
*C.cariosa Z*351-6	AT	A/5
*C.cariosa Z*415-15	AT	SW/1
*C.cariosa Z*422-4	AT	SW/2

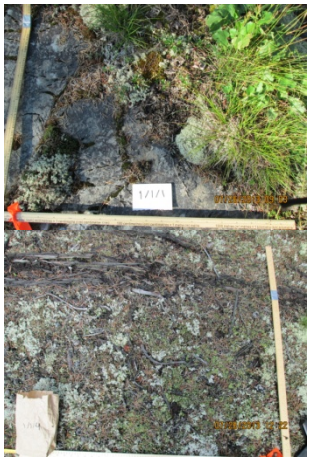
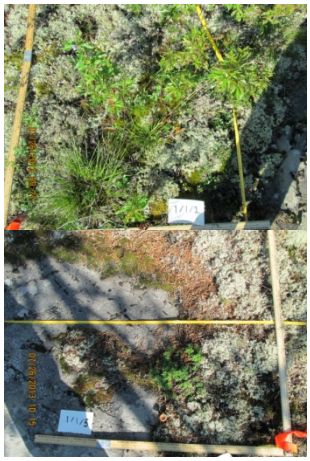
*C.cariosa Z*454-8	AT	SW/5
*C.cariosa Z*454-10	AT	SW/5
*C.cariosa Z*525-9	AT	SA/2
*C.cariosa Z*542-8	AT	SA/4
*C.cariosa Z*543-7	AT	SA/4
*C.cariosa Z*545-4	AT	SA/5
C.rangiferina Z112-13	FUM-AT	S/1
C.rangiferina Z131-12	FUM-AT	S/3
C.rangiferina Z133-4	FUM-AT	S/4
C.rangiferina Z155-7	FUM-AT	S/5
C.rangiferina Z432-4	FUM-AT	A/3
C.rangiferina Z434-9	FUM-AT	A/3
C.rangiferina Z435-7	FUM-AT	A/3
C.rangiferina Z442-6	FUM-AT	A/3
C.rangiferina Z523-5	FUM-AT	SA/2
C.rangiferina Z531-2	FUM-AT	SA/3
C.rangiferina Z541-13	FUM-AT	SA/4
C.rangiferina Z553-11	FUM-AT	SA/5

Appendix B. Coordinates, elevation, geographic location, and site description for the location of the transects in this study.

Transect	Latitude/Longitude	Elevation(m)	Geographic Location	Site description
1.1	54°86'74.30"N 101°47'37.50"W	362	Northern Manitoba	Sandy Jack pine stands over the Precambrian Shield.
1.2	54°79'72.30"N 101°46'21.40"W	341	Northern Manitoba	
1.3	54°76'77.40"N 101°48'93.30"W	352	Northern Manitoba	
1.4	54°74'76.30"N 101°50'37.50"W	350	Northern Manitoba	
1.5	54°70'67.50"N 101°54'83.90"W	318	Northern Manitoba	
2.1	54°77'69.10"N 101°61'71.00"W	332	Northern Manitoba	Granite Precambrian Shield)
2.2	54°75'68.70"N 101°63'82.70"W	324	Northern Manitoba	
2.3	54°74'48.50"N 101°65'19.60"W	309	Northern Manitoba	
2.4	54°73'24.00"N 101°66'57.60"W	306	Northern Manitoba	
2.5	54°69'75.40"N 101°68'12.30"W	334	Northern Manitoba	
3.1	54°51'14.30"N 101°54'36.20"W	319	Northern Manitoba	Boreal forest over limestone in
3.2	54°51'58.80"N	313	Northern Manitoba	

	101°53'19.10"W			
3.3	54°52'52.80"N 101°50'75.80"W	301	Northern Manitoba	
3.4	54°53'14.80"N 101°49'13.60"W	312	Northern Manitoba	
3.5	54°53'63.80"N 101°47'50.50"W	304	Northern Manitoba	
4.1	49°73'34.10"N 99°28'84.55"W	374	South Western Manitoba	
4.2	49°72'04.52"N 99°28'83.04"W	370	South Western Manitoba	
4.3	49°68'28.40"N 99°27'01.34"W	366	South Western Manitoba	Open, stabilized sand dunes and river bottom forest
4.4	49°68'29.10"N 99°26'72.22"W	340	South Western Manitoba	
4.5	49°69'58.43"N 99°26'63.24"W	354	South Western Manitoba	
5.1	49°32'69.00"N 96°28'10.32"W	387	South Eastern Manitoba	
5.2	49°32'89.16"N 96°27'29.17"W	380	South Eastern Manitoba	
5.3	49°33'56.36"N 96°24'65.45"W	389	South Eastern Manitoba	Sandy till forest, rolling landscape interspersed with low lying black spruce stands
5.4	49°39'39.16"N 96°23'11.05"W	384	South Eastern Manitoba	
5.5	49°39'39.16"N 96°23'11.05"W	388	South Eastern Manitoba	

Appendix C: Site and quadrat photos taken from study transects listed in Appendix B.

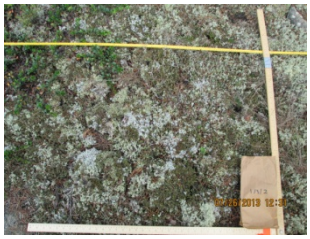




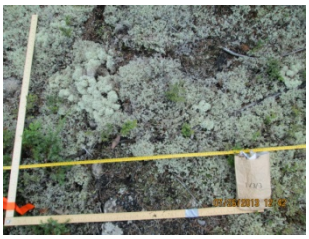
Transect 1.1: Site photo; Quadrat 1/1/1, 1/1/2, 1/1/3, 1/1/4, 1/1/5



