Sterol And Galactolipid Composition of the Lichen Photobiont Genus *Trebouxia* Compared with a Potential Host Lichen in the Genus *Usnea*

By

Henry Whittemore

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Thesis committee:

Dr. Jeffrey D. Leblond, chair

Dr. Donald M. Walker

Dr. Jeffrey Walck

Abstract

Lichens are a diverse group of cosmopolitan fungi that are notable for their close symbiotic relationship with single-celled photosynthetic organisms. These symbionts are typically chlorophyte algae, though some groups host cyanobacteria, which provide the fungal component with photosynthesized nutrients. This relationship is so close that the fungus cannot grow properly without its symbiote, and as a result biochemical analysis of lichens typically looks at both the fungal and algal components together. Understanding these organisms separately and identifying the nature of their symbiosis helps us understand both lichens and symbiosis better, and while the fungus's reliance on its algal partner complicates this goal, there are ways around it, particularly if we focus on the algal component. Using unialgal cultures of *Trebouxia*, a common chlorophyte algae symbiote genus which can grow in media without a fungal component, this study identifies the sterols and galactolipids found in isolated *Trebouxia erici* and *Trebouxia excentrica* algae and compares them to those of wild *Usnea* lichens in middle Tennessee, using gas chromatography/mass spectrometry analysis to identify the sterol lipids and positive-ion electrospray/mass spectrometry to identify galactolipids. *Trebouxia* species are a broadly used lichen symbiont typically found in species of *Usnea* lichen, which allows us to distinguish which sterol structures in the whole lichen samples are synthesized by the algae and which by the fungus. While there is evidence in the literature that symbiosis changes the metabolism of algae, which could potentially affect sterol synthesis, eliminating the sterols synthesized by the algae from the sterols of the

lichen leaves sterols that are either synthesized by the fungus or require its presence to form in the algae. This allows for separate biochemical analysis of these closely paired organisms - for example whole lichen samples contain ergosta-5,8,22E-trien-3β-ol (lichesterol) and 24-methylcholesta-5,7,22E-trien-3β-ol (ergosterol), common lichen sterols, but these are not present in unialgal samples, connecting their synthesis to the fungal symbiont. The greatest percentage of sterols found in algal samples, however, are also found in whole lichen samples in smaller proportions relative to the fungal sterols. These sterols, 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol) and 24-methylcholest-5en-3β-ol (campesterol), are synthesized by algal symbiont species regardless of the presence of a fungal partner, so are of algal origin. Galactolipids are only found in chloroplasts, which the fungal component of lichens lacks completely, but these lipids are compared between 2 species within an algal genus and between cultured and lichenized algal samples as a lipid type known to belong only to the algal partner. This serves as a baseline for lipid variation between algal sample groups, as well as providing galactolipid data for a group of green algae. This study finds a common monogalactosyldiacylglycerol (MGDG) with an 18:3/16:4 *sn*-1/*sn*-2 fatty acyl structure shared by algae across all groups, symbiotic or cultured. *T. erici* algal groups also shared all their smaller percentage MGDG and digalactosyldiacylglycerol (DGDG) molecules. *T. excentrica* showed only the 18:3/16:4 MGDG found in both algal groups and a set of 3 DGDG molecules, of which one was not shared by the *T. erici* lines - an 18:2/16:2 DGDG. Only 2 galactolipids were identified in the *Usnea* lichen samples, the 18:3/16:4 shared by all algal groups and an 18:3/18:3 DGDG shared by both *T. erici* lines. This study shows the lipid profiles of *T. erici* culture lines 911 and 912 are highly similar, with identical

galactolipids and only small differences in sterol composition, while *T. excentrica* has a distinct sterol profile with sitosterol and lacks an unidentified C_{29} sterol found in both algal groups examined here. *T. excentrica* also has fewer galactolipids overall compared to *T. erici* and contains an 18:2/16:2 DGDG not found in any other sample groups. Comparison with whole *Usnea* lichen samples confirms the presence of lichesterol, ergosterol, and an unidentified C_{28} sterol in the fungal *Usnea* that are absent in all algal sample groups.

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Chapter I - Introduction

Lichen symbiosis

Symbiosis can be broadly defined as any relationship between 2 organisms of different species that live together and interact. Because of the breadth of this definition there are traditionally 3 subcategories within symbiosis - parasitism, mutualism, and commensalism (Redman et al. 2001). There is an implication of intimacy and constant/regular contact when a relationship is symbiotic, however there is some ambiguity in defining a threshold of 'closeness' between 2 organisms, so this qualifier is often not included in the strict definition (Martin and Schwab 2013). Terms like 'obligate' are useful to denote the closeness of a relationship if one or both species require the other to survive, but beyond that more specific subcategories, defined by what the organisms involved lose or gain in the interaction, are more useful to characterize the nature of a symbiosis (Martin and Schwab 2013).

While symbiosis describes a biological relationship, whether beneficial, detrimental, or neither to the parties involved, for much of the time since the term was first coined it has been used interchangeably to refer either to this definition or to a relationship where both species gain a survival benefit (Leung and Poulin 2018). While this definitional confusion is understandable, many classic examples of symbiotic relationships are mutually beneficial, and unambiguous definitions are crucial to clear scientific communication. This form of symbiosis is referred to as mutualism, to distinguish it from other forms of symbiosis where one of the species involved gains nothing or loses something (Leung and Poulin 2018). Taxonomically, mutualism often creates a mirrored phylogeny between the organisms involved, with ancestral partners speciating at the same

rate. This is because evolutionary changes in a population of one organism involved in a mutualistic symbiosis puts evolutionary pressure on their partner organisms to adapt along with them (Hecht et al. 2018). This can be helpful in identifying mutualistic relationships, although other long lasting symbiotic relationships such as parasitism look phylogenetically similar in some cases as the host develops defenses which the parasite evolves in turn to resist (Divakar et al. 2015, Hecht et al. 2018).

Parasitism is an example of symbiosis that is detrimental to one of the organisms involved. In parasitism, one species gains a benefit at the cost of the other, often by extracting nutrients from its host species (Leung and Poulin 2018). This is similar in terms of the cost/benefit relationship to another ecological relationship, predation, and many parasitic organisms do absorb nutrients partially or completely on their host's tissue, but parasitism is differentiated by a couple of key features. First, parasites typically do not kill hosts (directly at least), but feed in small amounts over time on specific tissues such as blood or sap, maintaining a longer-term relationship (Westwood et al. 2010). Parasites often feed off a single host organism for their entire life, making their own survival dependent on that of their host (Westwood et al. 2010). Second, while nutrition is often the benefit gained by the parasite in this relationship, there are many parasitic relationships that have nothing to do with food. Brood parasites in birds are an example of reproductive parasitism, in which a bird species saves the time and energy involved in raising chicks by laying their eggs in the nest of another species and tricks or forces the host to care for their chick at the cost of the host's own chicks (Hamilton and Orians 1965).

Both forms of symbiosis discussed so far are defined by the effect they have on each member's survival, positive or negative. However, relationships also exist in which one member is unaffected by the species gaining a benefit from it. These relationships are commensal, and often involve one organism taking advantage of a byproduct of another's behaviors or processes (Redman et al. 2001). Many species have a commensal relationship with trees for example, from vascular plants to arthropods to lichens. These massive plants provide animals with shelter out of reach of predators, photosynthetic epiphyte species with greater access to sunlight in the upper branches, and fungi with shade and fertilizing leaves (Nadkarni 1986). If these organisms do not burrow into the tree or eat parts of it, the relationship often has no appreciable effect on the tree (Leung and Poulin 2018). This example shows how commensalism can be difficult in practice to separate from the other 2 forms of symbiosis discussed so far, since an apparent commensal epiphyte can easily confer some small harm (damaging bark, stealing minerals from the tree tissue) or small benefit (adding a layer of protection to the bark, producing volatiles that deter pests) which is not easily observable to researchers (Nadkarni 1986).

Lichens are a group of fungi defined not by membership in a particular clade, but by their symbiotic relationship with photosynthetic species of algae and cyanobacteria. This lifestyle has evolved multiple times, primarily in ascomycetes, although there are a small number of species from the basidiomycetes and other fungal phyla that have evolved it as well (Honegger 1996). A lichenized fungus holds colonies of single-celled photosynthesizers, typically green algae, in a specialized layer within its body, and feeds on carbohydrates produced by them (Richardson 1973). While the photobiont in the

relationship can be a eukaryotic or prokaryotic alga, just as the mycobiont can be an ascomycete or a basidiomycete fungus, eukaryotic photobionts are much more common and representative in lichens (, Honegger 1996, Jahns 1973) and specifically ascomycete/green algal pairings are the focus of this study. The relationship between photobiont and mycobiont is considered a mutualistic symbiosis, as the algae get a home and the fungus gets food, but this is likely not true of all lichens, particularly the more recently lichenized and less developed groups (Honegger 1991). The fungus is an obligate symbiote of its photobiont which it cannot develop normally without - many lichen groups can use a variety of algal genera as a photobiont so are not dependent on a single species or genera but cannot survive long term without some compatible symbiont (Hill 2009). The effects of symbiosis on the algal component are less clear however, with advantages and disadvantages. The fungus provides its lichenized symbiont a moist environment even during dry periods with water stored in its medulla, a safe space away from competition and grazing, and better access to sunlight from the lichen's large surface area and preference for growing on elevated sunny surfaces (Honegger 1991, Richardson 1973). However experimental evidence shows lichenization slows the reproduction rate of photobionts compared to growth as an algal culture, and the photosynthesizing partner does give up a significant portion of its carbohydrate production to its mycobiont, meaning there are potentially significant downsides for the photobiont as well (Richardson 1973). This suggests some lichen groups are closer to commensal symbiotes, with the fungus benefiting from the algae inside of it while the algae experience a net neutral effect. There are even cases of lichen parasitism in fungal species closely related to lichens (Divakar et al. 2015), so it is reasonable to assume there

are outlier species of lichen where the fungal component captures algae that may have been better off free living and feeds off the carbohydrates produced to their detriment.

Figure 1: Comparison of lichen anatomical types

A. Crustose lichen (no physical ID possible)
B. foliose lichen (likely genus *Parmellia*)
C. fruticose lichen (genus *Cladonia*)
Photos by author, IDs made using Phillips 1963

Anatomically, lichens are divided into 3 principal groups - crustose, foliose, and fruticose lichens (Figure. 1). As with the category of lichen itself, these divisions have been found to have little taxonomic significance but do indicate the level of complexity and differentiation in the lichen's external anatomy (Jahns 1973). Crustose lichens are the simplest form and grow directly on the surface they are attached to as a thin layer of fungi and algae (Büdel and Schiedegger 1996). Lichens with thin, leaf-like structures that grow out from the surface the lichen is attached to as it develops are called foliose lichens, while lichens with thicker outgrowths resembling bushes or buds are called fruticose lichens (Stenroos and DePriest 1998). It was initially hypothesized that foliose lichens are an intermediary stage of development and that fruticose lichens represent the

most highly differentiated form for a lichen - dissection and comparison of internal organ structures between these groups shows crustose lichens to be anatomically simpler than the other 2 groups, however foliose and fruticose lichens were found to be of comparable levels of complexity to each other (Büdel and Schiedegger 1996, Jahns 1973).

The 'body' or thallus of a lichen is composed of 3 main layers - the outer surface cortex, the algal layer, and the inner medulla (Jahns 1973). All 3 of these tissues are composed of hyphae, the fibers of which are typically woven together to form a plectenchymatous tissue structure throughout the thallus (Büdel and Schiedegger 1996). The outer layer, the cortex, is a protective layer of cells over the algal tissue of a lichen which in many groups helps to trap water, keeping the algae hydrated (Souza-Egipsy et al. 2000). The thickness of the cortex can vary, and a few lichens have a cortex that is partially or completely absent with algae residing on the lichen's surface, but in most lichens it is thin enough to allow light to pass through while thick enough to protect the algal layer (Jahns 1973). The middle layer of a lichen is the algal layer where the single-celled photobionts live, and where contact between the fungal and algal partners takes place. In some lichen groups the mycobiont and photobiont make no direct contact, while in others fungal hyphae surround the algal cells and hold them in place (Jahns 1973). This holding can be done through pressure alone with appressoria cells, but in some groups, such as basidolichens, they are pinned by penetrative fungal cells known as haustoria, which can damage or kill the algal cells they are penetrating (Sanders and Masumoto 2021). This is another example of potentially detrimental effects on the photobiont in some lichen groups, though the splitting of algal cells with haustoria can increase the fission rate of these algae, which could also benefit it by increasing reproduction rates

(Peveling 1973). The algal layer's position in the thallus is not permanently fixed but can move to better access sunlight if needed - if a lobe of the thallus on a lichen becomes twisted or bent so the reverse side now faces the sun, the algal layer will migrate to the reverse side over time (Jahns 1973). The innermost layer of lichen tissues is the medulla - here the hyphae is most loosely woven, forming a fibrous structure, and in lichen species which require more thallus support an axial strand of dense tissue runs through the center (Jahns 1973). The medulla is also an important storage space for the lichen, holding large quantities of water and nutrients (Souza-Egipsy et al. 2000). A lichen attaches itself to the surface it grows on, whether this be a living tree, a dead log, or a rock, with a root like system of hyphae called rhizines (Jahns 1973).

Within the algal layer, photosynthesizers replenish their numbers over time through mitotic division, as well as the production of aplanospores - most lichenized algae can also produce reproductive zoospores but many groups convert these into aplanospores as well when lichenized (Ahmadjian 1960). The lack of sexual reproduction may be a behavioral response by the algae itself to the environment of the algal layer (Jahns 1973) or the result of appressoria holding these cells preventing motile zooids from forming (Sanders and Masumoto 2021), and the exact mechanism likely differs between groups for both algal and fungal partners. The fungal symbiote in ascomycete lichens is also capable of both sexual and asexual reproduction, but unlike its symbiote typically reproduces using both methods (Letrouit-Galinou 1973). For a newly produced fungal ascospore of a lichenized group, the separate reproductive methods of these partner groups present a problem - resynthesis. To develop into a lichen, these fungi will have to acquire a new photosynthetic symbiote, in many cases from the environment it landed in

as a spore (Sanders and Masumoto 2021). If an acceptable partner species is not present the fungus will not develop normally, likely a major factor in many lichenized fungal groups' ability to use algal partners from different genera rather than specializing to a single partner (Ahmadjian 1973). There are also lichen groups that have developed strategies to pass their algal partners on to their offspring, bypassing the need to acquire a suitable photobiont. These lichens, for example *Pertusaria* and *Lecidea* species, produce ascospores that have been observed to carry a few symbiotic algal cells on their surface. These cells are found trapped in a gelatinous layer of their apothecium, having been picked up on transit through the ostiole before dispersal, and provide an initial inoculation of symbiotic partners for each new developing lichen (Ahmadjian 1973).

