The University of South Bohemia in České Budějovice
Faculty of Science

Counter Current Chromatography for enhanced recovery and characterization of intact polar lipids in soil organic matter

Master´s thesis

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Annotation:

The study aims to develop the method for the recovery of the phospholipid analyte from the environmental samples using Counter Current Chromatography. This novel method development thereby optimizes the enhanced recovery of the intact polar lipids, in comparison to the solid phase biased recovery. The developed method provides a promising data for the microbial lipids extracted from soil and organic biomass which will help in the understanding of the soil profile, soil respiration, and the assessment of nutrient concentration of the soil sample.

DECLARATION

I declare that I am the author of this qualification thesis and that in writing it, I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 12 April, 2024

Sakshi Ravindra Tripathi

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List of Abbreviations used:

PLFAs- Phospholipid-derived fatty acids

eDNA- Environmental DNA

C- labeled- Carbon labeled

PCR - Polymerase chain reaction

PC- Phosphatidylcholine

PE- Phosphatidylethanolamine

PS- Phosphatidylserine

PI- Phosphatidylinositol

PG-Phosphatidylglycerol

DGTS- Diacylglyceryltrimethylhomo-Ser

CCC- Counter Current Chromatography

CPC- Centrifugal Partition Chromatography

DNA- Deoxyribonucleic acid

SPE- Solid-phase extraction

HEMWat- hexane: ethyl acetate: methanol: water

UV-Vis- Ultraviolet–visible spectroscopy

LCMS- Liquid chromatography–mass spectrometry

mL- milliliter

P- Phosphorous

C- carbon

DCM- dichloromethane

MeOH- methanol

TLE- total lipid extract

UV- Ultraviolet

Mo-Blue- Molybdenum blue

mM- Millimetre

µl- microlitre

HCL- Hydrochloric acid

nm- Nanometer

QToF- Quadrupole time-of-flight

PL- Phospholipids

ppm- parts per million

% v/v- volume concentration

1. Introduction

1.1 Importance of soil for sustaining climate and life on earth

Soil is a complex, dynamic, and enormous source of life sustenance on earth. The soil performs wide variety of functions such as providing food, services, habitat, medium, nutrition, health, ecosystem and shelter to a variety of organisms and microorganisms (Al-kaisi et al., 2017; Banwart et al., 2019; Bhat, 2013; Raj et al., 2019). Soil is also known as the skin of the ecosystem (Bhat, 2013). The soil is a porous interface connecting the atmosphere, vegetation, shallow geosphere, groundwater and surface water (Banwart et al., 2019). Soil microbial organisms are an essential connecting link between the biotic and abiotic systems of the nature (Islam and Wright, 2003). The soil microorganisms perform many important functions in soil. The diversity of the ecosystem is indirectly maintained by the soil by being the reservoir of nutrients, carbon, phosphorus and other elements of the ecosystem (Islam and Wright, 2003; Nannipieri et al., 2003). These elements are stored in the soil which are transferred to the plants and is utilized for photosynthesis. The photosynthetic reactions carried out by plants perform the task of maintaining the balance of carbon between the ecosystem and soil (Islam and Wright, 2003). The microorganisms present in the soil also contribute to the soil respiration process of the ecosystem (Schlesinger and Jeffrey, 2000; USDA-NRCS, 2014)

The soil can be defined from the viewpoint of the different uses it delivers to the source, such as, farming, environmental uses or engineering. The soil environment and soil functions are greatly impacted by the soil composition, geographic features and other factors that contribute to the physical, chemical, and biological characteristics of soils. The inorganic composition of mineral soil is generally composed of sand, silt, and clay. The proportion of these inorganic component decides the soil texture, soil properties including its chemical, physical, and biological properties (Al-kaisi et al., 2017). Soil contains minerals, trace elements and metals that are utilised by the plants and soil organisms for their nutritional requirements along with soil water regulation for uptake by different vegetation, soil organisms and wild animals. These features of soil immensely support the essentiality of life sustenance activities performed by the soil for earth's life and ecosystem processes (Raj et al., 2019). The soil biological environment is formed of microbes and microflora such as bacteria, actinomycetes, fungi, algae, protozoans, soil worms, insects or arthropods along with microfauna as well as macrofauna. These organisms perform the

task of obtaining energy from soil and support their maintenance from soil (Russell, 1973). The physical complexity of the soil allows the soil microbes to acquire the essential resources from soil (Bhat, 2013). The importance of soil in sustaining life systems is depicted in Figure 1. Soil is an excellent source of phosphorus, nitrogen, sulphur and other elements which are essential and critical for the ecosystem functioning. The soil ecological fluxes and nutrient cycles are briefly described in this chapter in below sections.

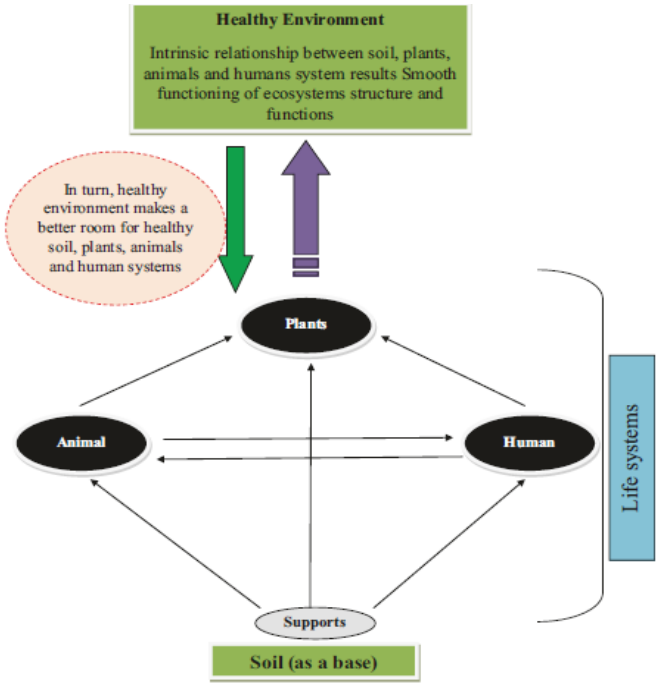

Figure 1: Soil as a source of life sustenance. Figure was adapted from (Raj et al., 2019)

1.2 Role of soil in sustaining the impact of climate change

It has been found that the parameters of climate change has both direct and indirect impact on soil microorganisms as well as plants and therefore indirectly also affecting the other life systems on the planet (Compant et al., 2010; Sergaki et al., 2018). Whenever there is any alteration in climate, there are structural changes, changes in abundance, composition and functionalities of the microorganisms associated to plant life (Lladó et al., 2017). Climate change has direct and indirect impact on plant–soil-microorganism interactions (Abhilash et al., 2013; Bojko and Kabala, 2017; Dubey et al., 2019a) and therefore it has the capacity to alter the community structure, abundance and function. Few indirect effects of the global climate change occur on the soil-microbial communities (Figure 2). These changes occur through the plants and are predicted to be way stronger than the direct effects. Any alteration occurring in the microbial community structure and composition causes changes in ecosystem functioning and affects the relative abundance of organisms

that are responsible in mediating the key and explicit processes which have a direct effect on the rate of those mediating processes (Schimel and Schaeffer, 2012).

With rising temperatures there has been a steady increase in the CO_2 levels (Jansson, 2019). The US national climate assessment has predicted a continual change in the weather patterns which is likely to become more erratic and extreme (U.S. Global Change Research Program, 2018). Soil microorganisms play a crucial role in organic carbon cycling and cycling of other nutrients. The soil microorganisms have an important role in the climate feedback inclusive of consumption of greenhouse gases such as carbon dioxide, methane and nitrous oxide (Bardgett et al., 2008). However, the role of soil becoming a source or a sink of greenhouse gases is still a mystery as there are many unknown processes related to nitrogen pool and differential microbial responses in soil (Friedlingstein et al., 2006; Wang et al., 2017). Hence, it can be stated that the understanding and study of microbial ecology is of utmost importance and is a challenge to completely understand it in a landscape scale model of climate study (Wieder et al., 2013).