Transect 1.2: Site photo; Quadrat 1/2/1, 1/2/2, 1/2/3, 1/2/4, 1/2/5







Transect 1.3: Site photo; Quadrat 1/3/1, 1/3/2, 1/3/3, 1/3/4, 1/3/5



Transect 1.4: Site photo; Quadrat 1/4/1, 1/4/2, 1/4/3, 1/4/4, 1/4/5



Transect 1.5: Site photo; Quadrat 1/5/1, 1/5/2, 1/5/3, 1/5/4, 1/5/5





Transect 2.1: Site photo; Quadrat 2/1/1, 2/1/2, 2/1/3, 2/1/4, 2/1/5



Transect 2.2: Site photo; Quadrat 2/2/1, 2/2/2, 2/2/3, 2/2/4, 2/2/5



Transect 2.3: Site photo; Quadrat 2/3/1, 2/3/2, 2/3/3, 2/3/4, 2/3/5





Transect 2.4: Site photo; Quadrat 2/4/1, 2/4/2, 2/4/3, 2/4/4, 2/4/5



Transect 2.5: Site photo; Quadrat 2/5/1, 2/5/2, 2/5/3, 2/5/4, 2/5/5



Transect 3.1: Site photo; Quadrat 3/1/1, 3/1/2, 3/1/3, 3/1/4, 3/1/5





Transect 3.2: Site photo; Quadrat 3/2/1, 3/2/2, 3/2/3, 3/2/4, 3/2/5

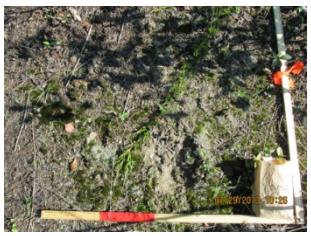


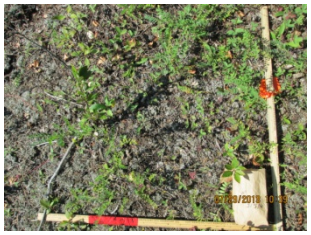
Transect 3.3: Site photo; Quadrat 3/3/1, 3/3/2, 3/3/3, 3/3/4, 3/3/5



Transect 3.4: Site photo; Quadrat 3/4/1, 3/4/2, 3/4/3, 3/4/4, 3/4/5






Transect 3.5: Site photo; Quadrat 3/5/1, 3/5/2, 3/5/3, 3/5/4, 3/5/5



Transect 4.1: Site photo; Quadrat 4/1/1, 4/1/2, 4/1/3, 4/1/4, 4/1/5



Transect 4.2: Site photo; Quadrat 4/2/1, 4/2/2, 4/2/3, 4/2/4, 4/2/5





Transect 4.3: Site photo; Quadrat 4/3/1, 4/3/2, 4/3/3, 4/3/4, 4/3/5



Transect 4.4: Site photo; Quadrat 4/4/1, 4/4/2, 4/4/3, 4/4/4, 4/4/5



Transect 4.5: Site photo; Quadrat 4/5/1, 4/5/2, 4/5/3, 4/5/4, 4/5/5



L



Transect 5.1: Site photo; Quadrat 5/1/1, 5/1/2, 5/1/3, 5/1/4, 5/1/5



Transect 5.2: Site photo; Quadrat 5/2/1, 5/2/2, 5/2/3, 5/2/4, 5/2/5



Transect 5.3: Site photo; Quadrat 5/3/1, 5/3/2, 5/3/3, 5/3/4, 5/3/5



M



Transect 5.4: Site photo; Quadrat 5/4/1, 5/4/2, 5/4/3, 5/4/4, 5/4/5




Transect 5.5: Site photo; Quadrat 5/5/1, 5/5/2, 5/5/3, 5/5/4(missing photo), 5/5/5

N

Appendix D: Summary of soil data from Chapters 2 and 4.