As lichens are not a monophyletic group but an ecological lifestyle for fungi, many lichen groups are more closely related to non-lichenized fungi than to other lichen groups (Gargan et al. 1995). Convergent evolution has created many similarities across lichen groups, but the fundamental diversity of lichens means there are exceptions to every general statement that can be made about lichens beyond their definition as a symbiotic association between a macroscopic fungal species and a single-celled photosynthetic species (Gargan et al. 1995). Considering this, the number of times the strategy of lichenization has convergently evolved in different fungal groups and the number of traits shared by most lichen groups despite their diversity is impressive. For instance, the metabolic processes of lichenized fungi and their photobionts across a wide range of lichen groups cooperate to create polyols such as arabitol and mannitol that neither organism can synthesize on their own (Honneger 1991). These polyols aid both lichen

and alga in water retention and resistance to desiccation and allow lichens to survive without water far longer than fungi or algae would alone (Honneger 1991).

Lichens are far from the only fungal lifestyle that relies on an ecological relationship with an autotroph for carbohydrates - saprotrophic fungal species rely on photosynthesizers in their environment for dead matter to consume (Boddy and Hiscox 2016), and mycorrhizal fungi have a mutualistic relationship with most terrestrial plants in which they promote root growth and nutrient uptake in exchange for sugars (Schüßler et al. 2006). The lichen relationship is built on this same autotroph/heterotroph pairing which provides the fungus with nutrients but is unique in the way the fungus cultivates its algal partner within its own body. The symbiosis between fungus and photobiont in many ways resembles human agriculture, or species of insect like ambrosia beetles that obtain their food by growing and tending fungi as a food source (Hulcr and Stelinski 2017). The fungus clearly benefits in the relationship, and because it is perpetuating a clonal line of algal cells and in some cases passing this same line on to its descendants, the algae benefit as well from an evolutionary perspective. In the alga's case, however, both positive and negative effects must be weighed against one another to find the net effect, meaning the label of mutualism should be applied with care to lichen groups rather than as a blanket definition for all lichenized symbioses (Sanders and Masumoto 2021).

Usnea is a genus of fruticose lichens found living in the upper branches of trees throughout much of the world (Eriksson et al. 2018). The distinct branching structure of fruticose lichens is useful for field identification of *Usnea* specimens in Tennessee, so this genus and its photobionts are the focus of this study. A common photobiont genus

used by many lichen groups (Sanders and Masumoto 2021) and specifically by members of the *Usnea* genus is *Trebouxia* (Ahmadjian 1967) - during photosynthesis *Trebouxia* species produce carbohydrates, using some to feed their own cellular processes and converting the excess into sugar alcohols (polyols) for storage and transfer (Tran et al. 2020). *Trebouxia* species produce a specific polyol, ribitol, to feed their fungal hosts (Richardson et al. 1967) - the fungal component of the symbiotic relationship must then convert the ribitol into new polyol forms including sorbitol, xylitol, and mannitol to use for their own metabolism (Tran et al. 2020). The fungus absorbs these carbohydrate structures and is unable to absorb related structures like glucose, sucrose, or ribose in the same way (Richardson et al. 1967). The specificity of the processing these fungi carry out in their thallus structures and their dependence on this molecule for nutrients demonstrate a specialization to the phytobiont's carbohydrate metabolism. Richardson et al. (1967) were working here on a lichen in the *Lobaria* genus that uses *Trebouxia* algae - this genus of algae is found in multiple lichen groups rather than each fungal genus using a specific genus of photobiont (Sanders and Masumoto 2021) - but the results suggest some degree of evolutionary adaptation to use particular symbiont groups in lichen (Richardson et al. 1967).

Lipid classes and utility as biomarkers

Lipids are a broad category of biomolecules found in many roles across the cell, defined primarily by their insolubility in water and solubility in organic solvents (Fahy et al. 2005). In eukaryotic cells, most lipid types are synthesized in the endoplasmic reticulum (ER) (Jacquemyn et al. 2017) and are typically composed of fatty acyl groups

forming long chains and/or in combination with sugars, alcohols, and other biomolecules (Schwarz and Blower 2016). This fatty acyl group cannot be used to define all lipids, as entire classes of lipids (i.e. sterols and prenols) are based on isoprene instead, but it is a common lipid component (Ohlrogge and Browse 1995). These synthesized lipids are distributed to other cell organelles via the secretory pathway and/or ER contact sites (Jacquemyn et al. 2017). Most lipid synthesis enzymes are transmembrane proteins, a type of protein that spans across the lipid bilayer of a cell membrane, with segments of the protein exposed to both the extracellular and intracellular environments. The ER's regulatory mechanisms modify the activity of these and other ER-localized enzymes to maintain cellular lipid homeostasis and respond to physiological and developmental stimuli, making the ER the central regulator of lipid levels across the cell (Jacquemyn et al. 2017). While some organelles contain lipids absent from the ER, such as the galactolipids found in chloroplasts (Sato and Awai 2016), most of these are derived from the enzymatic modification of ER-synthesized lipids after secretion, highlighting the importance of the ER in the synthesis and modification of lipids in eukaryotic cells (Jacquemyn et al. 2017). Multiple systems exist for categorizing types of lipid molecules, with slight variations in how they are grouped between them, so for clarity this thesis will use the categorization system proposed by Fahy et al. in their 2005 paper, as it fits closely with International Union of Pure and Applied Chemistry (IUPAC) nomenclature and organizes compounds clearly by biosynthetic structure and origin (Fahy et al 2005).

Fatty acids are an important lipid group primarily because they serve as a base for other lipid types to be built from. On their own, fatty acids are used in cells to maintain the cell membrane by modifying the structure of phospholipids, and polyunsaturated fatty

acids play a role in cell signaling, (Hou et al. 2016) but the majority of fatty acids in an organism are incorporated into larger lipid molecules such as phospholipids, sphingolipids, and other glycerolipids, which will be discussed later (Fahy et al. 2005). Fatty acid synthesis follows a consistent pathway across different groups of organisms: the enzymes and protein complex(s) involved vary by kingdom, but in all cases fatty acids are formed by carboxylation of the coenzyme acetyl-CoA into extender units which repeatedly condense an acyl-CoA derived starter unit, extending a chain of acyl units from the starter (Carvalho and Caramujo 2018).

Glycerolipids are a group of lipids composed of 1-3 fatty acid chains connected to a glycerol alcohol backbone (Fahy et al. 2005). Uncharged glycerolipids are primarily used for long term fatty storage in the form of mono-, di-, and triglycerides. Many glycerolipids also include a phosphate group attached to the glycerol backbone, which is typically negatively charged and renders the entire molecule amphiphilic (Raetz and Dowhan 1990). These charged glycerolipids, also called glycerophospholipids or simply phospholipids, are the primary component of the lipid bilayer of cell membranes along with a variety of catalytic proteins and sterols (Mannock et al. 2010). The differential charge of the phosphorylated glycerol head and hydrophobicity of the fatty acid chain tails in these glycerophospholipids maintains the alignment of the membrane bilayer. Phospholipids regulate the permeability of the cell membrane as a part of this bilayer, which also acts as a synthesis site for other membrane lipids (Raetz and Dowhan 1990). Some specialized glycerophospholipids such as diacylglycerol also act as cellular messengers (Fahy et al. 2005).

Another specialized glycerolipid group is the galactolipids - these lipids are notable in photosynthetic organisms as they are formed within the chloroplasts of algae and plant cells from base fatty acids produced by the ER (Jacquemyn et al. 2017, Sato and Awai 2016), and play important roles in chloroplasts, controlling grana arrangement and otherwise modulating their internal structure in response to light levels (Rocha et. al. 2018). Galactolipids contain one or more galactose molecules as their sugar/alcohol backbone, forming monogalactosyldiacylglycerol (MGDG) with a single galactose molecule or digalactosyldiacylglycerol (DGDG) with 2, and can be characterized by the structure of the attached fatty acid chain as well (Guella et al. 2003). In green algal and plant chloroplasts, both galactolipids are formed by the galactosylation of diacylglycerol, attaching the first galactose molecule to form MGDG and forming DGDG by an additional galactosylation. DGDG can be further galactosylated to form trigalactosyl-diacylglycerol (TGDG), but MGDG and DGDG are the most common galactolipids found in chloroplasts (Kobayashi et al. 2007). A mounting body of evidence supporting the endosymbiotic origin theory for these organelles, which means that shared biosynthetic pathways for galactolipid synthesis can be used to identify photosynthetic clades that diverged post-endosymbiosis. These organisms would all contain chloroplasts descended from that same endosymbiont, so typically share similar galactolipid molecules (Sato and Awai 2016).

Sphingolipids are a family of lipids unified by their use of a sphingoid backbone. This backbone is synthesized from the condensation of the amino acid serine with a long-chain fatty acyl-CoA, which is reduced to form the sphingoid base (Futerman and Riezman 2005). From this base ceramides, phosphosphingolipids, glycosphingolipids, and protein

sphingoid adducts can be constructed (Holthuis et al. 2001). Ceramides add a fatty acyl group to this backbone, while phosphosphingolipids and glycosphingolipids incorporate a phosphorylated hydroxyl group or glycerol molecule respectively into the sphingolipid structure in addition to the fatty acid group (Futerman and Riezman 2005). These more complex sphingolipids can also be classified with the fatty acids, phospholipids, and glycolipids according to the molecules added to the sphingoid base, but Fahy et al (2005) has grouped them together here as they serve similar functions in cellular metabolism. Sphingolipids can be commonly found as components of the cell membrane, but they work mainly as cellular regulators and messengers, inhibiting cellular growth at times by inhibiting protein kinases but also stimulating growth by promoting DNA binding/ activating kinases (Spiegel and Merrill 1996).

Sterol and prenol lipids are constructed from a different base molecule than all other lipid classes, being derived from the 5-carbon terpene unit isoprene – this technically makes both groups alcohols but they are still categorized as lipids based on their hydrophobic organic solubility (Fahy et al. 2005). All steroids are based on gonane – a 17-carbon molecule of 4 rings - and are characterized further based on the structure and positions of alkylation of the side chain (Mahdy et al. 2022). Sterols are the primary subcategory of steroids, defined as gonane molecules with a hydroxyl group substituted for the hydrogen atom at C3 on its ring structure (Mahdy et al. 2022, Vainio et al. 2006). The sterols cycloartenol and lanosterol are generally used by plants and other eukaryotes, respectively, to generate other sterols, as well as other steroids, by modifications and substitutions at other points along the ring (Hall et al. 1969). Steroids are commonly found alongside phospholipids in the cell membrane, where they modulate membrane

fluidity (Mannock et al. 2010), but more derived forms of steroids are often used in cell signaling and metabolic communication. Prenol lipids, or terpenoids, consist of a chain of isoprene units - molecules with less than 40 carbons in this chain are classified as isoprenoids/terpenoids while longer chains are polyterpenes (Fahy et al. 2005). Unlike other lipid classes, base terpenoids are manufactured in plastids, but further chain extension takes place in the ER (Fahy et al. 2005). Isoprenoids are biologically important as vitamins and precursors to vitamins such as A, E, and K, and the terpene class is notable for its role in interorganismal signaling in both plants and insects (Eisenreich et al. 2003).

Polyketides are synthesized in a very similar process to fatty acids and are constructed from the same enzymatic family - their final structure and functions, however, are quite different (Chen et al. 2018). Polyketides begin with an acyl starter group which is extended into a chain by acyl - derived extender units, just as fatty acid synthesis does, although in polyketides a variety of acyl - derived starter and extender units are used while fatty acid synthesis typically uses acetyl-CoA derived starters and malonyl-CoA - derived extenders. From here the polyketide synthases (PKS) begin to differ from fatty acid synthases (FAS) further, carrying out ketoreductase, dehydratase, or enoylreductase processes and different points along the molecule to leave portions unreduced while others are partially or completely reduced (Kwan and Schulz 2011). This results in a variety of complex polycyclic compounds as opposed to the chain structures of fatty acids, and these compounds are typically used as secondary metabolites by organisms. This means polyketides have applications as antimicrobial, antifungal, or antiparasitic agents (Fahy et al. 2005).

Saccharolipids are similar to glycerolipids, long fatty acid chains built off a molecular backbone, but in saccharolipids the glycerol molecule is substituted for 2 molecules of the monosaccharide *N*-acetylglucosamine (Fahy et al. 2005). This sugar backbone contains more attachment points for fatty acids than a glycerol molecule, so saccharolipids have more carbon chains in their structure than their glycerolipid cousins (Raetz and Dowhan 1990). Saccharolipids are primarily found in Gram-negative bacteria, where saccharolipids are found as the lipid component of the lipopolysaccharides of the outer cell membrane (Raetz and Dowhan 1990).

Sterols and related lipid groups are used by all eukaryotic organisms, as well as in some bacteria (Lee et al. 2018). They are associated with cell and organelle membranes, which they play a role in regulating the structure and permeability of (Bean 1973). While sterols are used by all kingdoms within Eukarya, different groups use different sterol structures (Boutte and Grebe 2009). For instance, animal sterols often have 27 carbons (C_{27}) in their sterols, while fungi use primarily C_{28} sterols, and green algae (members of phylum Chlorophyta) use both C_{28} and C_{29} sterols (Goad et al. 1997, Lenton et al. 1972) as well as C_{27} sterols, though these are an exception in chlorophyte algae and much more commonly associated with rhodophyte and glaucocystophyte algae (Kodner et al. 2008). While sterols can be grouped based on carbon number alone, they are typically easier to identify based on a combination of carbon number with their side-chain structure and the number and position of their double bonds (Volkman 2003). For example, 2 diunsaturated C_{27} sterols, cholesta-5,22E-dien-3β-ol and 27-*nor*-24-methylcholesta-5,22E-dien-3β-ol, share the same number of carbons and have similar mass spectra to one another but can be differentiated by their gas chromatography (GC) retention times due to

differences in side chain structure (Volkman 2003). The structure of the side chain can have a significant impact on the physical and biological properties of the molecule (Jones et al. 1994). The position and number of double bonds and alkylations in the side chain varies - for example phytosterol molecules typically have less double bonds compared to cholesterol and related animal sterols (Volkman 2003). This variation in the side chain structure is thought to play a role in the regulation of membrane fluidity and permeability, as well as in the biosynthesis of hormones and other signaling molecules (Bean 1973).

Sterols originally developed in eukaryotes as cell membrane reinforcers (Jones et al. 1994), but many are modified for more complex functions (Bean 1973), and these sterol derivatives such as bile acids in animals, oxysterols, and steroid hormones have been shown to serve a variety of roles in signaling and regulating cellular metabolism (Wollam and Antebi 2011). Sterols can be identified using gas chromatography/mass spectrometry (GC/MS) - retention time can help narrow the structure down by comparison with known retention times for various sterol compounds, but retention times can vary between devices or overlap between multiple sterols. This is why for positive identification, not only the retention time of the derivatized compound but the mass spectrometric fragmentation ions the sterol produces are important for determining structure, as these give us the masses (i.e. molecular weights) of both the sterol and the fragments it breaks down into (Gerst et al. 1997). To ensure the sterols being examined break into fragmentation ions of a consistent size, they are treated immediately before analysis with a trimethylsilyl ether which binds to the sterols forming a sterol-ether derivative. By comparing the molecular weights and locations of ion fragments of an unknown sterol

with those previously tested in the literature and known samples run on the same machine, it can be matched and identified based on structure as well as size (Jones et al. 1994).