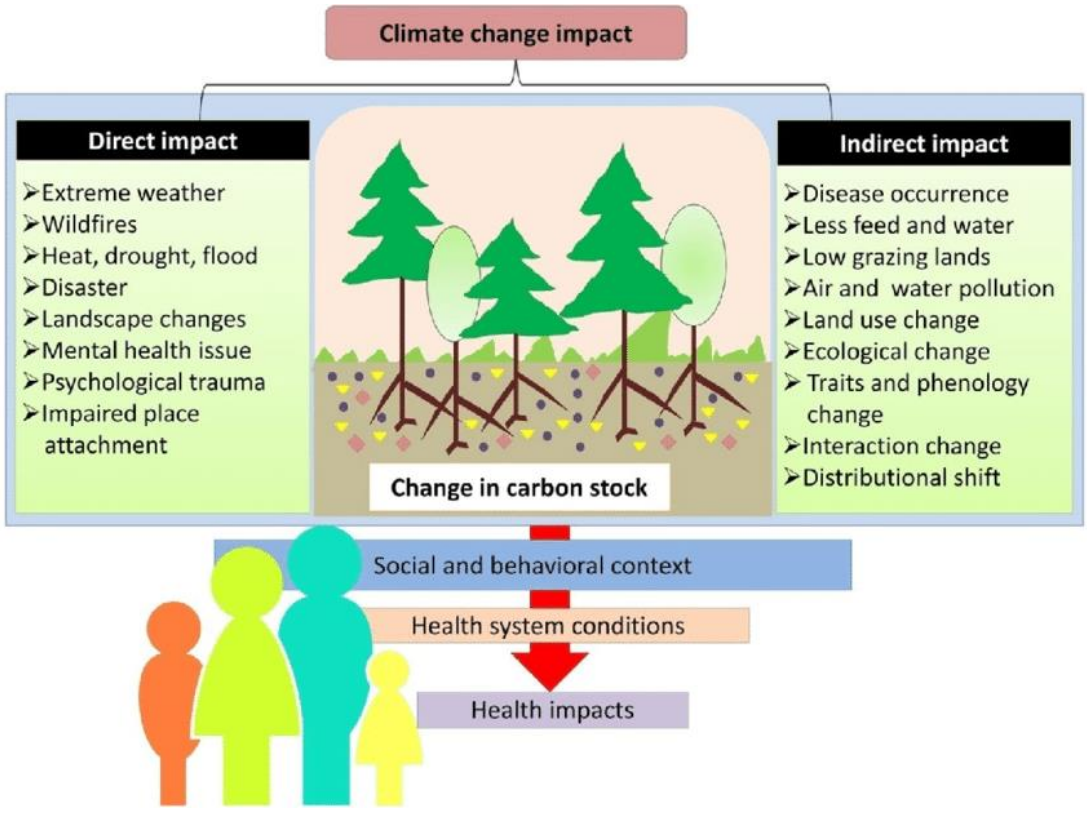

Figure 2: Role of soil in direct and indirect effects of climate change. Figure was adapted from (Dubey et al., 2019b).

1.3 Soil and nutrient turnover

The soil decomposition system comprises of a complex and diverse range of microorganisms which perform microbial activity that accounts to more than 90% soil decomposition (Condron et al., 2010). The soil organisms such as earthworms and mites act in regulation of decomposition. The soil organisms precondition the organic residues and modify the physical composition of soil by burrowing and other movements. All these organisms feed on the soil microbes (Doran and Werner, 1990). Soil ecosystem contains a huge amount of energy and that is stabilized by the proportionate balance of physical and chemical protection (Jensen and Magid, 2009). Biomass turnover, carbon, nitrogen, and phosphorus recycling plays an important role in maintaining soil fertility. The stoichiometric ratio of phosphorus, nitrogen and carbon in soil is a determinant of the soil nutritional state. Carbon content of the soil is related to the energy store of the soil where nitrogen and phosphorus are associated to the nutrient resource of the soil (Chen et al., 2019).

Soil microorganisms have a significant role in nutrient turnover of soil, i.e. bacteria and fungi, catabolize organic matter, mineralize and immobilize nutrients. The soil microfauna i.e. amoeba and nematodes, mites and earthworms help in regulating the fungal and bacterial population, alter the nutrient turnover of soil, stimulate microbial activity and breakdown plant residues in the soil. The soil activities change the soil structure by producing organic components, affecting aggregate formation, promoting soil humification and facilitating proper mixing of organic and mineral particles present in the soil (Doran and Werner, 1990).

1.4 Microorganisms in mediating the ecosystem

The diverse microbial community dwelling in soils are the drivers of the biogeochemical cycles that transform these elements in the ecosystem (Mackey and Paytan, 2009). Therefore, the environmental controls on soil structure and soil microbial communities determine the function and services provided by the belowground ecosystem, and thorough understanding of soil microbial dynamics can help to constrain ecosystem-level responses to environmental changes (Xue et al., 2018). Soil microorganisms spend their entire life cycle in the soil environment having mutual interaction and mutual relationships, showing varying associations among different levels of biological forms encompassing genetic variation, richness and evenness (Islam and Wright, 2003).

1.4.1 Biogeochemical cycles and the soil microorganisms

The earth's habitat consists of oxidised and reduced materials that are maintained by sun's energy, energy from earth's core and biological forces (microorganisms) which drive the different chemical and physical processes and evolution (Madsen, 2011). Microorganisms are a vital part of the biogeochemical cycling of nutrients by providing the facility of fixing both carbon and nitrogen into soil organic matter. The microbial driven processes very efficiently alter the chemical composition of the biosphere and its surrounding atmosphere for benefit of the higher forms of biodiversity (Merchant and Helmann, 2012). Microorganisms carry out the processes where an element circulates through biotic and abiotic systems of an ecosystem, such as carbon cycling and nitrogen fixation (Jensen and Magid, 2009). Soil organic carbon is the largest source of carbon in the terrestrial biosphere which is crucial for the carbon cycle. Similarly, soil respiration in terrestrial ecosystems is also important for the global carbon cycle regulation. The microbial decomposition results in a huge source of carbon dioxide which is derived from soil organic matter decomposition (Wang et al., 2017). Similarly, soil phosphorus is an essential element which mandatory to be maintained for balanced agroecosystem in soil, control soil productivity and also for a healthy aquatic ecosystem (Bünemann, 2015). The soil phosphorus dynamics is overseen by the physiochemical processes of nature and also the microbial immobilization, remineralization of immobilized phosphorus and mineralization of non-microbial organic phosphorus present in soil (Bünemann et al., 2012). The terrestrial biosphere and its organisms play an important role in the carbon cycling process as well, where the atmospheric carbon is captured by the microbes and higher life forms and is again returned back to the soil with the help of soil microbes (cyanobacteria) by breaking them into dissolved organic carbon compounds (Schimel, 1995).

The study of soil community structure has multiple applications, such as stabilizing ecosystem functioning (Wagg et al., 2021), carbon sequestration (Bhattacharyya et al., 2022), nutrient turnover and biogeochemical cycling (Gayan et al., 2023; Osburn et al., 2021; Perez-Quezada et al., 2021), enhancing soil fertility, boost plant tolerance towards biotic and abiotic stresses and increasing crop productivity (Gayan et al., 2023).

1.5 Respiration rate of soil and environment

The plants trap the carbon from soil through roots for their nutrition and the release of such CO_2 through soil and is known as soil respiration (Lloyd and Taylor, 1994). Soil respiration is the amount of CO_2 produced by the soil after converting the fixed organic carbon to CO_2 and returning to the atmosphere. The respiration rate of soil varies, among the ecosystems

as well between different communities, however, despite that the varying rates the respiration mechanism by the microorganisms remains the same in different ecosystems including terrestrial as well as marine (Schlesinger and Jeffrey, 2000). Increase in temperature has also shown latitudinal increase in soil respiration temperature sensitivity (Johnston and Sibly, 2018). Meanwhile, it was also observed that, the increasing deposition of nitrogen from the atmosphere is likely to lead to the sequestration of carbon in soil as well as in its vegetation (Schlesinger and Jeffrey, 2000). The amount of soil organic carbon content of the soil has been found to fluctuate depending on the environmental conditions of the soil such as in times of drought, fire, elevated carbon dioxide, increased temperature, increased precipitation and sea water intrusion in soil environment (Jansson, 2019)

1.6 Soil properties and soil microbial community

Microbial communities play important roles in soil health, contributing to processes such as the turnover of organic matter and nutrient cycling (Brewer et al., 2019; Naylor et al., 2022). Despite the huge contribution of soil biome and soil microbiome the understanding about the soil and subsoil microbial communities and their functionalities are yet under exploration (Dove et al., 2021; Xue et al., 2018). The wide variation in soil properties are stated to be likely due to the edaphic factors of soil, that vary with depth, varying organic carbon (C) availability, variation in soil nutrients, soil pH, and soil texture (Dove et al., 2020). Additionally, climate is also considered is also as a major driver of microbial community, microbial composition and microbial activity of soil (Thompson et al., 2017). The molecular structure of microorganisms, environmental factors of soil and biological factors also play an important role in controlling the soil organic matter stability (Schmidt et al., 2011).

Some factors, such as mean annual temperature, precipitation, environmental factors, etc., directly affect the soil microbial community (Dove et al., 2021; Jansson, 2019). Few indirect factors, such as soil chemistry on microorganism composition of a soil, its extracellular enzyme activity and the changes in the extracellular activity profile across elevation gradients. help in understanding depth of microbiome acclimatization with respect to its environmental factors (Dove et al., 2021). The microbial communities in soil have a direct effect on soil functionality which are observed by understanding their roles in the soil nutrient cycling as well as in carbon storage of soil (Xue et al., 2018). Variation in microbial communities have been observed temporally as well as spatially and even the subsoil microbiome communities also play important roles in soil carbon content and soil nutrient cycling (Dove et al., 2021). Study of the diversity in soil microbial community is

important to understand the soil properties, soil functionality and its applicability with respect to the environment and its biological requirements.