Site	Trans.	Quad.	soil color	soil chart	granular b	structure	ragmens %	Sieve 10%	Sieve 35%	sieve 100%	>200%	bulk weight	%sub angle	%sub rounded	well rounded	OM%	pH
Sherridon	1	1	3/1	2.5Y	very dark g	very fine	5	32.2	42.2	19.9	5.7	10.6	100.0	0.0	0.0	65.82	5.3
	1	2	3/2	2.5Y	very dark g	very fine	5	35.7	38.9	19.4	5.9	9.3	25.0	75.0	0.0	56.94	5.4
	1	3	3/1	2.5Y	very dark g	fine	7	30.7	14.6	20.3	34.3	13.6	75.0	25.0	0.0	39.14	5.7
	1	4	3/1	2.5Y	very dark g	fine	5	28.6	35.2	27.3	8.9	8.7	28.6	71.4	0.0	72.18	5.6
	1	5	3/1	2.5Y	very dark g	fine	7	61.5	26.2	11.6	0.7	5.9	50.0	50.0	0.0	82.22	5.8
	2	1	6/4	2.5Y	light yellow	very fine	3	2.1	23.3	63.4	11.2	78.0	88.1	11.9	0.0	6.95	5.1
	2	2	6/4	2.5Y	light yellow	very fine	3	1.9	19.3	67.9	10.9	78.4	75.8	21.0	3.2	3.6	4.5
	2	3	6/1	2.5Y	gray	very fine	2	5.3	19.5	63.0	12.2	63.2	69.6	30.4	0.0	5.3	4.3
	2	4	6/2	2.5Y	light brown	very fine	2	5.5	20.8	62.0	11.8	38.7	77.4	18.9	3.8	2.91	4.4
	2	5	6/4	2.5Y	light yellow	very fine	5	3.9	23.9	64.6	7.6	66.2	82.9	14.3	2.9	6.2	4.4
	3	1	6/3	2.5Y	light yellow	fine	7	6.2	21.6	63.8	8.4	98.1	87.2	12.8	0.0	1.06	6.7
	3	2	6/3	2.5Y	light yellow	fine	5	9.4	23.1	60.0	7.5	109.3	70.0	28.0	2.0	1.09	6.8
	3	3	6/3	2.5Y	light yellow	fine	7	43.0	22.2	31.8	3.0	72.7	64.9	35.1	0.0	4.02	7.1
	3	4	6/3	2.5Y	light yellow	very fine	3	13.2	30.9	43.1	12.8	104.5	79.6	20.4	0.0	1.29	6.9
	3	5	6/3	2.5Y	light yellow	fine	7	14.9	29.2	48.0	7.9	98.8	79.3	20.7	0.0	1.78	6.8
	4	1	3/2	2.5Y	very dark g	very fine	5	27.6	32.6	27.5	12.3	11.7	25.0	75.0	0.0	51.86	5.1
	4	2	4/2	2.5Y	dark grayis	very fine	7	34.1	47.5	18.1	0.3	20.5	75.0	25.0	0.0	25.72	5
	4	3	4/3	2.5Y	olive brow	fine	7	35.8	37.2	21.4	5.6	11.0	50.0	50.0	0.0	63.84	5.2
	4	4	3/2	2.5Y	very dark g	fine	7	38.6	23.7	28.8	8.9	14.9	75.0	25.0	0.0	68.02	5.3
	4	5	5/2	2.5Y	grayish bro	fine	7	15.4	34.5	45.4	4.7	24.5	82.4	17.6	0.0	16.66	5
	5	1	7/4	2.5Y	pale yellow	very fine	1	2.1	22.7	58.2	17.0	90.1	83.0	13.2	3.8	2.14	5.7
	5	2	6/3	2.5Y	light yellow	very fine	3	0.9	6.2	65.3	27.7	82.4	85.2	14.8	0.0	4.19	5.6
	5	3	7/4	2.5Y	pale yellow	very fine	3	2.3	20.1	59.5	18.1	103.2	87.3	10.9	1.8	1.94	5.4
	5	4	7/4	2.5Y	pale yellow	very fine	2	2.0	6.7	69.6	21.7	71.4	80.8	15.4	3.8	3.43	5.7
	5	5	7/3	2.5Y	pale yellow	very fine	2	2.2	7.5	62.2	28.2	75.5	85.7	14.3	0.0	2.89	5.6
North Star	1	1	3/2	7.5 YR	dark brown	very fine	7	29.9	34.6	24.7	10.8	31.8	75.0	25.0	0.0	25.66	5.7
	1	2	3/2	7.5 YR	dark brown	very fine	5	31.4	43.3	18.3	6.9	32.3	71.4	14.3	14.3	15.71	5.5
	1	3	2.5/1	7.5 YR	black	very fine	5	41.9	34.9	20.1	3.1	12.6	100.0	0.0	0.0	50.04	5.7
	1	4	2.5/1	7.5 YR	black	very fine	3	30.4	50.7	13.3	5.6	9.2	100.0	0.0	0.0	63.99	5.9
	1	5	2.5/1	7.5 YR	black	very fine	3	34.9	32.8	21.0	11.3	12.1	81.8	18.2	0.0	73.65	6
	2	1	3/2	7.5 YR	dark brown	very fine	3	32.6	39.0	21.0	7.4	8.1	88.9	11.1	0.0	72.21	5.5
	2	2	2.5/1	7.5 YR	black	very fine	5	27.8	42.9	24.5	4.8	9.6	100.0	0.0	0.0	62.81	5.6
	2	3	3/2	7.5 YR	dark brown	very fine	5	21.9	48.2	25.2	4.7	11.4	100.0	0.0	0.0	75.28	5.7
	2	4	3/1	7.5 YR	very dark g	very fine	5	47.6	25.7	19.4	7.3	12.5	62.5	12.5	25.0	63.31	5.7
	2	5	3/1	7.5 YR	very dark g	very fine	5	34.6	29.9	24.0	11.5	7.2	100.0	0.0	0.0	77.02	5.9
	3	1	4/1	7.5 YR	dark gray	very fine	3	19.0	29.8	27.0	24.3	9.7	85.7	14.3	0.0	57.40	6.1