Some level of taxonomic identification in macroorganisms such as the fungal component of lichens can be made using morphological structures in the field, but chemical analysis in the lab is typically required to differentiate between species of the fungus (Crespo 2010, Phillips 1963). The algal component is a single-celled organism and morphological identification is often inaccurate for green algae due to limited vegetative characteristics (Lewis and Flechtner 2002), further reducing the available methods for accurate species differentiation. The ability to identify the fungal component by a combination of field observation and chemical analysis not only helps with the identification of specimens, but also in tracing the relationships and genetic history within the genera (Crespo 2010). While they are single-celled, chlorophyte algae possess a great range of specialized structures and adaptations in their cellular membrane and organelles as eukaryotic organisms, and differences in these structures are reflected in their biochemistry (Thompson 1996). These biochemical traits can be used for identification in the same way as physical traits are used on many macroscopic organisms (Patterson 1971) and are often more accurate than morphological identification (Lewis and Flechtner 2002). Additionally, the proportions of sterols present in these algae can vary with environmental factors, including light and temperature levels (Guschina and Harwood 2009) or whether the algae are free living or growing in conjunction with a fungal symbiont in a lichen (Ahmadjian 2001). This indicates that the symbiont affects these sterol compounds, and previous research has shown sterol- rich globules can form

in *Trebouxia* pyrenoids as a result of the fungal symbiont structurally modifying the algae's chloroplasts (Ahmadjian 2001).

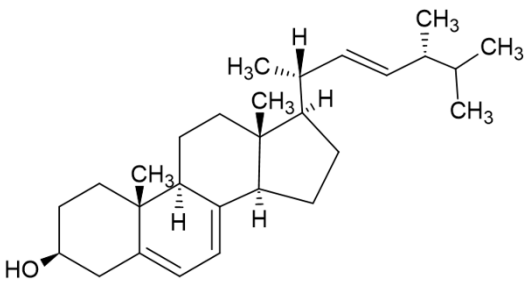
While it is possible to grow the full symbiotic *Usnea* lichen *in vitro*, the process is complicated by the slow growth rate of the fungal component of the lichen. *Trebouxia* algae can be grown separately from the fungal component in a suitable growth medium relatively quickly, however the fungus takes years to fully form a mature thallus structure (Yamamoto et al. 1985). Additionally, the fungus will not develop normally or survive to full development without a suitable alga to feed it (Rafat et al. 2015). The time required to grow an *Usnea* lichen to a large enough size for lipid extraction and analysis is generally around 3 months - longer than most algal or fungal cultures, but it has been done successfully (Behera et al. 2009, Rafat et al. 2015). Other issues include the difficulty in obtaining samples of *Usnea* fungus without symbiotic algae already contaminating the sample, and the fact that previous experiments in growing isolated *Usnea* fungus in controlled conditions resulted in abnormal growth and a lack of thallus development (Behera et al. 2009). However, while these 2 genera are always found together in nature, and the fungal component has difficulty growing on its own, the algal component can be grown as an isolate in media relatively quickly. Since the focus of this study is differentiating between lipid structures produced by the algal component and those produced by the fungus, variables were limited by analyzing lab grown unialgal samples of *Trebouxia* algae alone. Using these to establish more clearly the variations in sterol structures of related species within the *Trebouxia* genus allows these controlled samples to be compared with wild-collected samples of complete lichens likely to carry related algal species. Galactolipids were also analyzed - as this lipid class is produced by

chloroplasts, only the algal component was expected to carry them, providing a baseline data set of lipids of known origin. This baseline for biochemical variations between closely related species *in vitro* is used here to assess whether *Trebouxia* species are present in collected samples, but the primary focus is identifying lichen sterols of fungal origin by process of elimination.

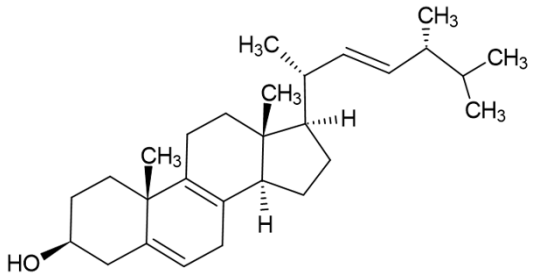
Lipids of *Trebouxia* and *Usnea* species

Green algae species such as *Trebouxia* members produce some larger sterol structures than fungi, with green algae containing primarily C_{29} sterols, and ascomycete fungi containing primarily C_{28} sterols (Lenton et al. 1973). This is a generalization however, as chlorophyte algae species such as *Trebouxia* typically contain a mixture of C_{28} and C_{29} sterols when analyzed, even when separate from any fungal partner (Kodner et al. 2008), with some groups containing C_{27} sterols as well (Kodner et al. 2008). This overlap makes it difficult to identify which organism synthesized which sterol when working with whole lichen samples. *Usnea* species contain the sterols ergosta-5,8,22E-trien-3β-ol (lichesterol, C_{28}, 2 double bonds) and 24-methylcholesta-5,7,22E-trien-3β-ol (ergosterol, C_{28}, 2 double bonds) as their 2 highest proportion sterols (Figure 2), both C_{28} sterols associated strongly with fungi (Lenton et al. 1973, Yokokawa and Mitsuhashi 1981). They also contain several smaller proportion sterols (Solberg 1987).

Figure 2: Skeletal formula of ergosterol and lichesterol



ergosta-5,7,22-trien-3-ol (ergosterol)



ergosta-5,8,22E-trien-3beta-ol (lichesterol)

An analysis of the sterols of the Norwegian *Usnea hirta* showed low concentrations of the sterols 24-methylcholesta-6,8,22-trien-3β-ol (fungisterol, C_{28}, 3 double bonds), 24-methylcholest-5-en-3β-ol (campesterol, C_{28}, 1 double bond), 24-methylcholesta-7,24(28)-dien-3β-ol (episterol, C_{28}, 2 double bonds), and 24-ethylcholest-5-en-3β-ol (sitosterol, C_{29}, 1 double bond)(Solberg 1987). Because both photobiont and mycobiont are being analyzed together in this study, it is unclear which of these are being produced by the algal photobiont (Solberg 1987). Fungisterol and ergosterol are likely fungal in origin (Yokokawa and Mitsuhashi 1981), but episterol and β-sitosterol are phytosterols. Both sterols are typically found in plants, with β-sitosterol produced from episterol, making these likely candidates for chlorophyte algal sterols from the symbiont (Bin Sayeed et al. 2016). To definitively separate the fungal sterols from the algal in a whole lichen sample however, we require data on at least one member of the symbiosis in isolation, and as *Trebouxia* species can be cultivated in media without a partner this data is available for this common *Usnea* partner in isolation.

Trebouxia species 213/3 from the Cambridge Culture Collection contains the sterols ergost-5-en-3β-ol (C_{28}, 1 double bond), 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol, C_{29}, 2 double bonds), and 24-ethylcholest-5-en-3β-ol (sitosterol, C_{29}, 1 double bond), 24-methylcholesta-5,22E-dien-3β-ol (brassicasterol, C_{28}, 2 double bonds) and cholest-5-en-3β-ol (cholesterol, C_{27}, 2 double bonds) (Goad et al. 1972). Other chlorophyte algae contain a wide range of sterols, but common sterols across non-symbiotic chlorophyte species include cholest-5-en-3β-ol (cholesterol, C_{27}, 1 double bond), cholesta-5,22E-dien-3β-ol (22-dehydrocholesterol, C_{27}, 2 double bonds), 24-methylcholest-5,22-dien-3β-ol (brassicasterol, C_{28}, 2 double bonds), 24-methylene-cholest-5-en-3β-ol (24-methylenecholesterol, C_{28}, 2 double bonds), poriferasta-5,25-dien-3β-ol (clerosterol, C_{29}, 2 double bonds), and (24Z)-stigmasta-5,24(28)-dien-3β-ol (isofucosterol, C_{29}, 2 double bonds)(Aknin et al. 1992, Cranwell et al. 1990, Fattorusso et al. 1980, Shevchenko et al. 2009).

Galactolipids are produced in photosynthetic cyanobacteria and chloroplast-containing organisms such as plants and algae (Sato and Awai 2016), so all galactolipids found in lichens are derived from the algal symbiont. Chlorophyte algae typically follow a similar galactolipid synthesis pathway to plants, using the MGD1 and DGD1 enzymes to synthesize MGDG and DGDG galactolipids (Sato and Awai 2016). The fatty acyl tails of both types of galactolipid in green algae typically contain either 16 or 18 carbons (C_{16} or C_{18}), although some chlorophyte algal genera have been found to contain trace amounts of galactolipids ranging from C_{13} to C_{24} (Sahu et al. 2013).

Objectives

The analysis of the sterol lipids and galactolipids present in a set of unialgal *Trebouxia* cultures in this study not only provides a lipid profile for *Trebouxia* species, but also allows the fungal *Usnea* partner's sterol lipids to be separated from sterols potentially produced by their symbiont. By comparison of the unknown algal symbiont species in a set to related *Trebouxia* species in isolated culture, lipids that do not match with any present in the isolated *Trebouxia* will likely be produced by the fungal *Usnea* symbiont. Wild collected samples may contain other species beyond the primary algal and fungal symbionts, however- lichens in the wild often carry other species of fungi on their surface, or even within the thallus structure in the case of endolichenic fungi, and these fungi which live closely with lichens but are not a part of the primary symbiosis between alga and *Usnea* fungus may also contribute to whole lichen sterols (Hawksworth and Grube 2020). The primary objective of this study is the identification of these fungal sterols in lichen samples, demonstrating their origin by showing which sterols found in this genus are produced only by or in the presence of fungal symbionts. This data set accomplishes a secondary objective of this study as well, by providing descriptive data on the lipids used by multiple *Trebouxia* species. By comparing the sterol lipids of multiple known *Trebouxia* lines against an unknown alga, which is likely also a *Trebouxia* species due to its symbiosis with an *Usnea* species (Rafat et al. 2015), a baseline is established for expected lipid variation both between lines of the same species of *Trebouxia* and between different species in the genus. To support this data, galactolipid data was also collected for each sample group for comparison alongside sterol lipids. Galactolipids are found in chloroplasts, which out of the 2 symbiotic organisms involved in a lichen only

chlorophyte algae contain, and therefore belong to the algal symbiote alone. Other algal or cyanobacterial species not directly involved in the *Usnea*/*Trebouxia* symbiosis may have been present on or in collected whole lichen specimens, potentially producing a high enough concentration of their own lipids to show up in sterol and galactolipid analysis - if this was the case we expected to see galactolipids not present in any unialgal *Trebouxia* groups appear in the whole *Usnea* lichen samples.

Chapter II - Materials and Methods

For a baseline of sterol variation between *Trebouxia* species, unialgal lab-grown samples - 2 from different lines of *Trebouxia erici* and one from a sample of *Trebouxia excentrica* - were grown in triplicate in *Trebouxia* media. The sterol lipids in each were then compared via gas chromatography/mass spectrometry (GC/MS) against isolated samples of common phytosterols, while galactolipids were analyzed via positive-ion electrospray/mass spectrometry (ESI/MS) and identified by the molecular weight of the whole molecule and of their fatty acyl groups. 3 samples of lichens in the *Usnea* genus, identified in the field by their distinctive branching fruticose thallus structure, containing an unknown algal symbiote, were also collected from a farm in Warren County, Tennessee (lat. 35.8017764, long. -85.9027691) with permission from the owners.

Culturing

3 lines of unialgal *Trebouxia* cultures were obtained from the Culture Collection of Algae at the University of Texas Austin - *Trebouxia erici* (UTEX 912), *T. erici* (UTEX

911), and *T. excentrica* (UTEX 1714). These were selected as 2 species of the most common algae genus found in *Usnea* lichens available from culture collections (Goldsmith 1997), with 2 lines of *T. erici* selected to control for sterol variation between lines within the same species, and one *T. excentrica* to control for interspecies variations across the genus. All cultures were grown in triplicate in 1 L of growth medium. The growth medium used was the standard University of Texas medium used for maintenance of *Trebouxia* species (*Trebouxia* medium, UTEX.org, https://utex.org/products/trebouxia-medium?variant=30991736635482), a modified version of Ahmadjian's photobiont media for lichen culturing (Ahmadjian 1967). Cultures were left for 30 days in a growth chamber at 20°C under LED/ fluorescent lights producing 50 micromoles of photons per m^2s. The lights were on for 14 hours and off for 10 each day to simulate daylight conditions. Algal cells were filtered from medium after one month of growth through precombusted 110 mm-diameter glass fiber filters (Whatman grade 934-AH, pore size 1.5 µm), and frozen at -80°C for storage before extraction.

Lichen collection

As well as for its close association with *Trebouxia* species, the *Usnea* genus was selected for its distinctive fruticose structure, which aids both in identification and collection (see Figure 2). While many lichen groups require some form of biochemical analysis to identify (Crespo 2010) as many foliose and crustose lichens have little difference in external thallus structure consistent within genera, the more complex fruticose lichens are anatomically distinct enough for a confident identification in the field, at least when mature. *Usnea* species found in North America have a thallus structure composed of several large, tubular thalli covered in a profusion of small hairlike

sub-thalli. Other genera of fruticose lichens that could potentially be confused with *Usnea* found in Tennessee are *Alectoria, Anaptychia, Baeomyces, Cetraria, Cladonia,* and *Ramelina* (Phillips 1963, Skorepa 1972), but none are anatomically identical. *Ramelina* and *Centraria* resemble *Usnea* when small but develop a distinct flattened structure with pointed branches as they grow, compared to *Usnea's* round tubular thallus (Brodo et al. 2001). *Cladonia* and *Alectoria* are the only genera found in Tennessee that share this tubular shape (Brodo et al. 2001, Skorepa 1972), but *Cladonia* species grow low to the ground on rotting logs or soil while *Usnea* species in Tennessee are epiphytes of living trees typically found in the upper branches, and *Alectoria* species produce long, thin hanging networks of thalli while *Usnea* thalli are considerably shorter and thicker (Brodo et al. 2001).

Figure 3: *Usnea* lichen sample (photo by author)

Usnea is also an advantageous genus to sample from in this study because contamination of wild collected samples by foreign organisms that produce sterols and galactolipids needs to be minimized. Ideal results will show only lipids originating from one of the 2 symbiotes, but wild specimens may contain microorganisms such as yeasts, protists, and non-symbiotic terrestrial algae, as well as growing closely and in many cases intermingling with other macroscopic epiphytes such as mosses, fungi, and other lichens. *Usnea* species produce usnic acid, which has been extensively studied in a medical context for its antifungal and antibacterial properties, and this acid likely helps protect the lichen from overgrowth by non-symbiotic microorganisms (Cansaran et. al. 2006). This is another benefit of studying this host genus, but the presence of usnic acid does not guarantee *Usnea* specimens collected from nature will be entirely free of microorganisms; it only increases the odds of harvesting samples that contain only the photobiont species.