1.6.1 Soil bacteria and soil fungi

Bacteria found in the soil are prokaryotic unicellular organisms of varying shapes and sizes. There are around 20,000 different species of bacteria found per gram of soil. These bacteria are adapted to varying life environments (Tate, 1995). Soil bacteria account for 20% of organic substances that is usually decomposed from the soil (Adu and Oades, 1978). Similarly, actinomycetes are filamentous structures and are often found to be profusely branched in form. Considering their mycelia, the actinomycetes threads are much smaller compared to that of fungi. Initially, actinomycetes were classified along with fungi, but eventually they have been separated and classified as bacterial cells. The actinomycetes lack nuclear membrane, and disassemble spores which resemble bacterial cells. Actinomycetes often play unique roles in the soil compared to bacteria (Tate, 1995). Even though, actinomycetes grow best in a warm, moist, and well-aerated conditions but they are uniquely functionally important in arid areas and salt-affected soils (Islam and Wright, 2003).

The fungal communities found in the soil are eukaryotic organisms that form macroscopic aggregates in soil. The soil fungi comprises of a very diverse range of microorganisms with tens and thousands of fungal species present in soil (Islam and Wright, 2003). The abundance of fungi is usually found to be lesser than bacteria in soil. However, their large size and branched structure makes them dominate in the soil microbial community. The size and structure of fungal colonies also contribute to have a higher metabolic activity in different types of soils. Soil fungi are known to be energy efficient (approximately 30 to 50% of the decomposed organic substances may become bacterial tissue) (Brady and Weil, 2002). A brief description of carbon cycle, nitrogen cycle and phosphorus cycle are described below.

1.6.1.1 Role of soil microbes in Carbon cycle

The terrestrial microbiota comprise the terrestrial biosphere which has an important role in global carbon cycle, quantifying to approximately twice as much organic carbon as the atmosphere (Schimel, 1995). Carbon storage in soil is mediated by microbes that use plant primary production from above- and below ground litter and soil organic matter as their sources of carbon (Brant et al., 2006). Carbon content of global soil organic matter contains

three times higher carbon concentration in comparison to the carbon content of the atmosphere or terrestrial vegetation (Schmidt et al., 2011).

1.6.1.2 Role of soil microbes in Nitrogen cycle

Nitrogen cycle is a part of the nutrient cycling and nutrient turnover process. It is of high biological significance and three major processes (nitrogen fixation, nitrification and denitrification) are performed on the basis of different functions of various types of microbes (nitrogen fixers). In the process of nitrogen fixation, the inert dinitrogen gas is converted to reactive nitrogen (the usable form of nitrogen) by the microbial activities of nitrogen fixing bacteria (*Azotobacter sp, Bacillus sp, Klebsiella sp and Clostridium sp* etc.) (Stein and Klotz, 2016).

1.6.1.3 Role of soil microbes in Phosphorus cycling

The global phosphorus cycle is formed of the different phosphorus sources, sinks, and the pathways of phosphorus transport in the environment. Phosphorus is a crucial element in soil nutrient regulation. Phosphorus forms an essential link between earth's biotic and abiotic components. The amount of phosphorus affects the primary production process (the method of fixing inorganic carbon into cellular biomass through photosynthesis) and therefore, it has an vital role in understanding the global carbon, assessing the impact on global biogeochemical cycle and understanding climate influenced changes in environment (Mackey and Paytan, 2009). Microorganisms help in soil P immobilization and remineralization (Bünemann, 2015).

1.7 Study of soil microbial community

Study of soil microbial community is performed in order to understand the microorganisms in soil, their abundance, distribution and diversity which allows a clear understanding of its effects on varying faunal diversity, gradient as well the vegetation (Mackey and Paytan, 2009; Xue et al., 2018). The mechanism by which the spatial distribution soil microbes takes place is largely unknown, and in order to understand the variations in soil microbial community, different markets of soil composition are being studied.

Soil community structure is a wide subject to study and it is affected by a variety of reasons such as temperature, chemical content of soil, environmental factors, water content etc (Jansson, 2019). It was found that changing climatic, environmental and physiochemical factors of land area has a profound impact on its soil microbiome structure (Jansson, 2019), such as experiments have revealed that, transplanting soil samples from

one core to a new environment has an impact on its soil microbial community. When core soil of an environment is transplanted from another location, the communities change rapidly (Waldrop and Firestone, 2006). Drenovsky et al., (2004) suggested that the soil communities changed over time in the same soil sample with changing vegetation or cropping and with treatment of soil with organic carbon and water inputs. Changing microbial density has also been observed with a change in soil depth (Fierer et al., 2003). Physicochemical factors like heat stress, soil type and chemical structure of soil have been found to have a profound effect on the soil community composition and its microbial physiology (Griffiths et al., 2008). Changing climatic conditions such as elevated CO_2 levels, drought, temperature changes are found to have an impact on the changes in soil microbial structure (Mekala and Polepongu, 2019).

1.7.1 Significance of phospholipid study in terms of soil microbial study

Microorganisms have a membrane bound structure which often contains phospholipids, glycolipids and fatty acids in their membrane structure. The lipids present in the membrane of microorganism are the determiners of microbial presence in a soil sample (Papadopoulou et al., 2011; Wu et al., 2009; Zelles, 1999). Phospholipids contain phosphorus and is an important element of nature due to its diverse role in different life forms and systems (Mackey and Paytan, 2009). The amount of microbial activity and microbial presence in a soil sample have a major role in understanding the microbial activity of the soil. The presence of microbial community is responsible for abundance, functionality, energy reserve, nutrient turnover and soil respiration (Chen et al., 2019; Islam and Wright, 2003; Jensen and Magid, 2009; Pajares and Bohannan, 2016).

Lipid estimation in soil is informative of the presence of microbial activity and microbial presence of a soil (Frostegård et al., 2011; Zelles, 1999). The active as well as inactive lipid forms can infer the knowledge about the microbial diversity of a soil sample. The diversity of microorganisms can be studied by identifying the type of phospholipid and its uniqueness to determine the microbial diversity and identify the soil micro-organisms in a sample (Barnes and Turner, 2016; Piotrowska-Seget and Mrozik, 2003; Wu et al., 2009).

Phospholipid-derived fatty acids (PLFAs) is one of the method for identification and estimation of phospholipids and its residues from soil samples (Papadopoulou et al., 2011; Wu et al., 2009). Phospholipid-derived fatty acids have been used widely in efficient determination of significant phospholipids present in soil samples for microbial estimation, but the limitations of PLFA method makes it less efficient in identification of soil microbial

communities (Joergensen, 2022). The PLFA method of lipid estimation has limitations in terms of specificity, correct assignation and recycling of microorganisms (Frostegård et al., 2011; Joergensen, 2022). There has been recent advancements in the process of microbial lipid identification and counter current chromatography has been found to be a beneficial technique for estimation and identification of microbial lipid molecules (Hubert et al., 2012).

The methods used for studying the soil microbial community relates to the applications of soil microbial biomass with respect to the soil type, soil microbial colonization and land use characteristics of a soil. Usually there are several methods to study the soil microbial community structure that range from eDNA analysis, lipid extraction, studying phospholipid fatty acids, studying the fatty acids and lipopolysaccharides for microbial characterisation, rRNA gene analysis, lipid biomarker analysis and solid phase analysis etc. (Barnes and Turner, 2016; Joergensen, 2022; Padmanabhan et al., 2003; Papadopoulou et al., 2011; Picariello et al., 2023; Piotrowska-Seget and Mrozik, 2003; Wu et al., 2009; Zelles, 1999; Zelles and Bai, 1993).

Phospholipid-derived fatty acids are the major components of most cell membranes which usually do not accumulate in the soil organic matter while the cells are rapidly dividing and synthesizing. Therefore, PLFA is an excellent indicator for the study of microbial biomass as it contains viable organisms and lipids which can be used to derive information about the soil microorganism (Joergensen, 2022). The prediction of the soil and other environmental factors that control the microbial distribution also helps in predicting the future soil and environmental change in spatial distribution of microbes (Xue et al., 2018). PLFA is a significant method for understanding the microbial diversity as it is not only indicator for soil microbes but it also provides an estimation of total soil microbial biomass (Joergensen, 2022).

1.7.2 Current approaches to study soil microbial biomass and activity

1.7.2.1 Study of eDNA

Environmental DNA (eDNA) is the genetic material extracted from bulk environmental samples like water, soil and air. The eDNA estimation method is used to identify the different species and perform genetic analyses to conserve, manage, and research on samples or aggregates where collecting an entire organism is not feasible. The successful use of eDNA detection has shown significant increase in recent years but the emphasis on the ‘‘ecology’’ of eDNA. The ecology of eDNA refers to the understanding of the

interactions among the organism and its genetic material and the environment along with its influence on the detection on eDNA, quantification of eDNA, eDNA analysis, and its applications on conservation and research. The study of eDNA has been found to be useful for the estimation of population size, population genetics and different analyses using the eDNA. The study of eDNA can also include other indicator biomolecules such as environmental RNA or proteins along with a diverse range of environmental samples (Barnes and Turner, 2016).