	3	2	3/2	7.5 YR	dark brown	very fine	2	19.8	37.7	29.8	12.7	14.4	88.9	11.1	0.0	45.29	4.2
	3	3	3/2	7.5 YR	black	very fine	3	9.9	42.4	36.2	11.4	12.5	100.0	0.0	0.0	62.06	4.2
	3	4	3/2	7.5 YR	dark brown	very fine	2	22.5	35.4	29.2	12.8	10.6	90.0	10.0	0.0	49.09	4.2
	3	5	6/3	7.5 YR	light brown	very fine	5	28.5	41.0	22.5	8.0	27.1	76.9	15.4	7.7	48.01	4.2
	4	1	3/1	7.5 YR	v. Dark gra	very fine	3	18.0	30.4	30.8	20.8	12.1	75.0	16.7	8.3	52.35	4.5
	4	2	4/2	7.5 YR	brown	very fine	3	27.1	40.3	24.4	8.2	20.3	78.6	14.3	7.1	35.78	4.4
	4	3	4/2	7.5 YR	brown	very fine	3	23.5	36.7	26.8	13.0	8.5	87.5	12.5	0.0	68.46	4.5
	4	4	4/1	7.5 YR	dark gray	very fine	3	26.0	41.9	23.8	8.3	26.9	76.9	15.4	7.7	19.60	4.4
	4	5	5/3	7.5 YR	brown	very fine	2	12.3	33.8	39.8	14.2	22.7	68.8	25.0	6.3	32.90	4.3
	5	1	3/1	7.5 YR	v. Dark gra	very fine	2	19.7	39.3	27.2	13.8	12.3	83.3	16.7	0.0	34.56	4.8
	5	2	3/3	7.5 YR	dark brown	very fine	3	30.8	43.0	19.1	7.2	9.2	82.4	17.6	0.0	55.90	4.6
	5	3	3/1	7.5 YR	v. Dark gra	very fine	2	22.1	46.7	23.9	7.2	10.2	77.8	22.2	0.0	68.26	4.5
	5	4	3/2	7.5 YR	dark brown	very fine	2	31.1	40.4	23.1	5.4	15.5	83.3	8.3	8.3	46.41	4.4
	5	5	3/1	7.5 YR	v. Dark gra	very fine	3	38.7	34.3	20.7	6.3	6.8	87.5	12.5	0.0	74.34	4.2
Athapap	1	1	3/2	7.5YR	dark brown	fine	3	33.9	19.5	24.3	22.3	15.1	16.7	83.3	0.0	25.47	6.7
	1	2	3/2	7.5YR	dark brown	fine	3	25.8	36.2	27.4	10.6	4.8	25.0	75.0	0.0	79.29	6.1
	1	3	3/1	7.5YR	v. Dark gra	fine	5	24.3	40.6	31.8	3.3	31.7	20.0	80.0	0.0	21.17	6.4
	1	4	2.5/2	7.5YR	v. Dark bro	very fine	3	40.3	27.9	22.1	9.8	15.4	60.0	40.0	0.0	28.40	6.5
	1	5	3/2	7.5YR	dark brown	very fine	3	51.1	16.7	20.3	11.9	4.5	42.9	57.1	0.0	84.90	6.1
	2	1	3/1	7.5YR	v. Dark gra	very fine	2	38.4	23.2	22.2	16.2	18.1	27.3	72.7	0.0	38.40	6.9
	2	2	2.5/1	7.5YR	black	very fine	3	47.7	26.1	17.9	8.4	7.5	25.0	75.0	0.0	61.46	6.6
	2	3	3/2	7.5YR	dark brown	very fine	7	49.3	17.6	16.3	16.8	30.0	22.2	77.8	0.0	23.22	6.8
	2	4	3/1	7.5YR	v. Dark gra	fine	5	49.3	21.2	18.3	11.2	16.6	55.6	44.4	0.0	49.53	7
	2	5	2.5/3	7.5YR	v. Dark bro	very fine	5	25.7	36.0	28.0	10.3	13.7	80.0	20.0	0.0	52.86	6.6
	3	1	3/2	7.5YR	dark brown	fine	5	31.2	25.3	27.7	15.9	20.9	39.2	60.8	0.0	19.03	6.9
	3	2	2.5/1	7.5YR	black	very fine	3	35.5	28.2	24.9	11.4	8.6	44.1	55.9	0.0	78.53	6.5
	3	3	2.5/1	7.5YR	black	very fine	3	57.1	20.0	15.9	7.0	9.6	41.2	58.8	0.0	57.13	6.6
	3	4	3/1	7.5YR	v. Dark gra	very fine	3	32.2	26.2	26.5	15.1	6.2	25.0	75.0	0.0	74.01	5.9
	3	5	4/2	7.5YR	brown	fine	5	23.3	33.6	39.2	3.9	20.0	35.4	64.6	0.0	68.91	5.9
	4	1	3/1	7.5YR	v. Dark gra	fine	3	42.9	32.4	21.1	3.6	18.5	41.2	52.9	5.9	19.50	7.1
	4	2	5/4	7.5YR	brown	fine	5	32.8	31.4	24.7	11.0	41.3	45.0	53.3	1.7	7.21	7
	4	3	6/3	2.5Y	light yellow	fine	5	38.3	33.0	25.8	2.9	50.7	38.4	60.3	1.4	3.47	7
	4	4	4/3	7.5YR	brown	fine	7	33.1	37.3	27.2	2.4	21.8	45.0	52.5	2.5	11.52	7
	4	5	6/3	2.5Y	light yellow	fine	2	47.6	21.9	19.4	11.2	48.5	33.3	66.7	0.0	13.20	6.6
	5	1	5/2	2.5Y	grayish bro	very fine	2	38.1	30.7	26.9	4.3	28.9	66.7	33.3	0.0	7.43	6.3
	5	2	5/1	2.5Y	gray	fine	10	23.1	44.2	31.7	0.9	63.1	64.9	32.4	2.7	3.31	7
	5	3	5/2	2.5Y	grayish bro	fine	3	29.1	33.8	29.0	8.1	32.4	56.8	37.8	5.4	10.39	6.6
	5	4	5/1	2.5Y	gray	fine	10	23.9	40.2	33.3	2.6	37.4	64.4	33.3	2.2	5.17	6.5
	5	5	5/1	2.5Y	gray	fine	7	23.2	40.0	34.0	2.9	52.8	46.7	53.3	0.0	4.54	6.8