Of particular concern is contamination by mosses which likely contain some plant sterols similar to green algae, or by other lichenized or non-lichenized ascomycete fungal groups, as these would be difficult to distinguish from target organisms in final lipid results if accidentally collected along with *Usnea* samples (Cranwell et al. 1990, Divakar et al. 2015). Epiphytic foliose and crustose lichen species typically grow so tightly against the bark of their tree that removal brings bark tissue with the lichen and are often partially covered by or covering mosses and fungi, which similarly are difficult to remove from the rhizoidal hyphae that anchors the lichen. Some fruticose species also have many close hyphal connections to the surface they grow from, but *Usnea* species develop several long thalli as they grow, all branching from a small central rhizoidal cluster at the

very base. Figure 3 shows an inverted specimen, with the rhizoidal attachment point visible as a small brown spot on the upper right-hand portion of the lichen. By cutting above this rhizoidal hyphal cluster and above the level of any nearby mosses or fungi, a clean sample with no plant or fungal tissue caught in the hyphae at the base can be assured. Additionally, *Usnea* is an ideal genus for ensuring a consistent sample, because members reproduce primarily by fragmentation of relatively large thallus pieces in which the photobiont is retained and passed on unchanged (Keon and Muir 2002). This means individuals growing in the same immediate area are likely to be clones of a common parent carrying the same photobiont strain, an important consideration since multiple individuals from the sample area were required for each replicate test.

Whole lichen samples were collected from deadfall branches in a stand of 6 trees - 3 red oaks, 2 poplar, 1 white oak - over 2 winters (2020-2021 for sample 1 and 2021-2022 for samples 2 and 3) with owner's permission on a private property in Warren County, Tennessee at 35.801, -85.901. Samples were divided into 3 replicate groups - multiple individual lichens from the collection area were required to achieve sufficient biomass for analysis, so each sample group represents an aggregate of *Usnea* lichens in the collection area. Whole lichen samples weighed 2.69 g on average (±1.24 g). Each replicate group was stored in the same growth chamber conditions used to culture the isolated algae (20°C under LED/ fluorescent lights producing 50 micromoles of photons per m^2s) with no media for 30 days before processing to standardize light and temperature based metabolic conditions across samples. During processing lichens were debrided manually to remove any visible dirt or fragments of bark and moss, rinsed under deionized water, and then ground via a blender to expose internal algae for extraction. All lichen samples

were then frozen, and lipids extracted and analyzed in the same way as the isolated algal cultures, described below. There are likely a variety of microscopic organisms in these wild samples that could not be removed prior to processing despite debriding and rinsing (He and Zhang 2012), but as prokaryotes typically do not produce sterols bacterial contamination will not affect results as plant or fungal tissues would (Wei et al. 2016). Yeasts, non-symbiotic algae, and other microscopic sterol producers must still be accounted for as potential contaminates in these wild collected samples (Torres et al. 2003), but the proportion of any contaminant lipids will be low compared to those produced by the algae/lichen, and no growth in media or other techniques that may allow single-celled contaminates to grow disproportionately was used for the whole lichen samples. It is unlikely contaminant lipids will be detectable using current methods, but any lower proportion lipids found only in the *Usnea* samples could potentially originate from microorganisms not participating in the symbiosis (He and Zhang 2012).

Lipid processing

Total lipids were extracted via a separatory funnel, using the procedure described by Leblond & Chapman (2000) - total biomass was suspended in a mixture of 75 mL chloroform, 150 mL methanol, and 60 mL of a 50 mM phosphate buffer to separate lipids from cells. An additional 75 mL of both chloroform and de-ionized water were added and allowed to separate in the funnel overnight after which the chloroform layer containing lipids and related compounds was removed, leaving the aqueous layer behind to be discarded. The sample was then placed under rotary evaporation to remove the chloroform and resuspended in 3 mL methylene chloride. Algal lipids had a dry weight of 15.45 mg (± 2.18 mg), while whole lichen lipids weighed 309.20 mg (±160.6 mg). Lipid

extracts were separated into 5 component lipid fractions using activated Unisil silica columns (1.0 g, 100 - 200 mesh, activated at 120°C, Clarkson Chromatography, South Williamsport, PA, USA). Samples were loaded into silica columns, and a series of solvents were used to separate lipids according to polarity - fraction 1 eluting the least polar lipids and fraction 5 the most polar (Leblond & Chapman 2000). Fractions and solvents were as follows: (1) 12 mL methylene chloride (sterol esters), (2) 15 mL 5% acetone in methylene chloride with 0.05% acetic acid (free sterols, tri - and diacyl glycerols, and free fatty acids), (3) 10 mL 20% acetone in methylene chloride, (4) 45 mL acetone (chloroplast-associated galactolipids), and (5) 15 mL methanol with 0.1% acetic acid (polar lipids, including betaine lipids and phospholipids). All solvents were purchased from Fisher Scientific (Hampton, NH, USA) at their highest available purity, Optima grade. Before further processing, all fractions were evaporated under nitrogen and suspended in 1.5 mL of methylene chloride.

GC/MS, ESI/MS, and ESI/MS/MS techniques

The first and second fractions, containing sterol esters and free sterols respectively, were saponified and derivatized to form trimethylsilyl (TMS)-ether derivatives of sterols according to Leblond and Chapman (2002). For saponification, samples were suspended in 0.1 mL of toluene and 1.9 mL of a solution of 5% KOH/ 80% methanol (v/v) and heated at 80 °C for 2 hours. After heating, 0.5 mL glacial acetic acid and 1 mL deionized water were added and samples vortexed, and 3 2 mL extractions were made from the top layer of the sample using a 1:1 hexane/methyl-tert-butyl ether mixture. Solvent was removed by nitrogen evaporation, and the sterols were derivatized by dissolving in 0.5 mL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and

heated for 0.5 hours at 80 °C before evaporating under nitrogen and resuspending in 20 µl methylene chloride. The derivatives were then analyzed via GC/MS with a Thermo TSQ Quantum GC/MS (Austin, TX) using a DB-5 column (30 m × 0.25 µm film thickness; J&W Scientific Incorporated, Folsom, CA) in positive-ion electron impact (EI) mode under the following conditions: 2 µl injected via splitless injection with injector set at 280 °C, transfer line set at 275 °C, helium carrier at 28 cm/s, 70 eV with a scanning range 50–600 *m/z*, and a cycle time of 1.1 s. The GC temperature was 50 °C for 1 min, 50–170 °C at 15 °C/min, and 170–300 °C at 10 °C/min with a hold time of 11 min. Relative retention times (RRT) were calculated using retention times (RT) of samples and retention times of standards for cholesterol and stigmasterol run on the same machine according to the methodology of Jones et al. (1994). Results were compared with the TMS-ether derivatives of standards of a range of sterols commonly found in chlorophyte algae or ascomycete fungi - 8,24-5α-cholestadien-3β-ol, 5,22-cholestadien-3β-ol, 5,22-cholestadien-24-methyl-3β-ol, 5,22Z-cholestadien-3β-ol, 5α-cholesten-3β-ol, 24-ethylcholesta-5,24(28)E-dien-3β-ol (fucosterol), 24-5α-cholesten-9,19-cycloartenol, and 7-5α-cholesten-3β-ol from Steraloids Inc. (Newport, RI), cholesta-5,24-dien-3β-ol (desmosterol), ergosta-5,22-trien-3β-ol (ergosterol), 4,4,14-trimethylcholesta-5,7-dien-3β-ol (lanosterol), 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol), cholesta-5,7-dien-3β-ol (7-dehydrocholesterol), and (+)-dihydrocholesterol from Sigma-Aldrich (St. Louis, MO), and cholest-5-en-3β-ol (cholesterol) from Alfa-Aesar (Tewksbury, MA). After extraction, all samples were stored in a −20 °C freezer rated for storage of solvents.

Fraction 4 of each sample, containing the galactolipids, was mixed with methanol, chloroform, and 50 mM sodium acetate according to Gray et al. (2009) to create sodium

adducts with a positive charge ($M+Na^+$). The resulting adducts were analyzed via positive-ion electrospray/mass spectrometry (ESI/MS) using a Finnigan DecaXP ion trap mass spectrometer (Waltham, Massachusetts, USA). A 5 µl sample was injected directly into a methylene chloride carrier solvent at a flow rate of 0.5 ml min^{-1}. The ESI/MS scan was conducted from *m/z* 210–1200, following the procedure outlined by Gray et al. (2009). The relative percentage distributions were also calculated using a method developed by Gray et al. (2009), which relies on the combined intensity of all relevant galactolipid ions and is commonly used in metabolomic research (Leblond et al. 2019). When many analytes are being studied in one sample, it is practically impossible to quantify each one by analytical standards, so this method is used to identify relevant lipids. To examine these relevant galactolipids individually, ESI/MS/MS was carried out using a collision energy ranging from 37.5 to 48%. Analysis involved identifying major cleaved fatty acids by comparing the masses of the original ions and their fragments. The relative percentage compositions of the acyl chains (*sn*-1 or *sn*-2) were used to determine their positions. This was accomplished using a modified version of the procedure outlined by Guella et al. (2003) and following the protocol established by Gray et al. (2009).

Chapter III - Results

Table 1 shows GC/MS results for the sterols in the 3 lines of *Trebouxia* examined, identified by standards and comparison with Jones et al.'s 1994 work on relative retention times of sterol lipids, as well as the sterols detected in the whole *Usnea* lichen samples. While the mass peak of a sterol can be used to calculate carbon number and number of double bonds, which can narrow down what sterol structure we have, for positive

identification we look at the ion fragments of the spectrogram. Take ergosterol (Figure 5 B) as an example, we observe strong ion fragment peaks before the mass peak (468 *m/z*, the peak furthest along the x axis) at *m/z* 131, 143, 157, 183, 195, 207, 237, 253, 337, 363, and 378 (all rounded to nearest whole number), among others- the higher mass fragments tend to be most useful for structural identification, as they represent larger pieces of that structure. If we compare these peaks to typical ion fragment peaks produced by known sterols (Jones et al. 1994), we see that ergosterol produces distinctive ion fragments at *m/z* 131, 253, 337, 363, and 378- all of which match a peak observed in our spectrogram. There are other peaks that appear in this sterol spectrogram that can make these identifying peaks more difficult to pick out, as is often the case- but if the total mass matches and all identifying ion fragments are present, sterols are structurally complex enough for these matching fragment peaks to act as an identifying 'fingerprint' for a specific sterol compound.

The 4 largest proportion sterols found in this study - 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol, C_{29}, 2 double bonds), 24-methylcholest-5en-3β-ol (campesterol, C_{28}, 1 double bond), 4-methyl-5α-cholest-7-en-3β-ol (ergost-7-en-3β-ol, C_{28}, 1 double bond), and 24-ethyl-5α-cholest-7-en-3β-ol (stigmast-7-en-3β-ol, C_{29}, 1 double bond) - were present in all 3 lines of isolated *Trebouxia*, although proportional differences were seen between the 2 species (Table 2), with the *T. erici* samples containing more stigmasterol and campesterol, relative to ergost-7-en-3β-ol and stigmast-7-en-3β-ol, than the *T. excentrica* samples. All samples were grown and analyzed in triplicate and results for the higher proportion sterols were consistent across replications.

Table 1: Sterols present in *Trebouxia* and whole lichen *Usnea* (x indicates sterol is present in this sample)

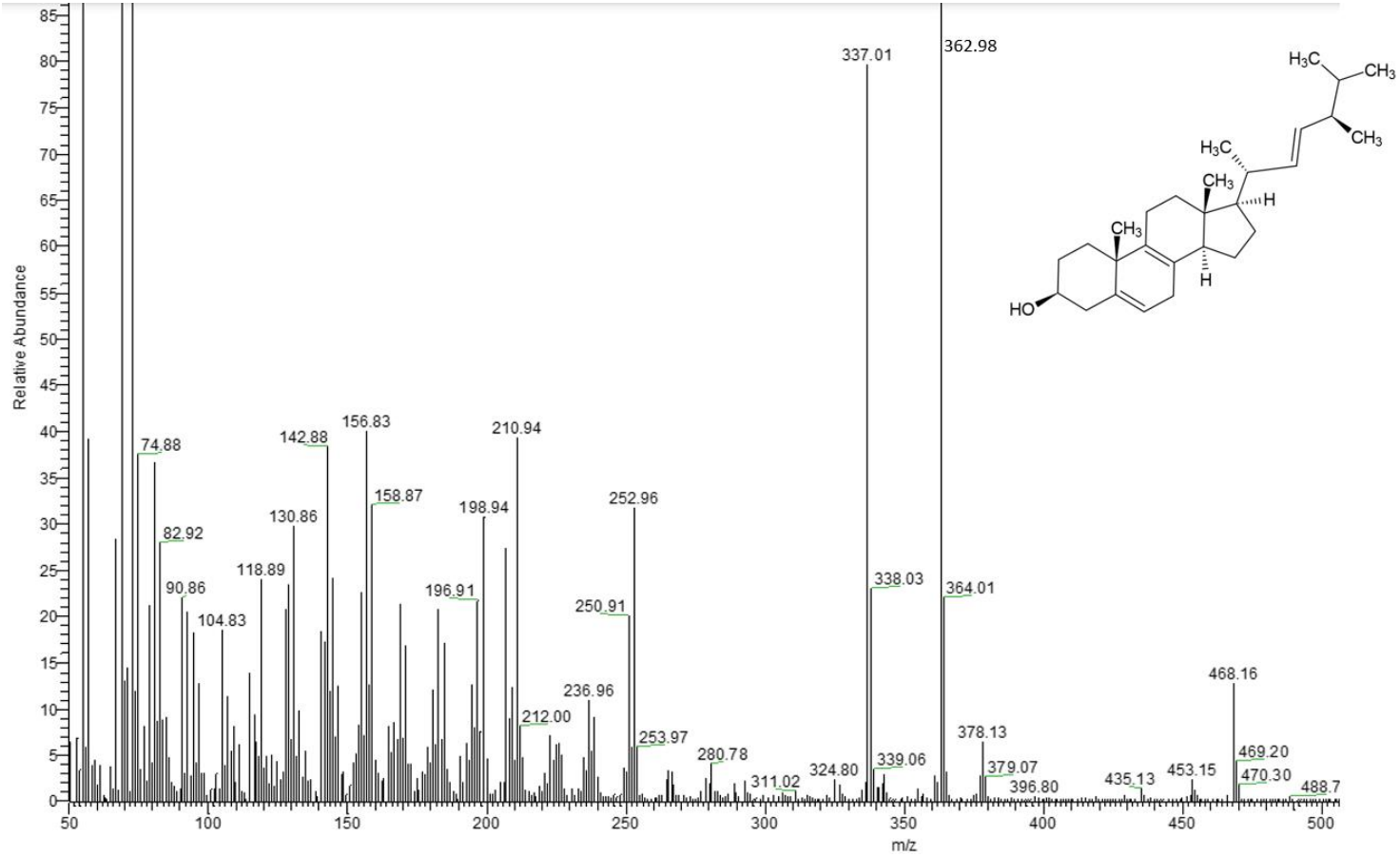
sterol	common name	RT	carbon #	double bonds	RRT	mass	*T. erici* 911	*T. erici* 912	*T. excentrica*	*Usnea*
ergosta-5,8,22E-trien-3β-ol	lichesterol	37.89	28	3	1.15	468				x
24-methylcholesta-5,7,22E-trien-3β-ol	ergosterol	38.3	28	3	1.27	468				x
24-methylcholesta-5,22E-dien-3β-ol	brassicasterol	37.92	28	2	1.16	470		x		
unknown C_{28} sterol		39.26	28	1	1.53	472				x
24-methylcholest-5en-3β-ol	campesterol	38.79	28	1	1.31	472	x	x	x	x
4-methyl-5α-cholest-7-en-3β-ol	ergost-7-en-3β-ol	39.55	28	1	1.61	472	x	x	x	
24-ethylcholesta-5,22E-dien-3β-ol	stigmasterol	39.18	29	2	1.43	484	x	x	x	x
24-ethylcholest-5-en-3β-ol	sitosterol	39.72	29	1	1.66	486			x	
unknown C_{29} sterol		39.88	29	1	1.7	486	x	x		x
24-ethyl-5α-cholest-7-en-3β-ol	stigmast-7-en-3β-ol	40.69	29	1	1.98	486	x	x	x	
unknown C_{30} sterol		40.87	30	2	2.07	498		x		