1.7.2.2 Estimation of C labeled soil DNA

C-labeled DNA analysis is a method that employs C-labeled DNA for the study of live bacterial population in soil sample. A study by Padmanabhan et al., (2003) focusses on to develop a method based on stable isotope probing of soil microorganism identification using C-labeled compounds. The method mainly emphasized on field soil biodegradation assay where 13C labeled compounds were used to identify the active microorganisms by analysing 16S rRNA genes in soil-derived C-labeled DNA. This method of biodegradation approaches to ensure least number of microbiological artifacts that is usually present in soil due to some physical or nutritional imbalance of soil. This method incorporated the use of C-labeled compounds (glucose, phenol, caffeine, and naphthalene) to soil plots before collection of soil samples, installing glass chambers with an open bottom to ensure the covering of the soil, and analysing the samples of headspace gases to check for carbon dioxide respiration. The carbon dioxide emission from naturally occurring soil organic matter to chambers inserted into our field soil test plots and the respired carbon dioxide in the soil was studied. Field respiration assays were performed and were compared with the background respiration from soil organic matter to document the in-situ respiration of caffeine and naphthalene compounds. The transient peaks of carbon dioxide released more than background were studied and Caesium-chloride separation of C-labeled soil DNA was performed, which was followed by PCR amplification of 16S rRNA genes from microbial populations. The sequencing results in obtaining the full sequences of bacteria revealing the active bacterial populations present in the soil sample (Padmanabhan et al., 2003). The use of carbon source in DNA extraction is performed with labeled DNA as well as unlabeled DNA of microorganisms in soil sample. Separation of carbon and C-DNA has been done using density gradient centrifugation where the active microbial pool in a soil sample can be successfully determined (Nannipieri et al., 2003).

1.7.2.3 Phospholipid fatty acid analysis (PLFAs)

Phospholipids are essential component of cell membranes that provide them their structural integrity and activity. The physical characteristics and roles of phospholipid molecules within biological membranes are largely determined by the diversity of their headgroups. Phospholipid diversity mainly consist of Phosphatidylcholine (PC); Phosphatidylethanolamine (PE); Phosphatidylserine (PS); Phosphatidylinositol (PI); Phosphatidylglycerol (PG); Diacylglyceryltrimethylhomo-Ser (DGTS). One of the most prevalent phospholipids in cellular membranes, especially in those of eukaryotic cells, is PC. It is characterized by a choline headgroup linked to the glycerol backbone by two fatty acid chains and a phosphate group (Ali and Szabó, 2023). Another important phospholipid found in cellular membranes is PE. It mainly has two fatty acid chains and a phosphate group connecting the ethanolamine headgroup to the glycerol backbone. PE is involved in membrane curvature and fusion processes, as well as membrane stability (Morita and Ikeda, 2022). PS is found in small amounts in cellular membranes, primarily localized to the inner leaflet of the plasma membrane. It has a serine headgroup that is joined to the glycerol backbone by two fatty acid chains and a phosphate group (Vance, 2015). PI is an essential component of cell signalling mechanisms, despite being a small phospholipid. It is made up of two fatty acid chains and a phosphate group that connect the inositol headgroup to the glycerol backbone (Katan and Cockcroft, 2020). phosphatidylglycerol (PG) is found in many biological membranes and is crucial to cellular signalling, structure, and function. PG is characterized by its glycerol backbone, two fatty acid chains, and a phosphate group, to which a glycerol moiety is attached (Shulga et al., 2011) and lastly Diacylglyceryltrimethylhomo-Ser (DGTS) is a unique phospholipid that is mostly present in bacteria, algae, and some plants, among other microorganisms. It differs structurally from the more widely distributed phospholipids, such phosphatidylcholine and phosphatidylethanolamine, that are present in eukaryotic membranes.

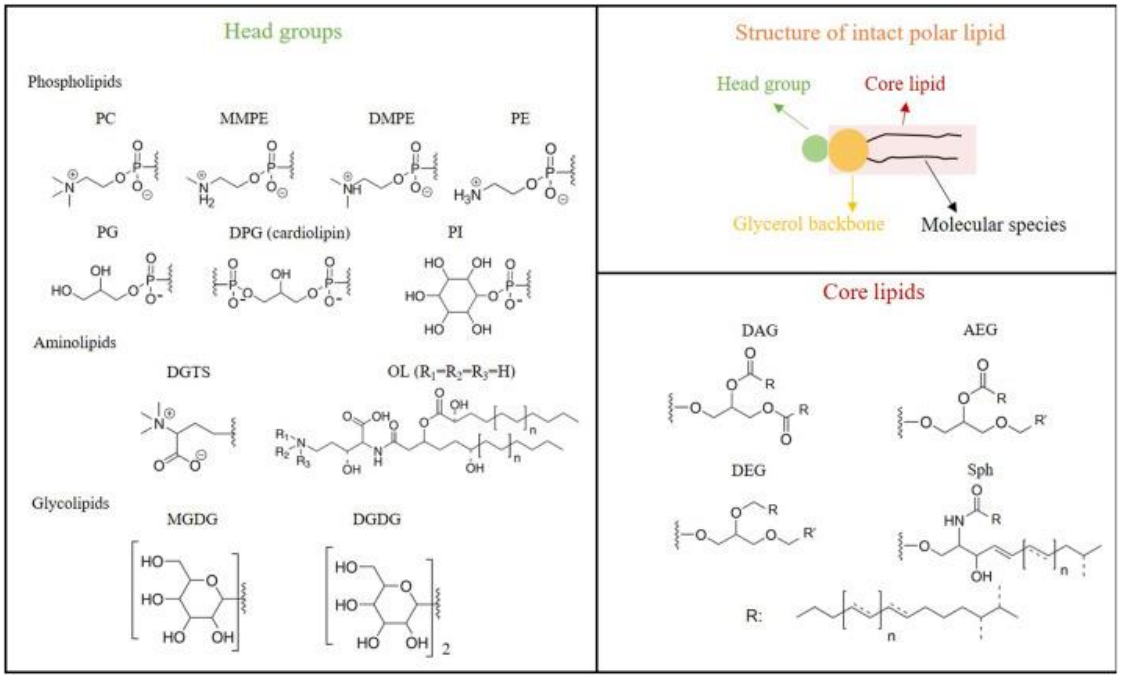

Figure 3. Head groups and core lipids with a glycerol backbone are seen in the chemical structures of intact polar lipids, respectively, on the left. DAG, diacylglycerol; DEG, dietherglycerol; AEG, acyletherglycerol; DGTS, diacylglycerylhydroxymethyltrimethyl-(N,N,N)-homoserine; OL, ornithine lipid; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; MMPE, phosphatidyl-(N)-methylethanolamine; DMPE, phosphatidyl-(N,N)-dimethylethanolamine; Sph, sphingolipid; MGDG, monoglycosyldiacylglycerol; DGDG, diglycosyldiacylglycerol; OH, monohydroxy; Me, methylated. Figure was adapted from (Ding et al., 2020).

Phospholipid fatty acid (PLFA) analysis is the method to assess the amount of fatty acids in the form of lipids, fatty alcohols or other complex compounds such as peptidolipids and glycolipids (Wu et al., 2009). It is a robust method of characterizing soil microbial communities in natural ecosystems (Papadopoulou et al., 2011; Wu et al., 2009). The PLFA analysis is based on the analysis of ester linked fatty acid profile of phospholipids in the membrane of microbial organisms. The distinction between the eukaryotes and prokaryotes and many prokaryotic groups along with their rapid degradation after cell death enable the PLFA technique to be a useful indicator in determining living microbial community (Papadopoulou et al., 2011). PLFA therefore helps in assessing the microbial community structure in soil and the microbial biomass (Zelles, 1999).

PLFA analysis provides information about the phospholipid type and therefore, it helps in identification of bacterial or fungal type and groups. The PLFA helps in

identification of bacterial groups such as gram-negative bacteria, gram positive bacteria as well as fungal groups which is a benefit of utilizing this process for soil microorganism identification and assessment. Solid phase extraction is a procedure to separate the lipids and fatty acid methyl esters present soil samples and assist in determining the ester linked fatty acids in phospholipids and hydroxyl fatty acids in lipopolysaccharides of soil microorganisms. This is one the most sensitive and reliable methods to determine microbial biomass and understand the microbial community structure (Quideau et al., 2016; Zelles and Bai, 1993). Furthermore, recent developments in liquid chromatography analysis of intact phospholipid molecules have demonstrated a large, diverse array of phospholipid (and other) headgroups connected to variable fatty acid chains in nature (Wörmer et al., 2017).

1.7.2.4 Counter current chromatography

Counter current chromatography (CCC) is a process to separate particles on the basis of charge and/or hydrophobicity (Leive et al., 1984). A counter current chromatographic solvent system consists of two immiscible phases. This kind of solvent system is known as a biphasic solvent system. Any specific compound will have a different relative solubility in each of the phases and this causes an effective distribution of the compound between the two different phases (Friesen and Pauli, 2015). This method has been found to be efficient and reliable in terms of lipid separation in the form of fractionation, making it a more intricate and detailed separation process of microbial lipids (Vetter et al., 2017). The counter current method is a newly introduced method in estimation of glycolipids from crude extracts (Hubert et al., 2012), where the glycolipids obtained from microorganisms are studied and analysed to understand their structure and identify them to assess the soil microbial organisms in the sample (Papadopoulou et al., 2011). The glycolipid mixtures from natural glycolipid sources have a high complexity and therefore it is essential to have proper purification techniques for biological identification purposes. Glycolipid extraction can be done using fractionation and purification methods such as centrifugal partition chromatography (CPC) and counter-current chromatography (CCC) techniques. These chromatographic techniques are beneficial as it can be used for the preparative or large-scale separation of glycolipids from complex crude extracts (Hubert et al., 2012) thereby helping in identification of diverse array of soil microorganisms in a soil sample (Padmanabhan et al., 2003).