Site	Trans.	Quad.	soil color	soil chart	granular b	structure	ragmens %	Sieve 10%	Sieve 35%	sieve 100%	>200%	bulk weight	%sub angle	%sub rounded	well rounded	OM%	pH
Spruce Wood	1	1	4/2	2.5Y	dark grayis	very fine	1	0.5	7.2	88.4	4.0	58.9	52.9	44.1	2.9	4.13	7.5
	1	2	3/1	2.5Y	very dark g	very fine	1	1.1	26.6	66.3	6.0	55.1	64.3	33.3	2.4	7.55	7.4
	1	3	4/2	2.5Y	dark grayis	very fine	1	0.3	23.0	74.7	2.0	57.5	52.9	47.1	0.0	3.88	7.4
	1	4	4/2	2.5Y	dark grayis	very fine	1	0.6	23.3	72.1	4.1	64.3	62.2	37.8	0.0	3.62	7.5
	1	5	4/2	2.5Y	dark grayis	very fine	1	0.2	12.8	82.7	4.3	75.1	55.8	39.5	4.7	2.61	7.4
	2	1	4/1	2.5Y	dark gray	very fine	1	0.6	6.0	88.3	5.1	83.5	61.1	36.1	2.8	2.62	6.4
	2	2	4/1	2.5Y	dark gray	very fine	1	0.4	9.5	84.2	6.0	62.1	68.9	31.1	0.0	3.17	6.6
	2	3	4/1	2.5Y	dark gray	very fine	1	0.7	15.8	79.2	4.2	71.9	54.9	45.1	0.0	3.79	6.3
	2	4	4/1	2.5Y	dark gray	very fine	1	2.3	13.4	79.8	4.5	49.1	56.4	43.6	0.0	5.68	6.2
	2	5	4/1	2.5Y	dark gray	very fine	2	1.6	16.6	77.5	4.4	69.3	60.0	40.0	0.0	4.12	6.3
	3	1	5/3	2.5Y	light olive t	very fine	1	0.4	5.0	90.4	4.3	95.2	71.4	26.2	2.4	2.38	7.5
	3	2	5/3	2.5Y	light olive t	very fine	1	0.2	4.1	88.8	6.9	94.2	55.9	32.4	11.8	1.04	6.2
	3	3	5/3	2.5Y	light olive t	very fine	2	0.5	4.8	90.0	4.7	87.5	62.5	35.0	2.5	1.48	7.4
	3	4	4/2	2.5Y	dark grayis	very fine	3	0.6	5.8	88.1	5.6	86.5	63.4	36.6	0.0	4.56	7.5
	3	5	4/2	2.5Y	dark grayis	very fine	2	0.6	5.9	88.9	4.5	82.9	53.5	44.2	2.3	1.67	6.8
	4	1	4/2	2.5Y	dark grayis	very fine	2	1.7	12.3	78.3	7.6	73.0	64.6	33.3	2.1	6.31	7.1
	4	2	5/3	2.5Y	light olive t	very fine	1	0.5	2.0	90.2	7.3	89.2	73.3	26.7	0.0	2.14	6.8
	4	3	4/2	2.5Y	dark grayis	very fine	1	0.5	2.7	89.5	7.2	59.4	48.7	46.2	5.1	3.10	7.3
	4	4	4/2	2.5Y	dark grayis	very fine	1	0.3	7.1	85.7	6.9	61.8	62.7	37.3	0.0	4.93	7.2
	4	5	4/2	2.5Y	dark grayis	very fine	1	0.6	4.9	84.5	9.9	49.2	65.2	34.8	0.0	4.80	7.4
	5	1	5/2	2.5Y	grayish brown			0.6	4.9	81.5	13.0	86.2	61.4	38.6	0.0	2.52	7
	5	2	3/1	2.5Y	very dark g	very fine	2	0.3	4.2	83.7	11.8	60.5	62.7	37.3	0.0	3.93	6.9
	5	3	3/1	2.5Y	very dark g	very fine	3	1.2	10.2	81.3	7.3	54.4	55.3	42.6	2.1	6.48	7.1
	5	4	5/2	2.5Y	grayish bro	very fine	1	1.2	9.9	78.7	10.2	97.3	54.0	44.4	1.6	1.54	6.7
	5	5	3/1	2.5Y	very dark g	very fine	2	2.5	11.9	77.0	8.7	55.9	58.0	42.0	0.0	5.96	6.8
Sandilands	1	1	5/4	2.5Y	light olive t	very fine	1	0.2	3.1	87.5	9.2	95.8	14.5	72.6	12.9	1.66	7.2
	1	2	5/4	2.5Y	light olive t	very fine	1	5.7	4.8	78.7	10.8	80.6	8.6	88.6	2.9	1.77	6.8
	1	3	6/6	2.5Y	olive yellov	very fine	1	2.8	5.1	81.9	10.3	95.9	21.2	69.2	9.6	0.95	7.3
	1	4	6/6	2.5Y	olive yellov	very fine	1	1.0	5.1	81.7	12.3	95.5	11.1	85.7	3.2	1.38	7.1
	1	5	7/6	2.5Y	yellow	very fine	2	0.5	5.1	81.1	13.3	94.9	9.8	85.4	4.9	1.01	7
	2	1	6/4	2.5Y	light yellow	very fine	3	1.4	1.6	47.8	49.2	83.5	48.7	46.2	5.1	2.10	5.8
	2	2	4/3	2.5Y	olive brow	very fine	2	2.8	7.7	57.6	31.9	63.6	28.6	60.7	10.7	5.07	6.9
	2	3	6/4	2.5Y	light yellow	very fine	1	1.7	4.4	42.8	51.1	90.1	44.7	42.1	13.2	1.70	5.9
	2	4	6/4	2.5Y	light yellow	very fine	1	0.9	1.2	43.5	54.3	90.9	25.0	62.5	12.5	1.11	7
	2	5	6/4	2.5Y	olive brow	very fine	2	0.9	1.8	48.5	48.8	95.6	5.0	92.0	3.0	0.75	6.7
	3	1	3/2	2.5Y	very dark g	very fine	1	0.8	7.7	85.4	6.1	47.7	10.0	86.7	3.3	5.16	7
	3	2	4/2	2.5Y	dark grayis	very fine	1	2.3	4.3	83.4	10.1	74.5	10.0	90.0	0.0	2.35	6.5
	3	3	3/2	2.5Y	very dark g	very fine	1	0.7	6.9	85.6	6.8	66.2	1.8	94.5	3.6	4.12	5.8
	3	4	4/2	2.5Y	dark grayis	very fine	1	3.2	8.0	78.6	10.2	91.5	12.5	78.8	8.8	1.28	7.2
	3	5	3/2	2.5Y	very dark g	very fine	1	0.7	6.5	86.5	6.4	40.8	19.4	65.7	14.9	8.00	5.6
	4	1	4/1	2.5Y	dark gray	very fine	3	3.5	16.4	57.7	22.4	32.2	14.8	83.6	1.6	8.22	6.1
	4	2	6/4	2.5Y	olive brow	very fine	1	4.3	17.0	58.3	20.4	82.0	22.2	76.2	1.6	2.87	6
	4	3	4/1	2.5Y	dark gray	very fine	3	5.2	17.4	58.0	19.4	34.3	17.0	80.9	2.1	10.34	5.8
	4	4	4/1	2.5Y	dark gray	very fine	3	8.8	15.8	54.2	21.2	17.9	9.1	89.1	1.8	26.60	5.6
	4	5	4/1	2.5Y	dark gray	very fine	2	5.8	15.2	61.0	18.0	50.4	17.8	82.2	0.0	8.79	5.5
	5	1	4/4	2.5Y	olive brow	fine	1	1.1	20.7	71.3	6.9	81.4	14.3	83.3	2.4	6.35	6
	5	2	6/4	2.5Y	light yellow	very fine	3	6.7	26.5	62.2	4.7	80.7	10.9	82.6	6.5	1.90	5.8
	5	3	4/4	2.5Y	olive brow	very fine	2	2.0	25.3	67.9	4.8	63.5	20.4	77.6	2.0	2.02	6.4
	5	4	6/4	2.5Y	light yellow	fine	2	2.0	17.4	77.6	3.0	69.4	17.5	80.7	1.8	2.95	6.1
	5	5	4/4	2.5Y	olive brow	very fine	1	2.2	18.6	75.1	4.0	59.5	13.0	84.8	2.2	1.79	6.2