Table 2: Proportions of free and esterified sterols by sample group (listed as average relative percent with standard deviation, sample groups in triplicate)

common name	*Trebouxia erici* 911		*Trebouxia erici* 912		*Trebouxia excentrica*		*Usnea*	
	sterol esters	free sterols	sterol esters	free sterols	sterol esters	free sterols	sterol esters	free sterols
lichesterol	0	0	0	0	0	0	30.88% ± 9.08	34.37% ±3.56
ergosterol	0	0	0	0	0	0	19.61% ± 3.13	29.74% ± 9.88
brassicasterol	0	0	0.61% ± 0	0.78% ± 0.11	0	0	0	0
unknown C_{28} sterol	0	0	0	0	0	0	8.72% ± 1.87	8.42% ± 3.63
campesterol	17.33% ± 4.76	18.88% ± 2.49	21.45% ± 5.39	29.4% ± 1.55	15.91% ± 6.74	51.21% ± 32.27	23.62% ± 8.00	8.21% ±4.29
ergost-7-en-3β-ol	6.33% ± 1.44	10.88% ± 1.86	5.65% ± 4.91	1.95% ± 0.28	2.40% ± 0.98	11.34% ± 1.2	0	0
stigmasterol	57.19% ± 3.82	52.57% ± 1.98	57.19% ± 7.00	59.57% ± 0.75	68.24% ± 8.49	45.03% ± 0	0	7.93% ± 6.18
sitosterol	0	0	0	0	10.49% ± 0.65	0	0	0
unknown C_{29} sterol	13.03% ± 8.66	9.03% ± 1.52	9.79% ± 1.07	4.56% ± 0.73	0	0	14.10% ± 4.84	11.32% ± 1.13
stigmast-7-en-3β-ol	6.12% ± 1.25	8.63% ± 0.09	6.39% ± 3.6	1.01% ± 0.81	2.95% ± 1.41	7.89% ± 3.92		0
unknown C_{30} sterol	0	0	1.34% ± 0	1.15% ± 1.04	0	0	0	0
% of total sterols	41.16%	58.84%	70.91%	29.09%	10.21%	89.79%	60.62%	39.37%

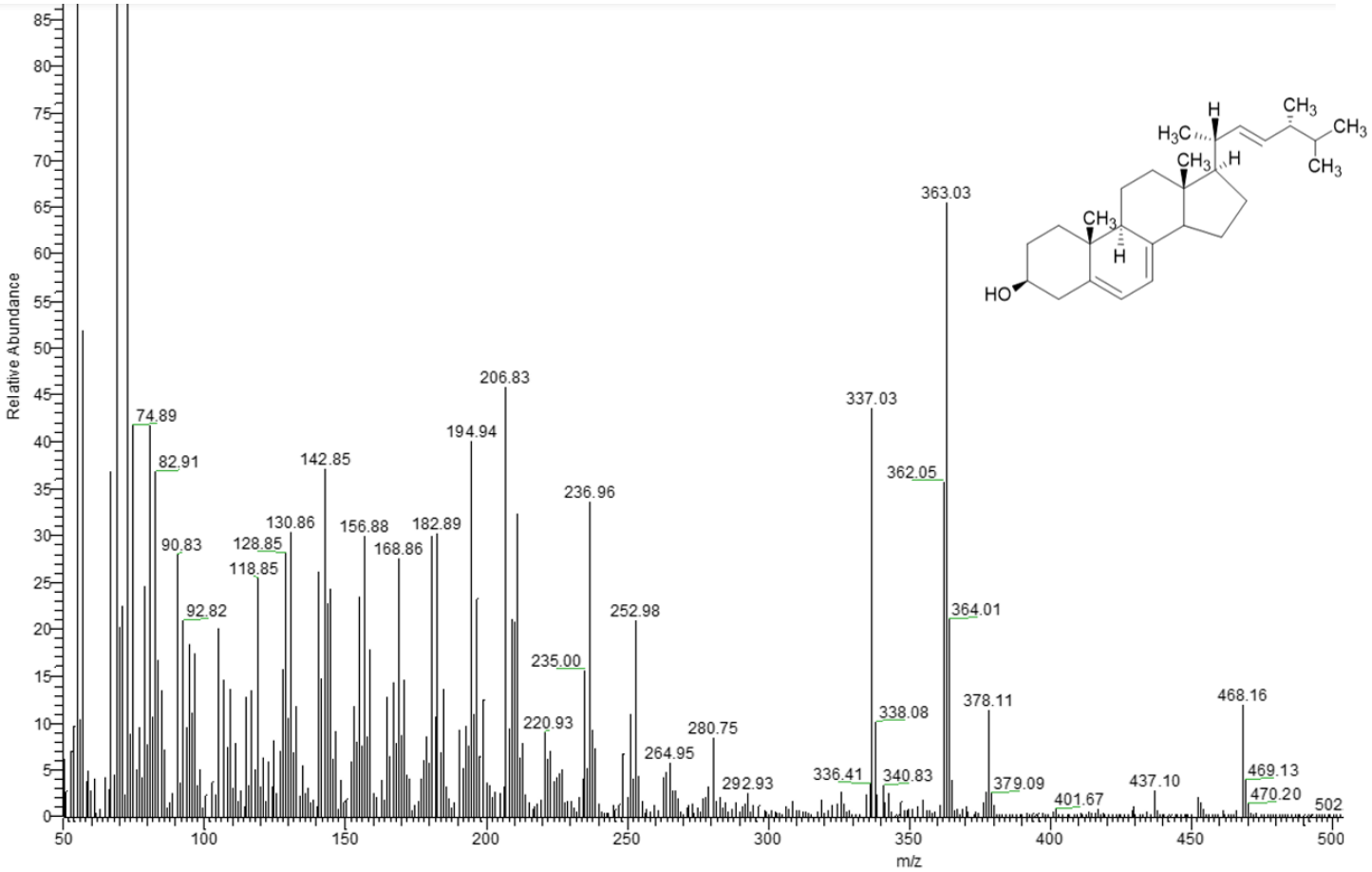
Analysis of collected wild *Usnea* specimens (Table 1 and 2) also showed consistent results for sterol composition between replicates - the 2 largest proportion sterols present were ergosta-5,8,22E-trien-3β-ol (lichesterol, C_{28}, 3 double bonds) and 24-methylcholesta-5,7,22E-trien-3β-ol (ergosterol, C_{28}, 3 double bonds), 2 fungal sterols commonly found in *Usnea* lichens (Solberg 1987). Stigmasterol and campesterol were also found in all *Usnea* replicates, and they had these 2 sterols in common with laboratory cultured *Trebouxia* specimens. *Usnea* samples also contained an additional C_{28} and C_{29} sterol, but these did not match stigmasterol, campesterol, or any standard or reference sterol, and are listed as unknown C_{28} and unknown C_{29} sterol, respectively. Lichesterol and ergosterol were the primary sterol lipids found in all whole lichen *Usnea* samples. Considering both are fungal lipids expected in *Usnea* lichens, and as neither of these sterols was detected in any algal isolate sample, they are clearly products of the fungal symbiote. Campesterol and stigmasterol are phytosterols (Almeida et al. 2020), presumably from the photobiont in the lichen rather than the mycobiont, and the fact that our isolated *Trebouxia* cultures also produced these sterols supports this. Stigmasterol and campesterol (Figure 4, D and F) are found in many chlorophyte algae groups (Lopes et al. 2011).

Figure 4: Sterols found



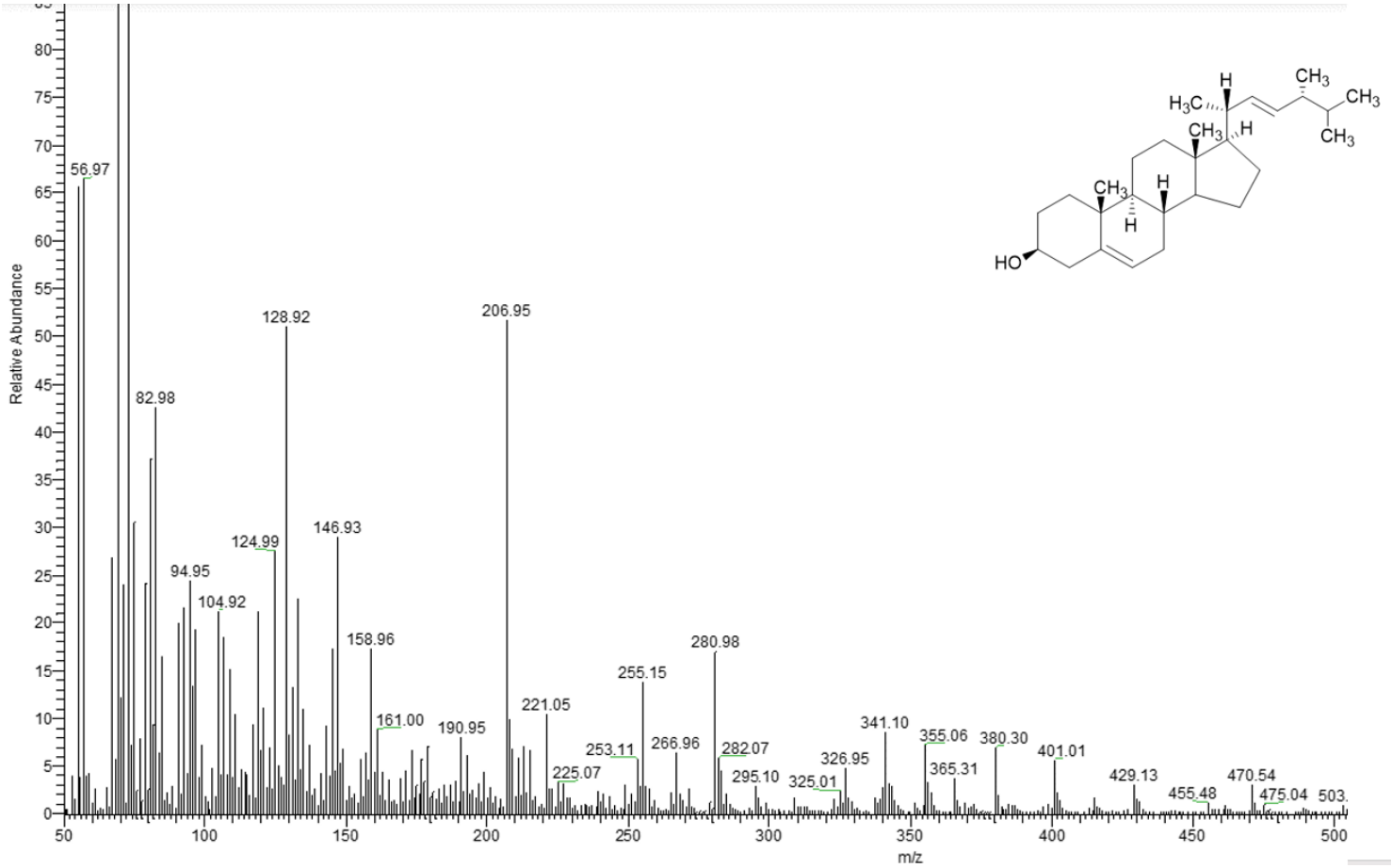
A: Skeletal formula and mass spectra of lichesterol

Peak at 468 represents *m/z* of sterol (C_{28}, 3 double bonds), peaks at 363, 337, 253, 237, 211, 183, and 157 represent ion fragments consistent with lichesterol (Solberg 1987)



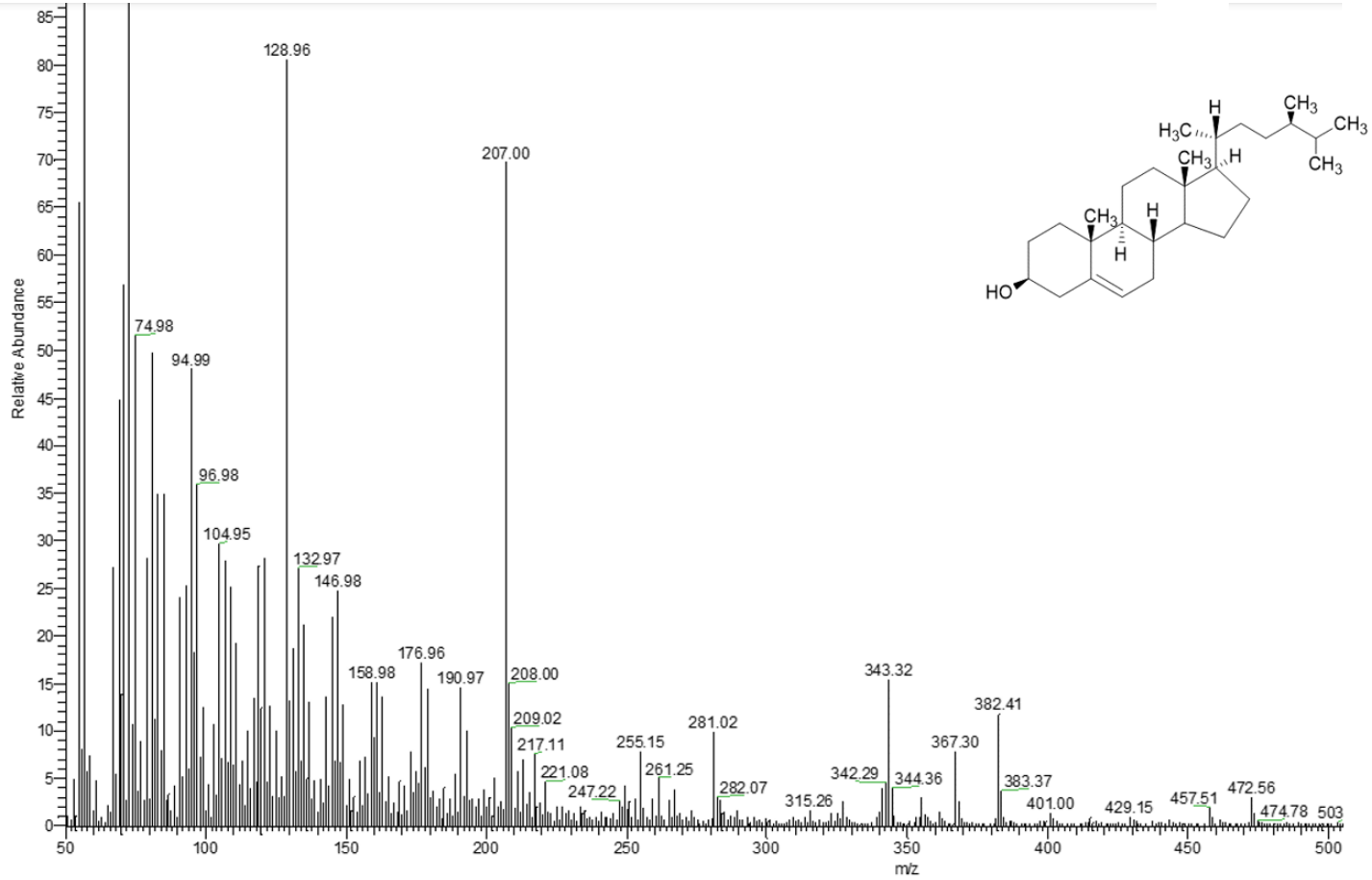
B: Skeletal formula and mass spectra of ergosterol