It was developed by Yoi chiro Ito in around 1970s. The counter current chromatography has therefore eventually become an important compound isolation

technique for natural products for its immense efficiency in compound separation (Ito and Bowman, 1970). Counter current chromatography is separation technique where its biphasic solvent system is of utmost importance (Friesen and Pauli, 2015). The modern counter current chromatography instruments utilize the J-type planetary centrifuge where a liquid phase is stationary by means of a fast planetary movement (in centripetal or centrifugal pattern, Figure 4). The mobile phase is transported through the coils using a pump like device. Considering other typical chromatographic solvents, the amount of sample in counter current chromatography are found to be approximately 100 mg to 10 g. The counter current chromatography technique offers a far higher amount of sample compared to the other classic chromatography techniques like high performance liquid chromatography and gas chromatography techniques (Vetter et al., 2017).

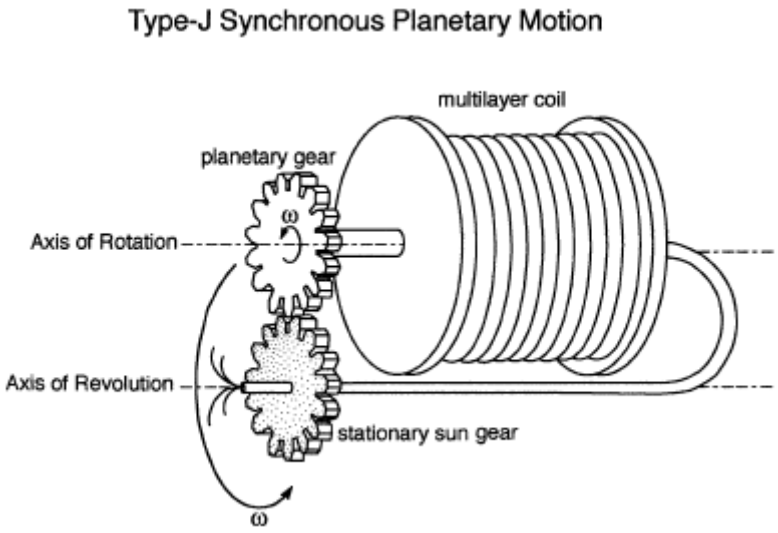

Figure 4. Type J planetary motion of a multilayer coil separation column. Figure was adapted from (Skalicka-Woźniak and Garrard, 2014).

1.7.2.5 Counter current chromatography and its benefits in lipid separation

Counter current chromatography offers multiple benefits for isolation of soil microorganisms (Friesen and Pauli, 2015; Hubert et al., 2012; Vetter et al., 2017). The soil microorganisms comprise of different microbial groups such as bacteria, fungi, actinomycetes etc. Each of these groups contain multiple sub groups and numerous types of microorganisms within these groups which makes the separation and understanding of their membrane lipids a complex process (Islam and Wright, 2003). The separation of the lipids of the microbial groups in different fractions may ensure efficient separation of the lipid compounds and therefore it will aid to a diverse and specific understanding of the

microbial organisms and groups (Papadopoulou et al., 2011; Wu et al., 2009; Xue et al., 2018).

The lipid content in soil contains both dead and inactive lipid samples as well as live microorganisms. In order to distinguish between the live microbial community in soil there are several processes that have been developed such as eDNA analysis, C labeled DNA analysis and signature lipid biomarker analysis etc (Barnes and Turner, 2016; Padmanabhan et al., 2003; Piotrowska-Seget and Mrozik, 2003). The counter current chromatography system allows for a systematic separation of lipid molecules based on the size of their hydrocarbon chains. The lipid molecules are separated in the counter current chromatography system on the basis of their molecular properties. The lipids having different molecular properties are eluted at different separation rates from the column. The separate elution volume and retention time of each lipid allows for a better separation of lipids from the chromatography column. The lipids β sitosterol, campesterol, cycloaternol, brassicasterol etc. separate at different rates when separated in different stationary phases (Vetter et al., 2017).

Glycolipid extraction of soil is performed in a three-layered process. The microbial cultures (containing the bacterial cells, fungi cells or residual sources) are grown in a fermentation media. The precipitate of the fermented colony is obtained and centrifuged to obtain the crude lipid extract. This extract is further converted to concentrated glycolipid mixture by using techniques such as membrane filtration, selective crystallization, foam fractionation adsorption etc. This concentrated glycolipid mixture is then filtered and further separated using techniques like thin layer chromatographic technique, Ion exchange chromatography, reverse phase column chromatography or other chromatographic techniques (Hubert et al., 2012). The lipid estimation methods presented in the other techniques apart from counter current chromatography are lengthy and less efficient in comparison to the counter current chromatographic system due to its unique and stringent fraction separation. This allows for a better lipid separation and therefore a more intricate process of determining soil microbial community in complex and diverse soil systems (Friesen and Pauli, 2015; Hubert et al., 2012; Vetter et al., 2017). The counter current chromatographic method of lipid recovery offers a high accuracy in terms of phospholipid recovery from soil samples (Hubert et al., 2012).

1.8 Objective of the study

Considering the existing research on glycolipid separation using counter current chromatographic techniques, the following objectives have been framed for the present study.

1. Develop counter current chromatography protocols for the separation of phospholipids from contaminants and background organic matter components in soil and sediments.
2. Measure total phosphorus content of polarity fractions and compare recovery yields of phospholipid standards using purification protocols, including solid phase extraction and counter current chromatography.
3. Determine total phosphorus content via colorimetric methods to estimate recovery yields of purified phospholipid fractions from biomass samples.

1.9 Significance of the study

The understanding of soil microorganisms and their diversity will contribute to the detailed understanding of carbon sequestration, nutrient turnover, soil fertility, soil efficiency and ecosystem structure and function of soil (Gayan et al., 2023; Müller et al., 2002; Osburn et al., 2021; Perez-Quezada et al., 2021). Study of soil microbial community enhances the understanding of soil microbial abundance and therefore the understanding of soil diversity, its vegetation and understanding the soil gradient across ecosystems (Mackey and Paytan, 2009).

The soil microbial study is performed by studying the DNA and lipid content in the soil by different methods (Barnes and Turner, 2016; Joergensen, 2022; Papadopoulou et al., 2011; Picariello et al., 2023). The PLFA analysis is the most widely used method but it has several disadvantages (Joergensen, 2022) whereas, counter current chromatography is a recently established process of lipid extraction by fractionation. The lipid extraction from soil microbial samples is more efficient and this process offers a systematic separation of lipid molecules based on their physical and chemical properties (Vetter et al., 2017). This method may thus also enhance sample recovery in comparison to other conventional methods, thereby improving assessments of soil microorganisms (Friesen and Pauli, 2015; Vetter et al., 2017).

The present study will therefore employ the counter current chromatography method to assess the recovery and isolation of lipids from the soil organic biomass compared to the conventional SPE method. The aim is to improve lipid proxies of soil organic biomass, which will be beneficial for understanding the soil profile, soil vegetation, soil fertility, soil respiration, and the assessment of nutrient concentration of the soil sample. The present study will provide a detailed overview of the recovery of the intact lipids from the soil and fungi samples. Therefore, the findings of this study will be helpful in vegetation gradient understanding and nutrient analysis of the soil biomass in the future.

2. Materials and Methods

2.1 Determination of the Partition coefficient

2.1.1 Preparation of solvents

One popular method of concocting a solvent system for CCC involves the mixing of a hydrocarbon solvent such as hexane with ethyl acetate, methanol and water (HEMWat) (Han et al., 2023; Liu et al., 2018). In the current study, these solvents were used to prepare the HEMWat solvent system in different ratios. Volume ratios were determined by adding appropriate volumes of hexane, ethyl acetate, methanol and water to a 500 mL separatory funnel to equal a (premixed) combined volume of 400 mL (Table 1). Each solvent system was thoroughly equilibrated at room temperature by repeated shaking and degassing. The solvent was later kept for few hours for the solvent mixture to settle into two distinct layers (biphasic upper and lower layers). The upper layer being mostly hexane and ethyl acetate, with some small proportion of methanol, and the lower layer being the methanol and water at the end of the separation of the entire solvent mixture.

2.1.2 Preparation of standard stock solution

A) L-α-Phosphatidylcholine (PC) 1.5 mg/mL

B) L-α-Phosphatidylinositol ammonium salt from Glycine max (soybean) (PI) 9 mg/mL

C) L-α-Phosphatidylethanolamine from Glycine max (soybean) (PE) 1.2 mg/mL

Each of the standard (A-C) was dissolved in 10 mL isopropanol. Aliquots of 1.5 mL PC (75 ppm), 2 mL of PE (100 ppm) and 1.6 mL PI (100 ppm) were used to prepare working solutions which was dried and resuspended in 20 mL methanol: water for two replicate partitioning experiments (2 x 10 mL).