Sieve number10 (2.00 mm opening) to collect the silt and clay grains,
Sieve numbers 35 (0.50 mm opening) and 100 (0.149 mm opening) to collect the fine and coarse sand, respectively.
Sieve number 200 (0.074 mm opening) for rocks and pebbles).
Soil color, soil chart, granular and crumb structure, estimating coarse fragments % are determined using soil analysis kit (Forestry Suppliers, Inc., Mississippi, USA).

Appendix E: Summary of sites profile Data from Chapter 2 and 4

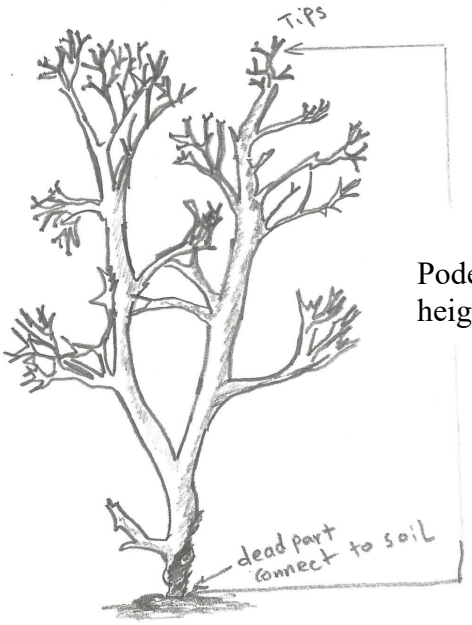
Site	Trans.	Quad.	Cup lichens	der lichens	ier lichens	ose lichens	ose lichens	reocaullon	Vulpicida	Mosses	Ferns	bare rocks	are ground	ody plants	s and grass	etter,	wood	opy cover%
Sherridon	1	1	0.5	22	0	0	0.5	0	0	22	0	35	0	0	20	0	0	
	1	2	0.5	55	0	0	0	0	0	20	0	0	0	0	25	0	0	
	1	3	0.5	65	0	0	0	0	0	15	0	15	0	0	5	0	0	
	1	4	0.5	68	0	0	0	0	0	15	0	0	0	0	17	0	65	
	1	5	0	32	0	0	0	0	0	40	0	25	0	0	3	0	60	
	2	1	5	35	0.5	0	0	0.5	0.5	0	0	0	0	0	0	56	40	
	2	2	5	60	0	0	0	0	0	0	0	0	0	20	0.5	15	80	
	2	3	2	36	0	0	0	0	1	0	0	0	0	12	0	49	90	
	2	4	5	48	0	0	0	0	0.5	0	0	0	0	25	0	22	80	
	2	5	1	47	0	0	0	0	0.5	2	0	0	0	20	0	30	50	
	3	1	66	8	0	0	0	1	0	0	0	0	0	0	0	25	25	
	3	2	60	8	0	0	0	15	0	0.5	0.5	0	0	10	0	6	15	
	3	3	0.5	86	0	0	0	0.5	0.5	5	0	0	0	4	0	5	5	
	3	4	2	42	0	0	0.5	2	0	5	0	30	0	4	2	10	0	
	3	5	3	32	0	0	0.5	5	0.5	3	0	30	0	0	0	25	0	
	4	1	3	2	0	0	0.5	0	0.5	8	0	0	0	0	0	85	80	
	4	2	15	2	0	0	0	0.5	0	8	0	0	0	5	0	70	80	
	4	3	3	8	0	0	0	0	0	2	0	0	0	2	0	86	80	
	4	4	42	5	0	0	0	0	0	0	0	0	0	2	0	50	70	
	4	5	2	2	0.5	0	0	0	0	3	0	0	0	10	0	80	80	
	5	1	60	7	0.5	0	0	0	0	25	0	0	2	5	0	0	0	
	5	2	65	15	0.5	0	0	0.5	0	5	0	0	0	0	4	11	0	
	5	3	65	8	1	0	0	0	0	7	0	0	0	1	2	15	0	
	5	4	42	20	0.5	0	0	0	0	25	0	0	1	0	10	2	0	
	5	5	16	20	0.5	0	0	0	0	40	0	0	0	3	1	20	0	
NorthStar	1	1	5	0.5	0	0	0	1	0	0.5	0	0	0	10	0	84	80	
	1	2	4	40	0.5	15	1	2	0	0	0	15	0	20	2	2	80	
	1	3	61	4	0.5	1	0	0.5	0	2	0	2	0	30	0	0	0	
	1	4	25	45	0.5	5	0	8	0	0	0	2	0	15	0	0	20	
	1	5	20	16	1	25	16	2	0	3	0	15	0	2	0	0	0	
	2	1	1	5	0	0	0	2	0	4	0	0	10	15	45	18	0	
	2	2	20	5	0.5	0	0	0	0.5	3	0	0	0	35	15	20	0	
	2	3	1	0	0	0	0	0	0	0	0	0	14	80	5	0	20	
	2	4	12	2	0	40	0.5	32	0	0.5	0	5	0	8	1	0	20	
	2	5	6	4	0	12	0.5	0	0.5	3	0	25	14	15	0	20	20	
	3	1	63	0.5	0	5	0	0.5	0	1	0	15	0	0	5	10	20	
	3	2	15	5	0.5	0	0	0	0.5	15	3	0	0	4	1	57	20	
	3	3	35	8	0.5	8	6	8	0	0	0	20	0	0	10	5	20	
	3	4	40	3	0.5	0	1	5	0.5	2	0	0	8	30	0	10	0	
	3	5	17	1	0	12	3	12	0	1	25	13	0	0	0	15	0	
	4	1	25	0.5	0.5	25	0.5	0.5	0.5	0.5	0	0	0	45	0	5	40	
	4	2	40	0.5	0.5	10	0	1	0.5	0	0	0	0	30	1	18	20	
	4	3	30	0	0	55	1	2	0	0	0	5	0	3	1	3	20	
	4	4	20	0	0	72	1	3	0	0.5	0	2	0	2	0.5	0	40	
	4	5	55	5	0	15	0	0	0	7	1	0	0	2	13	2	20	
	5	1	40	0.5	0	3	0	0.5	2	0.5	0	0	0	0.5	0	50	80	
	5	2	90	0.5	0.5	1	0	0	0	1	0	0	0	0	0	8	80	
	5	3	55	0	0	34	0	2	0.5	2	0	6	0	0	0	5	80	
	5	4	81	0.5	0	5	0	1	0.5	0	0	2	0	0	0.5	10	20	
	5	5	3	18	0	2	0	0	0.5	0	0	0	0	30	20	17	0	
Athapap	1	1	50	0	0	1	0.5	0	0	10	0	0	0	25	10	4	20	
	1	2	5	2	0	1	12	0	0	32	0	3	0	3	35	7	0	
	1	3	15	0.5	0	0.5	0	0	0	3	0	0	0	30	35	15	0	
	1	4	5	0.5	0	0	0	0	0	2	0	0	0	35	28	30	60	
	1	5	35	0.5	0	0	15	0	0	30	0	0	0	70	3	40	90	
	2	1	10	0	0	0	1	0	0	65	0	0	0	20	8	0	40	
	2	2	5	15	0	0	0.5	0	0	35	0	0	0	10	25	5	60	
	2	3	70	8	0	0	0	0	0.5	40	0	0	0	2	12	10	80	
	2	4	50	0.5	0	0	5	0	0	40	0	0	0	2	15	0	30	
	2	5	80	0	0	0	18	0	0	40	0	0	0	15	3	15	80	
	3	1	10	0	0	2	0	0	0.5	75	0	0	0	1	12	0	10	
	3	2	20	1	0.5	1	6	0	0	12	0	0	0	25	20	10	20	
	3	3	15	0.5	0	15	1	1	0	50	0	0	0	8	10	0	20	
	3	4	25	0.5	0	2	1	0	0	80	0	0	0	15	8	2	10	
	3	5	0.5	0	0	1	4	3	0	80	0	0	0	1	10	0	20	
	4	1	0.5	0	25	0	1	0	0	60	0	0	0	10	1	3	60	
	4	2	0.5	0	45	3	1	0	0	20	0	0	0	10	0	20	70	
	4	3	0.5	0	10	0	0	0	0	3	0	15	10	10	10	40	60	
	4	4	0.5	0	35	0	0	0	0	0.5	0	0	0	20	15	30	0	
	4	5	5	0	0	0	0	0.5	0	3	0	0	20	50	10	10	10	
	5	1	1	0	64	0	0	0	0	5	0	0	0	10	10	10	60	
	5	2	0.5	0	17	0	0	0	0	35	0.5	0	26	5	1	15	0	
	5	3	2	0	65	0	0	0	0	0	0.5	0	5	12	10	5	0	
	5	4	1	0	55	0	0	0	0	2	0	0	5	2	30	5	0	
	5	5	2	0	35	0	0	0	0	2	1	0	0	36	3	20	60	