Peak at 468 represents *m/z* of sterol (C_{28}, 3 double bonds), peaks at 378, 363, 337, 253, and 131 represent ion fragments consistent with ergosterol (Jones et al. 1994)



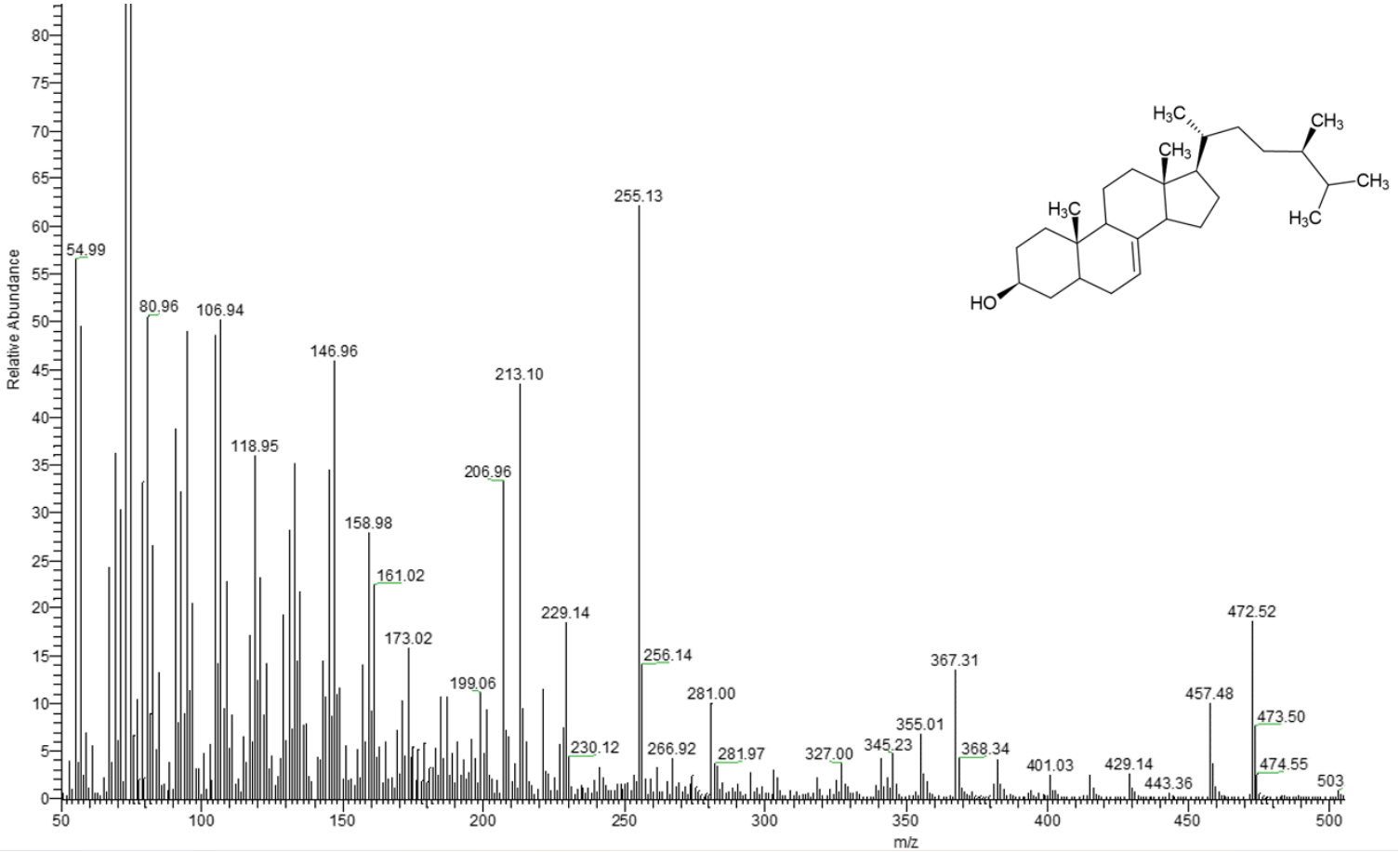
C: Skeletal formula and mass spectra of brassicasterol

Peak at 470 represents *m/z* of sterol (C_{28}, 2 double bonds), peaks at 455, 380, 365, 340, 255, and 129 represent ion fragments consistent with ergosterol (Jones et al. 1994)



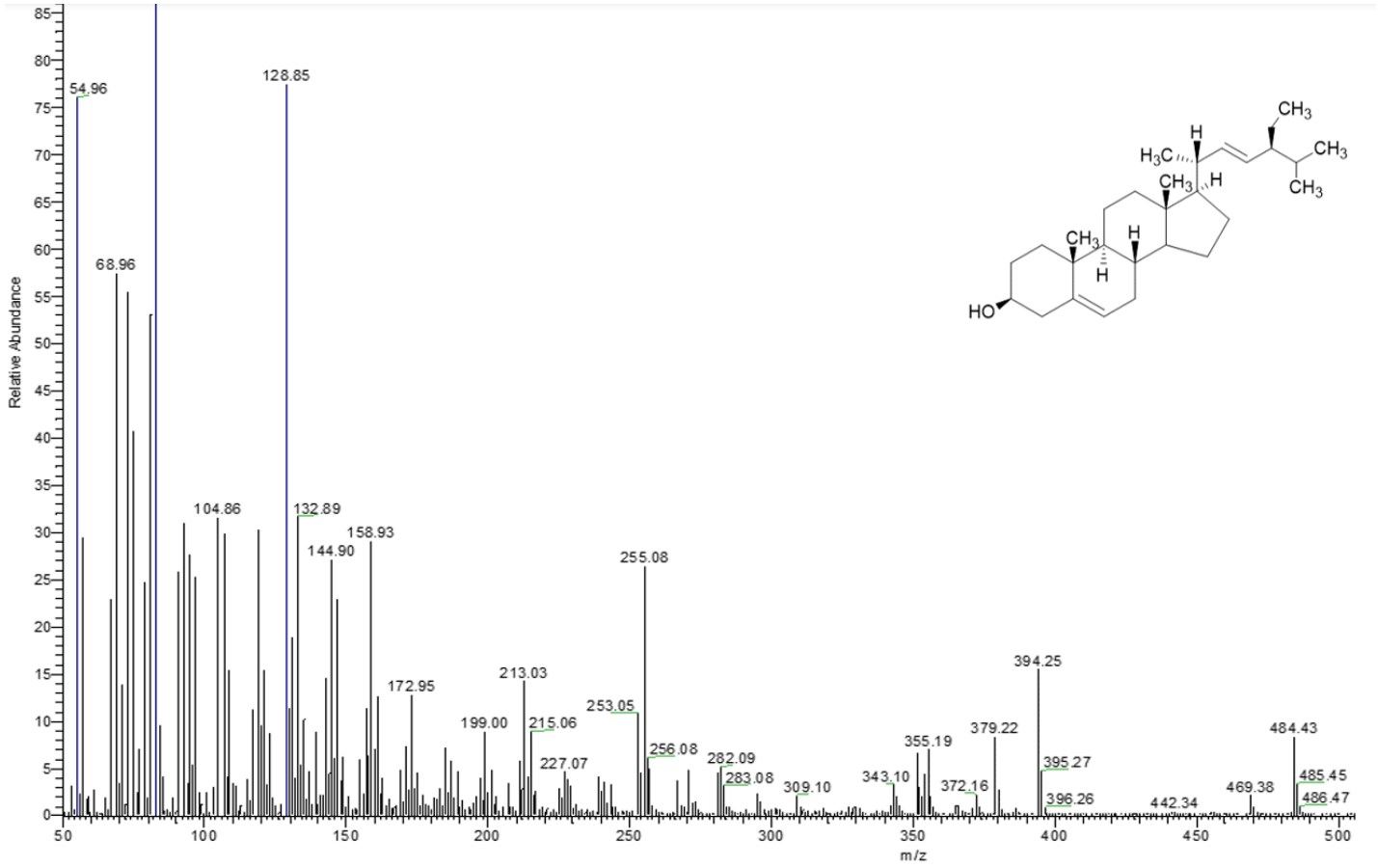
D: skeletal formula and mass spectra of campesterol

Peak at 472 represents *m/z* of sterol (C_{28}, 1 double bond), peaks at 382, 367, 343, 255, and 129 represent ion fragments consistent with ergosterol (Jones et al. 1994)



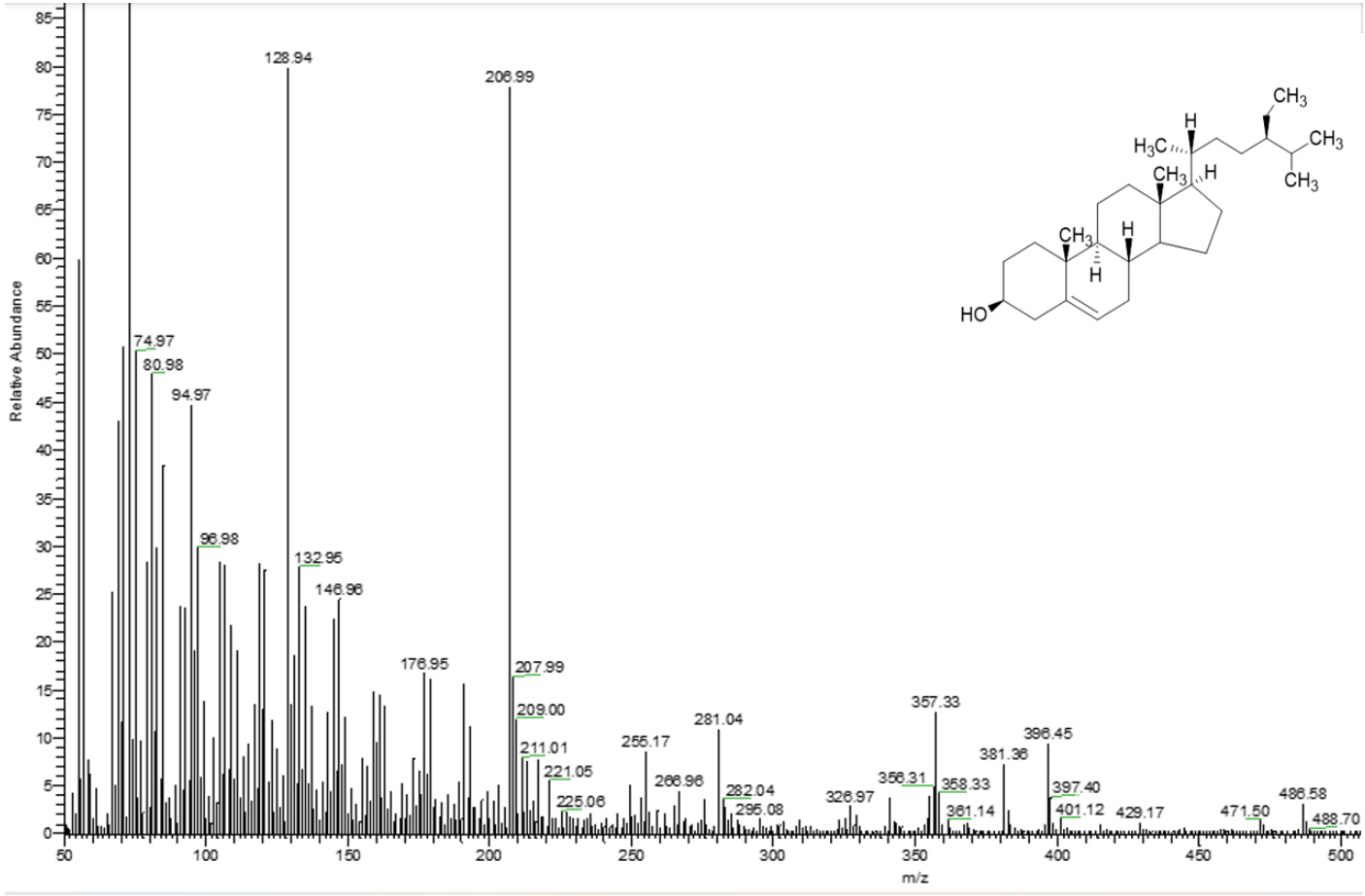
E: skeletal formula and mass spectra of ergost-7-en-3β-ol

Peak at 472 represents *m/z* of sterol (C_{28}, 1 double bond), peaks at 457, 378, 367, 343, 255, 299, and 213 represent ion fragments consistent with ergost-7-en-3β-ol (Jones et al. 1994)



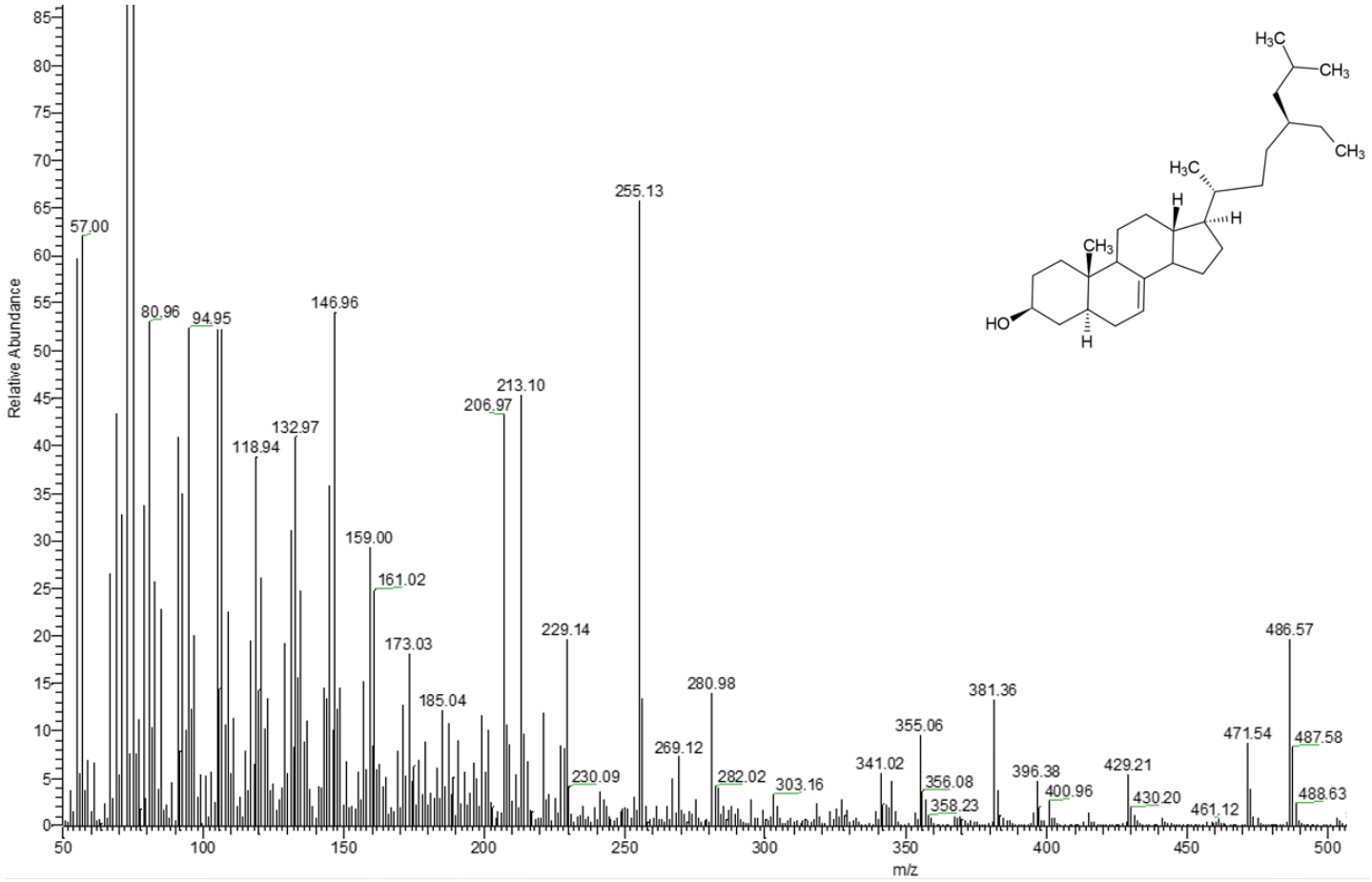
F: skeletal formula and mass spectra of stigmasterol