For determining the partitioning coefficient by UV-Vis spectroscopy, a small amount (typically a few milligrams or less, depending on its absorptivity) of each standard was added to a glass vial (13 mm × 100 mm) and the solvent was evaporated under a stream of nitrogen gas. For determining the partitioning coefficient by LC-MS, the three phospholipid standards were mixed into single, replicate vials. Then, 4 mL of each equilibrated solvent phases (upper and lower) were added and were thoroughly mixed with a vortex to emulsify the solution. After settling into upper and lower layers, a subsample (0.5 mL) was collected from each layer for analysis of total P (or LC-MS analysis). Collect the remaining layer into separate vials, without disturbing solvent interface. Inject 3×700

µL into UV-Vis is flow through detector using matching solvent as mobile phase. The absorbance was measured by a spectrophotometer at the suitable wavelength(s) to obtain the $K_{U/L}$ value. Temporarily express the partition coefficient as $K_{U/L} = C_U/C_L$, where C_U is the solute concentration in the upper phase and CL, that of the lower phase. If $K_{U/L} = 2$, the lower phase should be used as the stationary phase, which gives K= 0.5. It is important that this preliminary $K_{U/L}$ must be clearly distinguished from $K_{L/U}$ using the subscripts to avoid confusion (Figure 5).

HEMWat	Hexane (mL)	Ethyl Acetate (mL)	Methanol (mL)	Water (mL)
1:2:1:2	66.67	133.33	66.67	133.33
2:1:2:1	133	66.67	133.33	66.67
3:1:3:1	150	50	150	50
4:1:4:1	160	40	160	40
9:1:9:1	180	20	180	20
19:1:19:1	190	10	190	20

Table 1. HEMWat solvent system with the composition of each solvent prepared in different volume ratios.

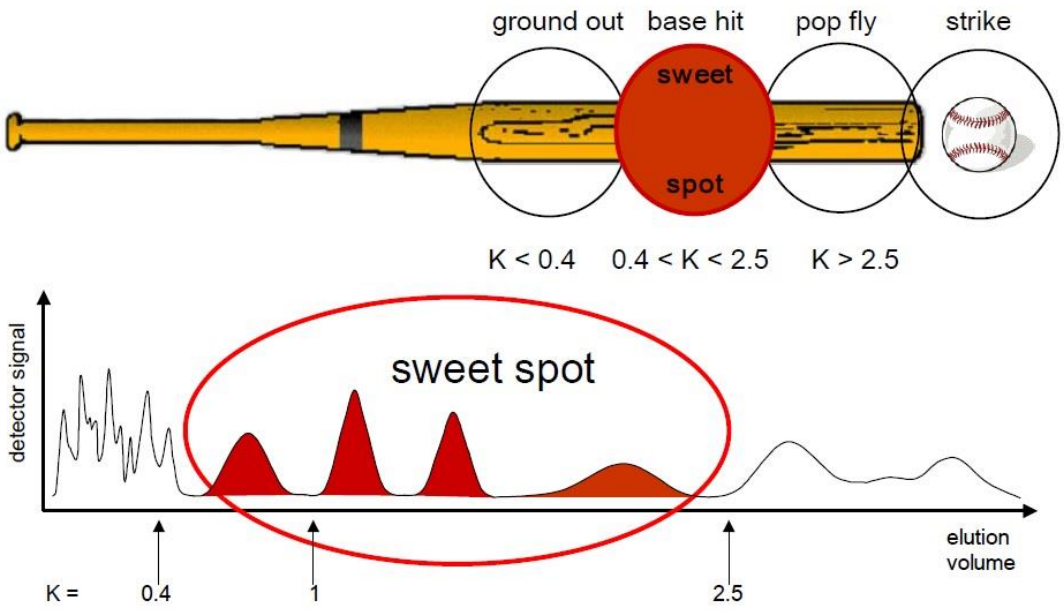

Figure 5. Schematic figure of the CCC sweet spot. The range around K=1 provides optimum resolution and in analogy to bat and racket sports, can be called the sweet spot of separation. Therefore, the process of choosing a solvent system aims to find a mixture, in which the analyte elutes in the range between K=0.4 and K=2.5, which is a working definition of the sweet spot range for the purpose of this study. Figure was adapted from (Brent Friesen and Pauli, 2005).

2.2 Extraction of lipids

Bligh & Dyer

Cordyceps farinosa cell biomass was extracted following the modified method Bligh and Dyer protocol (Bligh and Dyer, 1959; Sturt et al., 2004; Wörmer et al., 2017). Samples were sonicated for 10 min in four steps with a mixture of dichloromethane (DCM)/ methanol (MeOH)/ and an aqueous solution (1:2:0.8, v:v:v) by using 4 mL solvent per g biomass and extraction step. A phosphate buffer (8.7 g/L KH_2PO_4, pH 7.4) was used for the first two steps and a trichloroacetic acid buffer (50 g/L CCl_3COOH, pH 2) for the final two steps. After each extraction step, the samples were centrifuged at 2500 rpm for 5 min and the supernatants were collected in a separation funnel. Phase separation was induced by addition of DCM and water and the organic phase was drawn off and collected in a Schott bottle. The combined organic phase was then washed 4 times with de-ionized MilliQ water. Finally, the organic phase was collected as the total lipid extract (TLE), evaporated under a stream of N_2 and stored at -20 °C.

2.3 Preparation of samples

2.3.1. Preparation of biomass samples

The sample solution was prepared by dissolving ~ 100 mg of the crude Bligh and Dyer (TLE) of fungal *cordyceps* biomass or soil O-layer sample in 10 mL isopropanol, which was dried and resuspended in 11 mL methanol: water (3:1) solvent.

2.4. Counter Current Chromatography

The instrument was used in the Head to Tail configuration wherein the stationary phase was chosen to be the organic (3:1 hexane: ethyl acetate) and the mobile phase was chosen to be the lower aqueous (3:1 methanol: water). Out of the two coils present in the multilayer coiled CCC, only a single coil was used for the analysis of the samples, since we wanted to see the separation of the phospholipids with a sharp elution peak. The multilayer coil number two was filled with the upper organic phase (3:1 hexane: ethyl acetate) as stationary phase, pumping at 4 mL /psi (20 psi) for about 50 min while the apparatus was run at 600 rpm for a working volume ~160 mL. The next step was to equilibrate the coil with the bi-phase solvents (i.e., both the mobile and the stationary phase solvents), for this the mobile phase (3:1 methanol: water) was pumped at a flow rate of 2mL/ min at 600 rpm for a duration of 45 minutes until a working volume of~ 60 mL (ca. 40% of the coil volume, which is optimum for CCC to run) was eluted from the tail end coil. The effluent from the tail end coils was monitored with a UV-Vis detector. During this time, the eluted dead volume or the retention volume of the stationary phase was considered using a graduated measuring cylinder for collecting the eluted stationary phase from the tail end of the coil. Once, the coil was equilibrated, the next step was to check if the injection loop of the injector was empty and the injector was in INJECT position to load the sample suspended in the mobile phase. Once, the sample is injected into the CCC, the position of the injector is immediately shifted to LOAD position to load the sample into the coil of the CCC, during this time the apparatus was running at a flow rate of 2mL /min at 600 rpm for 6 minutes. Mobile phase was pumped at 2mL/ min at 600 rpm for 80 min (=160 mL, replaces mobile phase volume already filled in the coil of CCC). Exit flow goes to waste for 40 min, then to fraction collector, collecting new fraction every 4 min. (8mL per fraction; n = 21 fractions). After 80 min, the flow was changed to stationary phase at 4 mL/min at 600rpm for 40 min (=160 mL), collecting fractions for every 2 mins (8 mL per fraction; n=20). Out of the 8 mL collected fractions, 4 mL of each fraction was immediately transferred to 4 mL glass vials for total phosphorus measurement (Mo-Blue assay) and the remaining 4 mL was stored for LC-MS analysis of the intact phospholipid molecules.

2.5 Mo-Blue:

2.5.1 Preparation of Reagents

Fresh solutions of 4.86 mM ammonium molybdate tetrahydrate, 60 mM ascorbic acid, 0.2 mM potassium antimonyl tartrate trihydrate solution, 1.2 M sulfuric acid and 5% (w/w) persulfate stock solution (8 mL) were prepared. Persulfate solution was prepared using 0.3 g boric acid, 0.15 g sodium hydroxide and 0.5 g potassium di-sulphate oxide dissolved in 10 mL distilled water and the solution was heated at 55 °C until it was completely dissolved.

2.5.2 Formation of Molybdenum Blue Complex

450 µl (5%) stock persulfate solution was prepared by using the following ratios: Persulfate solution 0.75%, total volume of the sample 3 mL, 255 µl de ionized H_2O.

Later the samples were heated in heat block at 120 °C for 2 hr. This allows the formation of the molybdenum blue complex, which is proportional to the phosphate concentration. The sample was cooled to room temperature. Further the samples were acidified by adding 532µl of 0.75 M HCL. The inorganic phosphorous was measured using spectrophotometer.

2.5.3 Reduction with Ascorbic Acid

After the reaction time (i.e. two hours), ascorbic acid solution was added to each exetainer. This step reduces the molybdenum (VI) ions to molybdenum (V) ions, resulting in a blue colour indicative of the phosphate concentration.

2.5.4 UV-Vis Measurement

The absorbance of each solution was measured at an 890nm using a spectrophotometer (SPECORD 50 PLUS-233H1107F). The absorbance values for each standard solution and sample solution were recorded.