Site	Trans.	Quad.	Cup lichens	der lichens	lier lichens	ose lichens	ose lichens	reocaullon	Vulpicida	Mosses	Ferns	bare rocks	are ground	ody plants	s and grass	etter, wood	opy cover%
Spruce Woo	1	1	10	0	0	0	0	0	0	50	0	0	0	0	20	20	0
	1	2	15	0.5	0	0	0	0	0	2	0	0	15	0	60	7	0
	1	3	5	0	0	0	10	0	0	10	0	0	10	20	30	15	20
	1	4	63	0	0	0	0	0	0	2	0	0	0	10	20	5	0
	1	5	60	0	0.5	0	0	0	0	0	0	0	10	13	17	0	0
	2	1	40	0	0	0	0	0	0	10	0	0	8	0	52	0	0
	2	2	40	0.5	0	0	0	0	0	0	0	0	0	0	55	4	0
	2	3	65	0	0.5	0	0	0	0	2	0	0	0	3	30	0	0
	2	4	65	0	0	0	0	0	0	5	0	0	5	0	20	5	0
	2	5	5	0	0	0	0.5	0	0	5	0	0	20	0	27	43	0
	3	1	25	0	0	0	0	0	0	2	0	0	15	30	10	18	60
	3	2	40	0.5	0.5	0	0	0	0	3	0	0	4	0	43	10	0
	3	3	45	1	0.5	0	0	0	0	5	0	0	0	0	42	8	0
	3	4	40	25	0	0	0	0	0	0	0	0	0	20	12	2	0
	3	5	63	0	0.5	0	0	0	0	2	0	0	5	0	20	10	0
	4	1	15	0	0	0	1	0	0	40	0	0	2	25	13	5	0
	4	2	70	0	0	0	0	0	0	5	0	0	0	5	20	0	0
	4	3	25	0	0	0	0	0	0	5	0	0	0	60	10	0	0
	4	4	53	0	0	0	0	0	0	2	0	0	0	5	40	0	0
	4	5	73	0	0	0	0	0	0	2	0	0	0	13	10	2	0
	5	1	50	0	0	0	0	0	0	2	0	0	16	0	32	0	0
	5	2	55	0	0	0	0	0	0	0.5	0	0	2	0	37	5	0
	5	3	74	0	0	0	0	0	0	5	0	0	1	0	20	0	0
	5	4	65	0	0	0	0	0	0	0	0	0	2	29	2	2	5
	5	5	47	3	0	0	0.5	0	0	0	0	0	0	33	2	15	10
Sandilands	1	1	10	0	0	0	0	0	0	20	0	0	40	0	30	0	0
	1	2	15	0	0.5	0	0	0	0	10	0	0	30	0	45	0	0
	1	3	60	1	0	0	0	0	0	5	0	0	9	0	25	0	0
	1	4	15	0	0.5	0	13	0	0	2	0	0	30	0	40	0	0
	1	5	10	0	0	0	0	0	0	15	0	0	0	0	75	0	0
	2	1	30	0	0	0	0	0	0	2	0	0	0	0	58	10	20
	2	2	4	4	0	0	18	0	0	20	0	0	0	0	39	15	80
	2	3	15	15	0	0	0	0	0	10	0	0	0	0	50	10	60
	2	4	50	5	0.5	0	0	0	0	0.5	0	0	0	0	35	10	10
	2	5	25	0	0	0	0	0	0	30	0	0	10	0	7	28	10
	3	1	10	80	0.5	0	0	0	0	3	0	0	0	0	2	5	0
	3	2	3	15	0	0	1	0	0	0	0	0	50	5	25	0	0
	3	3	10	1	0.5	0	0	0	0	15	0	0	42	0	32	0	0
	3	4	30	0	0	0	5	0	0	0	0	0	25	0	40	0	0
	3	5	7	45	0	0	0	0	0	3	0	0	10	0	10	25	0
	4	1	10	20	1	0	5	0	0	8	0	0	0	35	15	7	20
	4	2	7	1	1	0	1	0	0	0	0	0	0	70	20	2	30
	4	3	5	23	0.5	0	0	0	0	25	0	0	0	0	10	37	20
	4	4	5	1	0	0	0	0	0	10	0	0	0	68	15	2	20
	4	5	20	15	1	0	0	0	0	0	0	0	0	20	15	30	20
	5	1	20	1	0.5	0	0.5	0	0	3	0	0	0	35	0	42	0
	5	2	25	2	0.5	0	6	0	0	1	0	0	10	20	15	20	0
	5	3	20	5	0.5	0	0	0	0	0	0	0	0	50	5	20	0
	5	4	55	25	0.5	0	3	0	0	0	0	0	0	10	5	2	0
	5	5	50	20	0	0	1	0	0	0	0	0	0	2	14	14	0

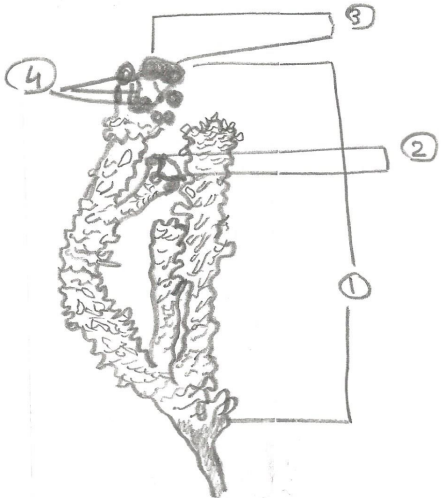
Soil color, soil chart, granular and crumb structure, estimating coarse fragments % are determined using soil analysis kit (Forestry Suppliers, Inc., Mississippi, USA).