Peak at 484 represents *m/z* of sterol (C_{29}, 2 double bonds), peaks at 394, 379, 355, 255, and 129 represent ion fragments consistent with stigmasterol (Jones et al. 1994)



G: skeletal formula and mass spectra of sitosterol

Peak at 486 represents *m/z* of sterol (C_{29}, 1 double bond), peaks at 471, 396, 381, 357, 255, and 129 represent ion fragments consistent with sitosterol (Jones et al. 1994)



H: skeletal formula and mass spectra of stigmast-7-en-3β-ol

Peak at 486 represents *m/z* of sterol (C_{29}, 1 double bond), peaks at 471, 396, 381, 255, 229, and 213 represent ion fragments consistent with stigmast-7-en-3β-ol (Jones et al. 1994)

There were 2 other sterols found in the collected *Usnea* samples, and if these could be matched to the ergost-7-en-3β-ol and stigmast-7-en-3β-ol (Figure 5) produced by the algal samples then a complete biochemical match could be made between the unknown photobiont and the *Trebouxia* genus, but while the carbon numbers of the unknown sterols were the same as ergost-7-en-3β-ol and stigmast-7-en-3β-ol each had an additional double bond in its structure which did not match any known sterols from standards or sources.

Lichesterol and ergosterol (Figure 4, A and B) were the highest proportion sterols present in all *Usnea* samples and were not detected in any unialgal samples, confirming these sterols were produced by fungal cells in the lichen, which aligns with previous studies on the group. The unknown C_{28} sterol was also only found in the *Usnea* samples, suggesting that it is of fungal origin as well. The sterols detected in all groups - campesterol and stigmasterol (Figure 4, D and F) - formed a higher proportion in the algal samples compared to the whole lichen samples, consistent with their being produced by the algal symbiont considering the high proportion of fungal sterols in the whole lichen group. The unknown C_{29} sterol is present in lower proportion in the unialgal samples compared to campesterol or stigmasterol, and was not found in any *T. excentrica* samples, but otherwise can be categorized as an algal sterol on the same grounds as these two. Of the remaining sterols - brassicasterol, ergost-7-en-3β-ol, stigmast-7-en-3β-ol, sitosterol (Figure 4, C, E, H), and the unknown C_{28} and C_{30} sterols - ergost-7-en-3β-ol and stigmast-7-en-3β-ol were only found in the unialgal groups, sitosterol was only found in *T. excentrica* samples, and brassicasterol was only detected in culture strain 912 of *T. erici*, and in trace amounts. These sterols, then, were also produced by algal symbionts of

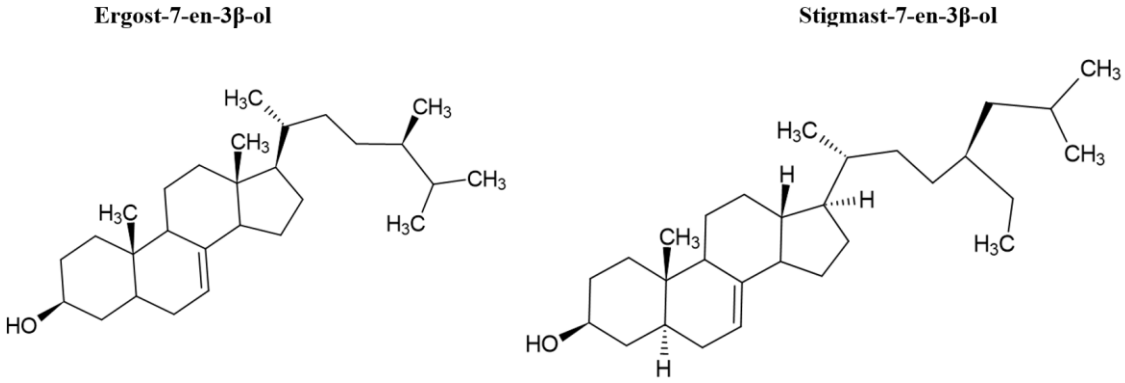
the genus *Trebouxia*, but not by the specific symbiont/s found in the set of collected *Usnea* samples (or if they are, they are produced in too small a quantity to be detectible with this method).

While stigmasterol consistently made up slightly more than 50% of the sterols found in all algal culture samples, in the whole Usnea samples it averaged less than 8% of the total sterols, with no esterified stigmasterol detected at all. Other sterols shared between the algal and whole lichen samples were proportionally similar between these samples, such as campesterol (the second highest proportion sterol in algal cultures), which Usnea samples showed a higher average percentage of than the *T. erici* 911 line but a lower average percentage of than the *T. erici* 912 line, as well as the unknown C_{29} sterol which made up a slightly higher percentage of the *Usnea* samples than the *T. erici* samples (Table 2). This was surprising considering that the relative percent of each sterol is calculated relative to the number of other sterols in the same sample and their concentration, and the whole *Usnea* samples contain on average 19-35% each lichesterol and ergosterol, which are fungal sterols that dilute the percentage of algal sterols in these samples. This means that for percents of campesterol and the unknown C_{29} sterol to be similar between the algal isolate and whole lichen sample groups, the algae in the *Usnea* lichen must be producing more of these sterols proportionally than any of the algal groups. This dilution by fungal sterols is not enough to account for the lower levels of stigmasterol that were present, as fungal sterols account for 50.49% of sterol esters and 64.11% of free sterols on average in the *Usnea* samples which would be expected to reduce the relative percentage of other sterols in these samples to less than 50% concentration, but these samples only contained 14.00% as much stigmasterol compared

to the algal samples. Thus, the algae living in symbiosis with collected *Usnea* lichens here produced more campesterol and unknown C_{29} sterol compared to the cultured algal groups, but significantly less stigmasterol.

The unknown C_{29} sterol found in all *T. erici* sample groups and the whole *Usnea* samples was absent in all *Trebouxia excentrica* samples, however these samples contained a known C_{29} sterol, sitosterol, which is only found in *T. excentrica*. Sitosterol, like the unknown C_{29} sterol, has 1 double bond and an atomic mass of 486 (Table 1). However, its relative retention time (RRT) and ion fragments differ from those of the unknown C_{29} sterol enough for it to be identified as sitosterol, while the RRT and ion fragments of the unknown sterol did not match with any reference sterol used here or found in the literature close enough for a positive identification. The similarities this unknown C_{29} sterol has to sitosterol, however, suggests that it is structurally close to sitosterol and/or follows the same synthesis pathway used for sitosterol in *T. excentrica* for much of its synthesis in *T. erici* and the *Usnea* algal partner. The relative percentages of sitosterol in *T. excentrica* also fall within the range of percents seen in *T. erici* and the *Usnea* algal partner for the unknown C_{29} sterol - 10.49% on average for sitosterol in *T. excentrica* while the unknown C_{29} sterol ranges from 9.03% to 14.10% in the averages found for the *T. erici* and *Usnea* samples.

Figure 5: Skeletal formula of ergost-7-en-3β-ol and stigmast-7-en-3β-ol



The galactolipid profile (Table 3) was similar between the 2 *T. erici* lines studied, but the galactolipids of *T. excentrica* were less diverse than those of either *T. erici* line, which is consistent with the sterol results for these 2 species. For both lipid types the *T. erici* lines contained some lipid structures that were not present in the *T. excentrica* or *Usnea* samples but shared the most representative lipids in each group. An 18:3/16:4 MGDG galactolipid was most abundant across all samples and was the only MGDG galactolipid detected in either the *T. excentrica* or *Usnea* samples, while the *T. erici* samples contained several more C_{18}/C_{16} MGDG lipids in smaller amounts.

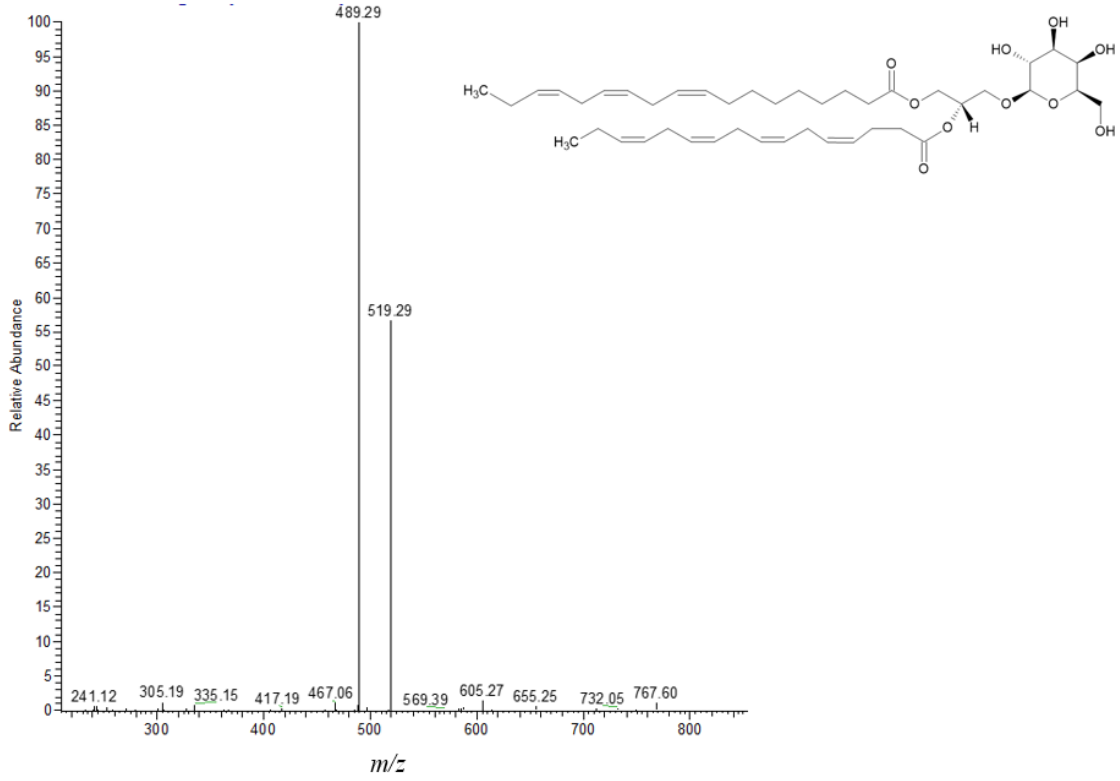
Table 3: Galactolipids of *Trebouxia* and whole lichen *Usnea* compared (listed as average relative percent with standard deviation, sample groups in duplicate)

lipid	fatty acids	mass	relative intensity (%)			
			T. erici 911	*T. erici* 912	*T. excentrica*	*Usnea* sp.
MGDG	18:3/16:4	767	35.26 ± 5.72	27.75 ± 9.60	59.59 ± 0.94	54.05 ± 4.1
MGDG	18:2/16:4	769	3.53 ± 0.42	7.16 ± 0.08	0	0
MGDG	18:3/16:3	769	12.15 ± 1.44	10.23 ± 2.51	0	0
MGDG	18:1/16:3	771	1.27 ± 0.41	1.79 ± 0.10	0	0
MGDG	18:2/16:2	771	8.87 ± 2.90	5.87 ± 0.33	0	0
DGDG	18:3/16:3	931	8.7 ± 1.41	15.64 ± 5.71	0	0
DGDG	18:2/16:2	935	0	0	14.29 ± 0.77	0
DGDG	18:1/16:1	939	9.26 ± 1.36	5.86 ± 2.33	1.14 ± 0.01	0
DGDG	18:2/16:0	939	2.16 ± 0.82	0.59 ± 0.12	25.56 ± 0.74	0
DGDG	18:3/18:3	959	11.44 ± 4.33	6.27 ± 1.22	0	40.95 ± 0.9
DGDG	18:3/18:2	961	7.57 ± 0.86	19.86 ± 10.28	0	0