2.6 Solid Phase Extraction (SPE)

Duplicate replicate samples of a synthetic mixture of phospholipids with PC, PI, and PE headgroups, soil and fungi samples (5% TLE) were dried and eventually resuspended in 500 mL chloroform. Supeclean LC-Si followed by confirmatory LC-MS analysis. Lipid

extracts were fractionated on silica SPE columns (500 mg, Discovery LC-Si, Supelco, Bellefonte, USA) using the same eluents and ratios of eluents to stationary phase as described previously (Mills and Goldhaber, 2010). In short, silica columns were equilibrated and activated with methanol and chloroform, lipid extract resuspended in chloroform was added, then fractions were collected by eluting columns with 2.0 mL chloroform (the "neutral lipid" fraction), 2.0 mL of acetone (the "glycolipid" fraction) and 6.0 mL of methanol (the "phospholipid" fraction) (Warren, 2019). Out of the 6 mL methanol fraction of phospholipids, 3 mL was retained for total P analysis (Mo-Blue assay) and the remaining was retained for LC-MS analysis.

2.7 Liquid chromatography mass spectrometry (LC-MS) analysis

For separation of phospholipids by liquid chromatography, we followed the protocol using MeCN:DCM (75:25) with 0.01% HCO_2H and NH_3 as eluent A and MeOH: water (50:50) with 0.4% HCO_2H and NH_3 as eluent B, as described by (Wörmer et al., 2015). The column (ACQUITY UPLC BEH Amide, 1.7 μm, 2.1 ×150 mm) was maintained at 40 °C, with a flow rate of 400 μm min^{-1}. The gradient started with 1% B (2.5 min hold), increasing to 5% B at 4 min, to 25% B at 22.5 min and to 40% B at 26.5 min. A one min washing step with 40% B is followed by the return to initial conditions and column equilibration (8 min). The entire chromatographic run was completed in 35.5 min and back pressure remained < 600 bar. Samples were dissolved in DCM:MeOH (9:1) prior to injection. Phospholipids were detected using a Bruker compact QToF mass spectrometer with electrospray ionization in positive ion mode. MS data were assessed by Data Analysis and Compass software (Bruker). Briefly phospholipids were tentatively identified by retention time and by diagnostic mass fragmentation patterns after collision induced dissociation of selected phospholipid masses (Meador et al., 2017). Phospholipid masses were then quantified by integrating the peaks of extracting ion chromatograms (± 0.05 Da).

3. Results

3.1 Choice of the solvent system

The most important and the crucial step of this study in terms of method development of the CCC was to select the right solvent system which was determined by the partition coefficient experiment. In this study, a range of 6 different volume ratios of the HEMWat (hexane: ethyl acetate: methanol: water) solvent system was being tested listed in (Table 2). The results of the partition coefficient (K) values measured by the spectrophotometer reveal that the K value for the HEMWat solvent system was very close K=1 for the volume ratio 3:1:3:1(K value for PE and PC= 0.96) (Figure. 6) which fitted well to the working definition of the sweet spot in CCC, wherein the K values lies between 0.4 and 2.5.

So, affinity of the distribution constant of the two synthetic phospholipid headgroups PC and PE for the HEMWat solvent system for decreased in the order 3:1:3:1>4:1:4:1>1:2:1:2>2:1:2:1>9:1:9:1>19:1:19:1.

To confirm the results of the UV-Vis measurement, the distribution constant for the phospholipid headgroups (PC, PE, and PI) were tested by the LC-MS analysis, through which we concluded that the HEMWat 3:1:3:1 solvent system fits well for the phospholipids separation (Table 3) The HEMWat 3:1:3:1 solvent system was finalized for further method development of the recovery of phospholipids via CCC.

HEMWat Volume ratios	% water	K value

		PC	PE
1:2:1:2	33.33%	0.51	0.26
2:1:2:1	16.67%	0.26	2.20
3:1:3:1	12.50%	0.96	0.96
4:1:4:1	10%	0.47	0.84
9:1:9:1	5%	0.05	0.14
19:1:19:1	2.50%	0.13	0.13

Table 2. Partition coefficient values (K value) of the synthetic phospholipid standards PC and PE for a range of different volume ratios of the HEMWat solvent system obtained from the experimental measurement by the spectrophotometer.

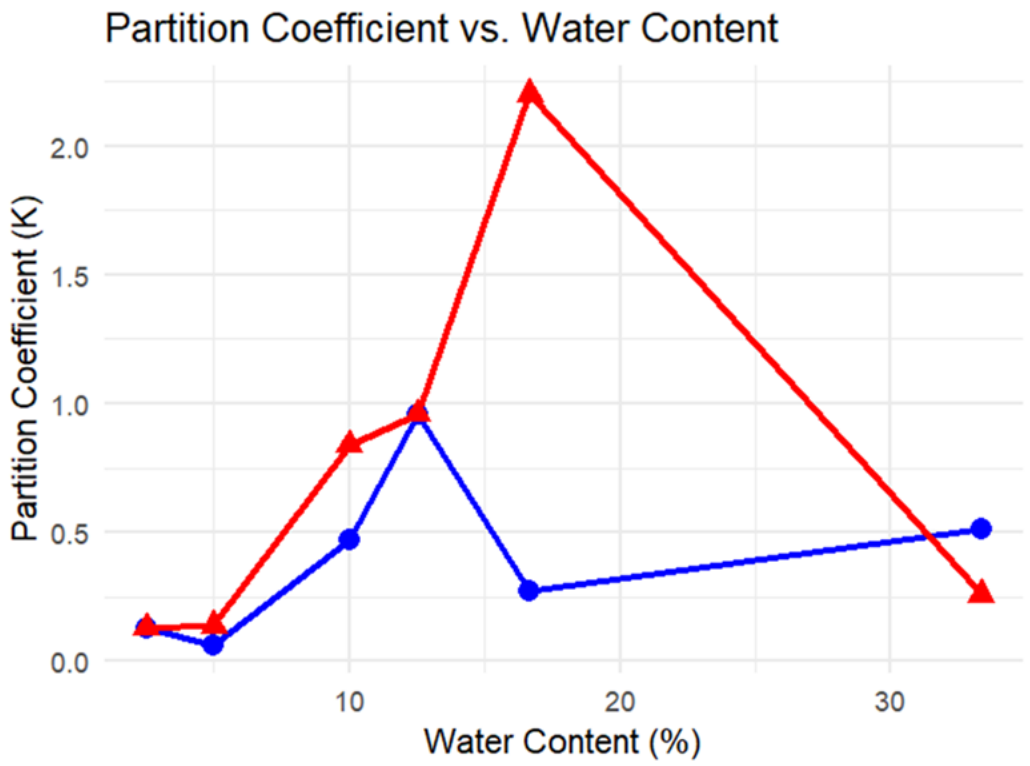

Figure 6. Distribution or the Partition constant (K) vs the water content (%) for the phospholipids PC (in red) and PE (in blue) for the HEMWat solvent system.

$K_{value=}$ (stationary/mobile)	m/z	Std mix Rep 1 (K_{value})	Std mix Rep 2 (K_{value})	Average (K_{value})
PC 16:0/ 18:1	760.5728	0.85	0.80	0.83
PE 16:0/ 18:1	718.5266	1.45	1.40	1.42
PI 16:1/ 18:1	835.5787	0.50	0.48	0.49

Table. 3 Distribution constant or the Partition coefficient for the synthetic phospholipid standards PC, PE, and PI of the final solvent system HEMWat 3:1:3:1 measured by the LC-MS.

3.2 SPE results

The Supeclean LC-Si cartridge showed a low recovery (~20%) for the synthetic mixture of phospholipids, and less than 20% for the soil TLE sample with PC, PI, and PE headgroups, whereas for the fungi TLE sample the recovery of the same phospholipid headgroups was more than 40% (Table 4., Figure 7.). The LC Si SPE blank was below the detection limit (i.e., < ~20 pg). The recovery of the phospholipids PC, PE and PI was lower than expected for all the samples (i.e., synthetic mixture of phospholipid standards, Soil TLE sample and Fungi TLE sample). The lower recoveries may indicate that retention capacity of the LC-Si cartridge was beyond its threshold, and/or, in case of Soil TLE sample, the high amount of interference of the organic matter with extraction or ionization of the phospholipid molecules.

Compound	m/z	P mix Std	Soil TLE	Fungi TLE
PC 16:0/ 18:1	760.5728	20%	13%	49%
PE 16:0/ 18:1	718.5266	23%	9%	45%
PI 16:1/ 18:1	835.5787	20%	7%	53%

Table 4. Recovery % for the phospholipid head groups PC, PE, and PI eluted by methanol from the LC-Si SPE cartridge for the synthetic mixture of phospholipid standards, Soil TLE and Fungi TLE samples.