R

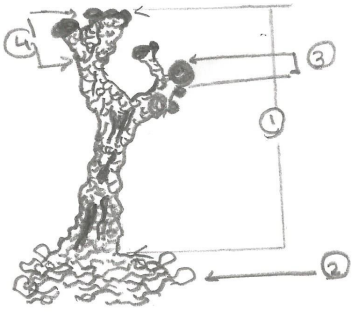
Appendix F: Morphological characteristics measured for six lichen species (*C. arbuscula, C. pyxidata, C. cariosa, C. gracilis* ssp. *turbinata, C. cristatella,* and *C. rangiferina*) in chapter 4.



C. arbuscula

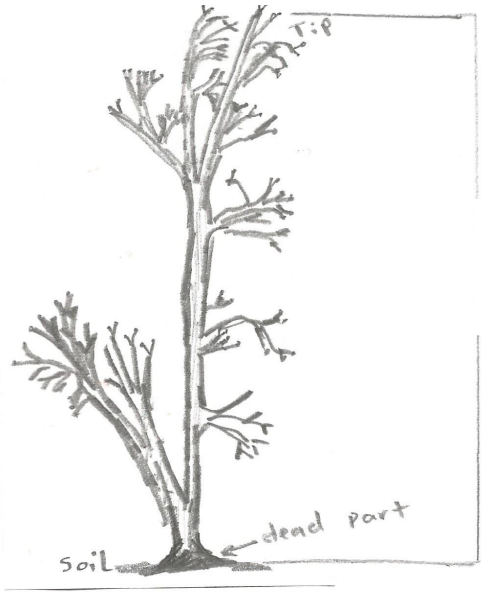


C. gracilis ssp. *turbinata*
1. Podetium height (mm)
2. Cup diameter (mm)
3. Apothecium diameter (mm)
4. Number of podetial branches



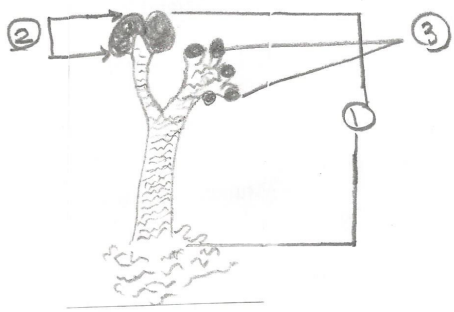
1. Podetium height(mm)
2. Squamule area (mm^2)
3. Apothecium diameter (mm)
4. Number of podetial branches

C. cariosa



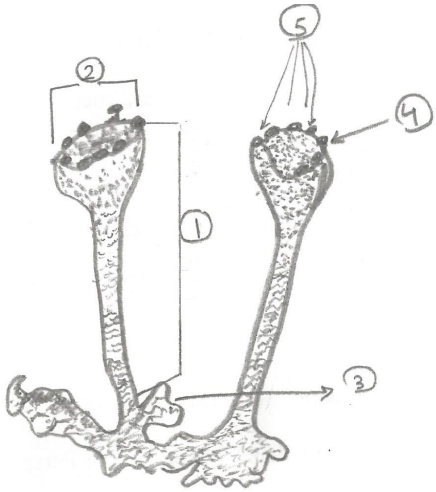
Podetium height(mm)

C. rangiferina



1. Podetium height (mm)
2. Apothecium diameter (mm)
4. Number of podetial branches

C. cristatella



1. Podetium height (mm)
2. Cup diameter (mm)
3. Squamule area (mm^2)
4. Apothecium diameter (mm)
5. Number of apothecia on cup

C.pyxidata

T

Appendix G: Comparison of the means of polyketide concentration for each variable (3 temperatures and 2 humidity values) using one-way ANOVA showing d.f., F ratio, and p-value for each of usnic acid, fumarprotocetraric acid, and atranorin and for each of the in vivo and in vitro experiments. The results show significance statistics for Figures 5.1 and 5.2.

Usnic acid	**d.f.**	**F Ratio**	**Prob > F**
In vivo			
D4	3	**29.1390**	**0.0001***
D18	3	4.7184	0.0353
D32	3	**5.5072**	**0.0024***
M4	3	0.4755	0.5478
M18	3	**10.5908**	**0.0037***
M32	3	1.4324	0.3035
In vitro			
D4	3	0.0878	0.9648
D18	3	2.3691	0.1465
D32	3	4.0919	0.0493
M4	3	0.5480	0.6633
M18	3	6.3548	0.0164
M32	3	**36.5070**	**<0.0001***
Atranorin	**d.f.**	**F Ratio**	**Prob > F**
In vivo			
D4	3	0.7170	0.5691
D18	3	**6.7875**	**0.0137***
D32	3	**31.6267**	**<0.0001***
M4	3	0.2058	0.8896
M18	3	**9.0232**	**0.0060***
M32	3	0.3749	0.7736
In vitro			
D4	3	1.5698	0.2708
D18	3	1.4980	0.2874
D32	3	0.5898	0.6387
M4	3	0.5841	0.6421
M18	3	1.0493	0.4222
M32	3	**13.4086**	**0.0017***
Fumarprotocetraric acid	**d.f.**	**F Ratio**	**Prob > F**
In vivo			
D4	3	0.0254	0.9941
D18	3	**25.2445**	**0.0002***
D32	3	**14.1807**	**0.0014***
M4	3	0.5743	0.6478
M18	3	3.7618	0.0595
M32	3	0.4247	0.7407
In vitro			
D4	3	0.1335	0.9374
D18	3	0.3586	0.7847
D32	3	1.0754	0.4126
M4	3	0.5418	0.6671
M18	3	1.3790	0.3175
M32	3	0.3832	0.7681

U

Appendix H: One -way ANOVA results for Figure 5.4, showing degrees of freedom (d.f.), F ratio and *P* value for treatment comparisons within each temperature.

Polyketides	d.f.	F Ratio	Prob > F
Fumarprotocetraric acid			
Temp. 4°C	3	85.6406	**<0.0001***
Temp. 18°C	3	57.3233	**<0.0001***
Temp. 32°C	3	37.4850	**<0.0001***
Usnic acid			
Temp. 4°C	3	30.6443	**<0.0001***
Temp. 18°C	3	89.0246	**<0.0001***
Temp. 32°C	3	56.1316	**<0.0001***
Atranorin			
Temp. 4°C	3	82.3459	**<0.0001***
Temp. 18°C	3	22.3565	**0.0003***
Temp. 32°C	3	22.4806	**0.0003***