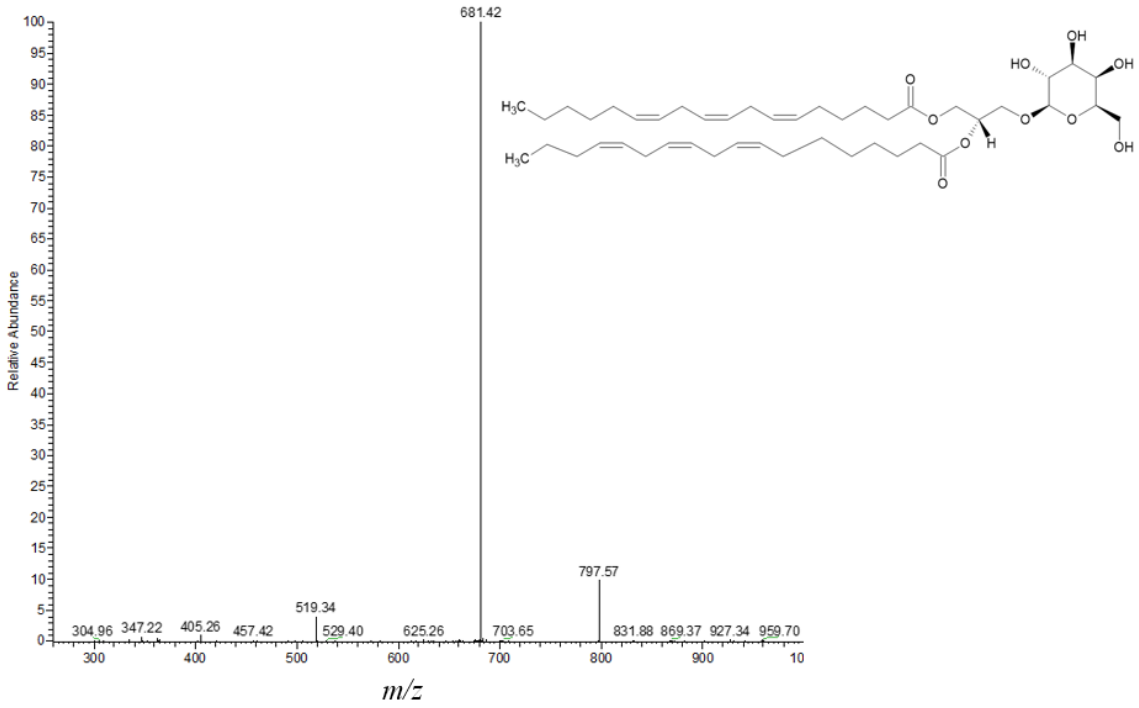
Galactolipid results show all samples to be dominated by an 18:3/16:4 MGDG, the highest proportion galactolipid across all groups (Figure 6, A). The *T. erici* samples displayed the greatest number of galactolipids, particularly MGDG - the 18:3/16:4 lipid previously mentioned was the only MGDG present in either the *T. excentrica* or *Usnea* sample sets, but several more were identified in both *T. erici* sample sets. While some variation of sterol lipids was found between the 2 *T. erici* lines, both line 911 and 912 of the *T. erici* samples had identical galactolipid content. *Usnea* samples only consistently showed one galactolipid beyond the 18:3/16:4 MGDG shared by all groups - an 18:3/18:3 DGDG also found in both *T. erici* groups (Figure 6 B). Galactolipids were identified by molecular weight (*m/z*) in an ESI/MS scan of each sample, and the fatty acyl structure for each was identified by an MS/MS scan focused on the molecular weight of the galactolipid (Figure 6). In the example MS/MS spectrograms below, the x axis represents

the mass to charge ratio, while the y axis (height of peaks) represents the relative abundance of the ions. The peaks with the highest mass represent total mass of the molecule, while the 2 high abundance peaks in each spectrogram represent the *sn*-1 and *sn*-2 fatty acid tails. Molecular weights for these fatty acyl fragments were compared to the molecular weight of the whole molecule, and fatty acyl structures calculated based off these relative weights. MGDG and DGDG were distinguished from one another by the greater molecular weights of DGDGs, irrespective to the fatty acid tails, due to the additional galactose molecule present. In figure 6 A, the total mass is 767 *m/z*, and the 2 peaks of interest to us are at 489 and 519 *m/z*- we subtract 489 and 519 from 767 to get 278 and 248 respectively for the acyl tails of this galactolipid. 278 corresponds with an 18:3 acyl structure, and 248 with a 16:4 structure (see skeletal formula figure 6 A), and a total mass in the 700s indicates an MGDG. Spectrograms with only one high abundance peak in the expected range for fatty acyls, as in Figure 6 B, indicate overlapping peaks for 2 fatty acyl tails of the same size. 959 minus 797, the only strong peak on this spectrogram besides the high abundance 681 peak, equals 162 which is too small for any acyl tail structure and most likely background noise. This leaves only the 681 peak, 959 minus 681 equals 278, which corresponds with an 18:3 acyl structure as in one tail of the 5A example above, but in this example both tails share this structure.

Figure 6: MS/MS spectra and skeletal formula of select galactolipids



A. 767 MGDG *sn*1/*sn*2 18:3/16:4



B. 959 DGDG *sn*1/*sn*2 18:3/18:3

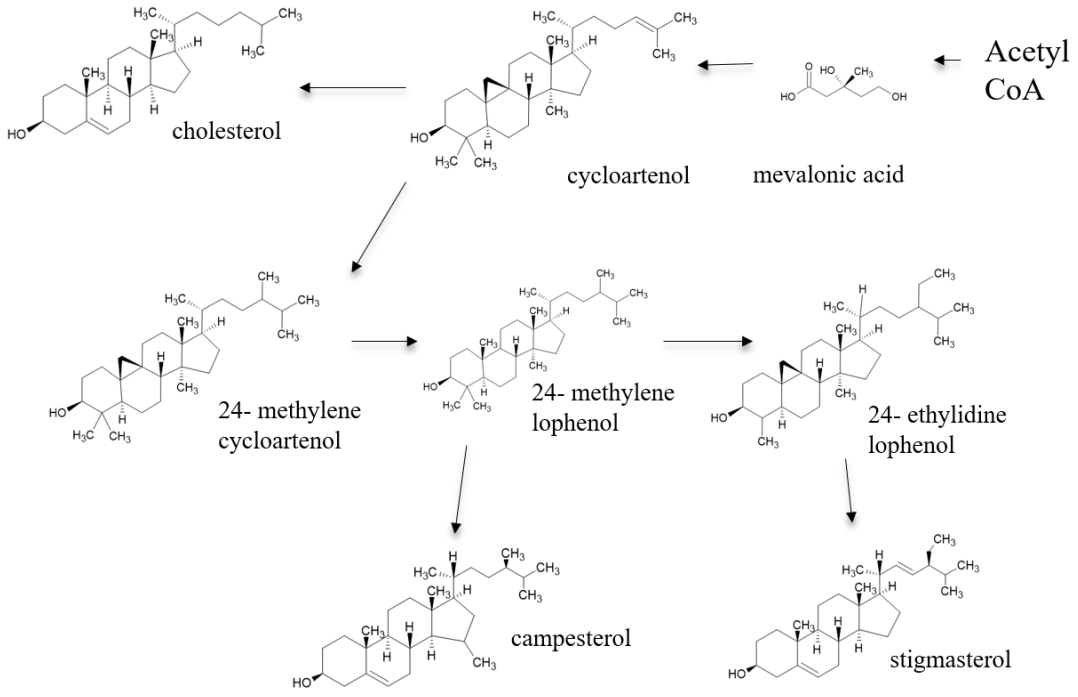
Chapter IV: Discussion and Conclusions

Sterols detected in the lab grown *T. excentrica* and *T. erici* algae as well as the wild collected *Usnea* lichens were all either C_{28} or C_{29}, as would be expected in phytosterols of chlorophyte algae (Kodner et al. 2008), except for one C_{30} sterol found in one of the two culture lines of *T. erici*. Stigmasterol and campesterol were found in all samples in this study and were the highest proportion sterols present in all unialgal *Trebouxia* lines - stigmasterol has been found previously in other *Trebouxia* species (Goad et al. 1972), and campesterol in whole *Usnea* lichens (Solberg 1987), so this aligns with what would be expected for these species. The *T. excentrica* sample set showed sitosterol as well, which has been found in *Trebouxia* species before (Goad et al. 1972). In the known *Trebouxia* lines of this study, sterol production is very similar to the

unknown *Usnea* photobiont it is being compared with, as the highest proportion of non-fungal sterols are stigmasterol and campesterol in both. However, several smaller proportion sterol molecules were found in the *Trebouxia* culture lines that do not match with the other sterols found in the literature sources, such as ergost-7-en-3β-ol and stigmast-7-en-3β-ol. In the whole lichen *Usnea* samples, a C_{28} and a C_{29} sterol were found that were very similar structurally to ergost-7-en-3β-ol and stigmast-7-en-3β-ol (Figure 5) respectively, sharing the same number of carbons, the same atomic mass, and the same number of double bonds. The same is true for campesterol and ergost-7-en-3β-ol, as well as sitosterol and stigmast-7-en-3β-ol - these 2 groups of 3 sterols each (C_{28}, 1 double bond and C_{29}, 1 double bond) can only be differentiated by differences in RRT and the different relative masses of their ion fragments. The unknown C_{29} sterol was only found in whole *Usnea* samples, but the unknown C_{28} was also found in very small quantities (less than 1% of total sterol lipids) in line 912 of *T. erici.* As the C_{29} sterol was only found in samples containing fungal cells, it could in theory be of fungal origin, but its similarity to phytosterols found in the same sample group and to phytosterols produced by the lab grown algal sample groups suggests that both these unknown sterols are products of the same algal sterol synthesis pathway as the campesterol and sitosterol in these samples. Sterol production takes place in multiple stages, with a portion of general use sterols being modified further after production for more specialized functions, and it is here that our *Usnea* derived unknown alga likely diverges in its sterol synthesis pathway from both the known *Trebouxia* species used in this study and the *Usnea* symbiote/*Trebouxia* species examined in the literature by Goad et al. (1972) and Solberg (1987).

End product sterols are synthesized from triterpene sterol bases (cycloartenol in the case of phytosterols and lanosterol in fungi and animals) by a series of enzyme mediated reactions (Figure 7). The enzymes used vary by group but modify the ring and side chain structures of the base sterol by one of a few methods (Benveniste 2002). Methyl group(s) may be added, removed, or modified by methylases, demethylases, or methyltransferases, and ring structure may be modified by enzymes like cyclopropyl sterol isomerase which converts pentacyclic sterols into tetracyclic sterols (the typical ring structure of end pathway sterols). Desaturases, reductases, and isomerases interact with one another to modify or replace portions of the ring or side chain at various steps (Benveniste 2002). A variation in one enzyme or group of enzymes involved in this process will result in a different end product sterol structure (Boguslaw and Goad 1982), which is why sterol molecules produced earlier in the pathway such as campesterol are more consistent and ubiquitous across genera, while more heavily modified end product sterols often vary within relatively small clades. Sterols such as campesterol and sitosterol are required to create a variety of end product sterols by modification to their structure, creating evolutionary pressure to retain the enzymes used in their manufacture unchanged (Benveniste 2002). End product sterols like stigmast-7-en-3β-ol and ergost-7-en-3β-ol, on the other hand, have more enzymatic steps involved in their creation leading to different pathways for synthesizing the same sterol molecule in different groups, as well as a variety of sterol molecule structures depending on the order and region of the molecule substitutions, reductions, etc. performed on them (Boguslaw and Goad 1982).

Figure 7: simplified major sterol biosynthesis pathway in plants (some intermediate steps have been excluded for brevity)



As expected based on previous studies (Sahu et al. 2013), only MGDG and DGDG with acyl tails of either 16 or 18 carbons in length were found in all samples tested in this study - primarily 16/18 (sn-1 tail C_{16}, sn-2 tail C_{18}), with 2 DGDG molecules with 18/18 tails also found. The 18:3/16:4 fatty acyl chain MGDG is common to all sample groups, with the *T. erici* samples sharing all galactolipids, and the *T. excentrica* and *Usnea* samples sharing some galactolipids with the *T. erici* samples or one another but missing galactolipids found in *T. erici*. As with the unknown C_{29} sterol found in both *T. erici* and *Usnea* samples, there is an 18:3/18:3 DGDG galactolipid common to *T. erici* and *Usnea* that is not found in any *T. excentrica* samples. Only 2 galactolipids -

the MGDG common to all sample groups and this DGDG shared by the *T. erici* samples - were consistently detected in *Usnea* samples, so while all galactolipids detected in *Usnea* samples match those in *T. erici*, many other galactolipids in the *T. erici* samples are absent in *Usnea*. This indicates that no contaminating galactolipid producing algae or cyanobacteria species were present on the whole lichen samples, or if any were present they were not abundant enough to be detected during analysis, however many galactolipids that were expected to be produced by the symbiotic algal partner of the *Usnea* were not detected during analysis either. The low proportion of algal symbiont to fungal symbiont is likely the limiting factor in analyzing *Usnea* galactolipids, as these lipids are found in the chloroplasts of plants and green algae and are heavily diluted by non-chloroplast containing fungal cells in the total biomass of whole lichen samples. There may be other galactolipid compounds present in the *Usnea* samples, but at too low of concentrations to be identified. However, the *T. excentrica* samples only contain 3 DGDG structures (along with the 18:3/16:4 MGDG found in all samples), and these samples are from concentrated algal cultures, so there is some variation in number of distinct galactolipids as well as fatty acyl structures between these groups - the unknown algal species in the whole lichen samples may simply produce fewer galactolipid types than either of the isolated *Trebouxia* species, and *T. erici* may be the outlier in the number of distinct galactolipid compounds it produces. The limited galactolipids in the whole *Usnea* samples could also indicate that algal cells produce smaller quantities of galactolipids or do not produce some galactolipids when paired with a symbiotic partner - algal cultures grown free of their fungal symbiont often show metabolic differences compared to symbiotically paired members of their species.

Both galactolipid data and sterol data confirm that as members of the same species, culture lines 911 and 912 of *T. erici* have the most similar lipid profile to one another out of all groups, with *T. erici* 911 and 912 containing the same galactolipids, and *T. erici* 912 containing small amounts of brassicasterol and an unknown C_{30} sterol that *T. erici* 911 lacks but otherwise sharing the same sterol profile. While *T. excentrica* shares the same major sterols as the other samples - campesterol, stigmasterol, ergost-7-en-3β-ol, and stigmast-7-en-3β-ol - it also contains sitosterol in place of the unknown C_{29} sterol present in the *T. erici* lines. *T. excentrica*'s galactolipid results also differ from the 2 *T. erici* lines more than these lines differ from each other, as far fewer galactolipids are present compared to the *T. erici* samples. Only one MGDG is present in *T. excentrica*, the 18:3/16:4 MGDG molecule that is the most abundant galactolipid in all the samples, and only 3 DGDG molecules are detectable - 2 of these the *T. erici* samples share, and one is only present in the *T. excentrica* samples. Comparison of the sterol composition of these isolated algal samples with the whole *Usnea* lichen samples confirms the presence of the fungal sterols lichesterol and ergosterol, as well as identifying an unnamed C_{28} sterol in this lichen. These 3 C_{28} sterols - 2 expected from the literature as common fungal sterols found in lichen, and one not matching any reference sterols used in this study close enough for positive identification - are the only sterols shown to be of non-algal origin, as they are absent in all algal culture groups. The unknown C_{28} sterol could also be interpreted as an algal sterol, however, as the C_{28} sterol ergost-7-en-3β-ol present in all unialgal sample groups is absent in the whole *Usnea* samples containing the unknown C_{28} sterol, suggesting the algal species found in these sample lichens could synthesize the unknown sterol in place of ergost-7-en-3β-ol. The whole lichen *Usnea* groups also lack

stigmast-7-en-3β-ol, a C_{29} sterol found in all unialgal groups, but the unknown C_{29} sterol it does contain (besides stigmasterol, found in all samples) is also found in both *T. erici* sample groups. A fungal sterol would not be expected to have 29 carbons in any case, but this sterol's presence in the unialgal samples as well confirms it as an algal sterol. All other sterols found in this study were found in the unialgal samples as well and are therefore produced by the algal partner in lichens. There is some variation in which sterols are used by the different species of *Trebouxia* - for instance *T. excentrica* uses sitosterol while *T. erici* and the *Usnea* lichen's symbiont species both use another C_{29} sterol, and the *T. erici* 912 line contained small amounts of brassicasterol and an unknown C_{30} sterol- but all *Trebouxia* photobionts used in this study produce campesterol, stigmasterol, and at least one other C_{28} and C_{29} sterol.

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