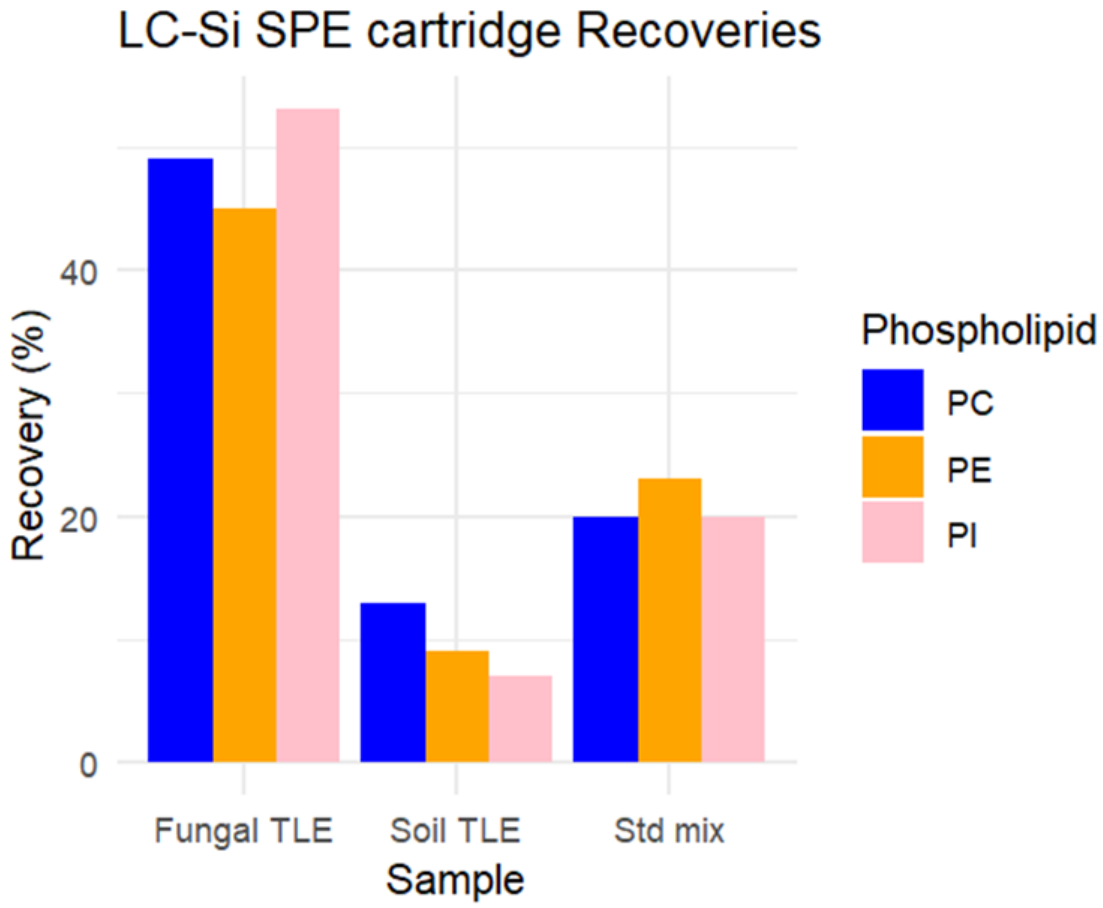

Figure 7. Percentage of the phospholipids recovered in the 6 mL fraction of the methanol from the LC-Si SPE cartridge for the synthetic mixture of phospholipid standards, Soil TLE and Fungal TLE.

3.3 Recovery of phospholipids with CCC (Counter Current Chromatography)

The synthetic mixture of the phospholipid standards sample was prepared as described in the methods section 2.1.2, and 30% of the soil and fungal TLE was resuspended in 11 mL of the mobile (aqueous phase i.e., methanol: water). Each sample was injected in duplicate (each 13.6% of TLE samples) into the CCC. The recoveries of the phospholipid headgroups in the different samples varied drastically depending upon the type of the sample injected. The 8 mL fractions collected by the CCC i.e., 21 fractions in the mobile phase (methanol: water) and 20 fractions of the stationary phase (hexane: ethyl acetate) were collected and tested by the colorimetric Mo-blue test for the testing the presence of the phospholipids in the form of inorganic phosphorus. The positive fractions for the

presence of phospholipids as well as the negative fractions for the Mo-blue test were further used for the LC-MS analysis.

3.3.1 Recovery of the Standard mixture

The injected sample into the CCC was a total 100% phospholipid content of each of the headgroups: PC, PE and PI (5000 ng of phospholipids each replicate). The Figure 8. shows the recovery of these headgroups over and shows the clear distinct separation of the phospholipids in the different fractions for the retention time of the collected fractions from the CCC. The results from the LC-MS analysis confirm the combined, average recovery of all the phospholipid standards PC, PE and PI to be ~100%, while individual recoveries of the standards PC, PE and PI were 143%, 200%, and 40% respectively.

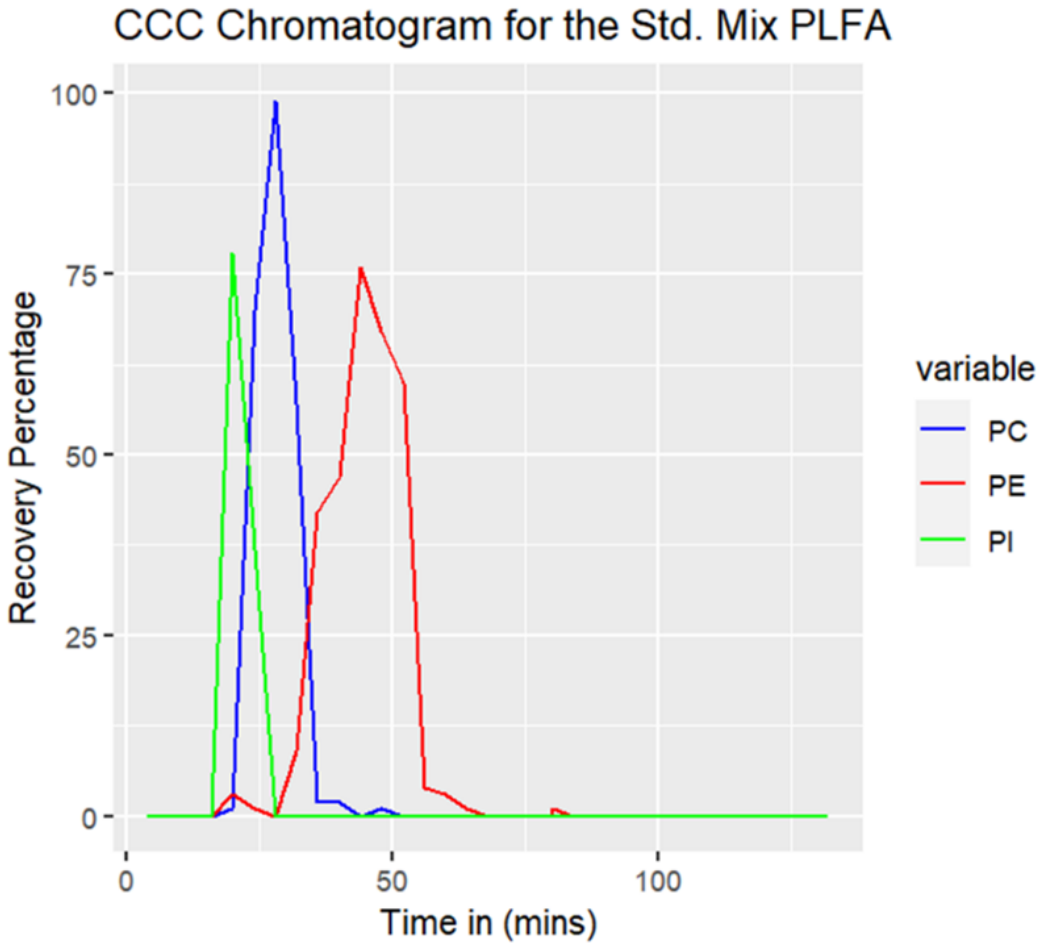

Figure 8. LC-MS data chromatogram of the phospholipids PC, PE and PI recovery from the fractions collected from the CCC both mobile and stationary phase obtained from the synthetic mixture of phospholipid headgroups PC, PE and PI (100 ppm each).

3.3.2 *Recovery of the Soil TLE sample:*

The environmental soil sample collected from the O- horizon, the 30% percent of the TLE sample was injected into the CCC in two replicates (625ng per injection). The injected sample when tested for the phospholipid content revealed a diversity of around 60 different lipids, which included glycolipids, phospholipids as well as the fragmented head groups. Out of the 60 diversified lipids, we selected 21lipids that were the most abundant lipids which accounted to the relative abundance of the lipid content~75%. The selected EIC (Extracted Ion Chromatograms) of 21 different lipids were taken into consideration to seek their recovery percentages in the collected fractions of the CCC (both mobile phase and stationary phase) in comparison to the injected sample of the pure soil TLE. The results of the LC analysis reveal that the combined average recovery was 953/2100= 45%. The individual recoveries of the lipids are listed in (Table 4). The tentative identification of the lipids was done in the injected sample based on the fractionating pattern, time of elution, and the pattern of the lipids observed in the heat map chromatogram of the LC-MS Bruker data analysis software (Figure 9.). The lipids detected in the injected sample were grouped to understand the overall recovery of a particular lipid. The overall individual recoveries of the lipids were ranging from quite high to quite low as well. The most abundant eluted lipid in the collected fractions of the CCC were: PE phospholipid ~498% followed by PI~173%, PG~137%, PI~127% and the least recovered lipids were the DGTS ~13% and MG-DAG~5%. The results suggests that although the overall recovery seems low (i.e., ~45%) but the fractionation technique of the CCC helps us in recovering the separated and purified lipids. The chromatogram in the (Figure 10.) shows the elution pattern of the different lipids with their recovery%.

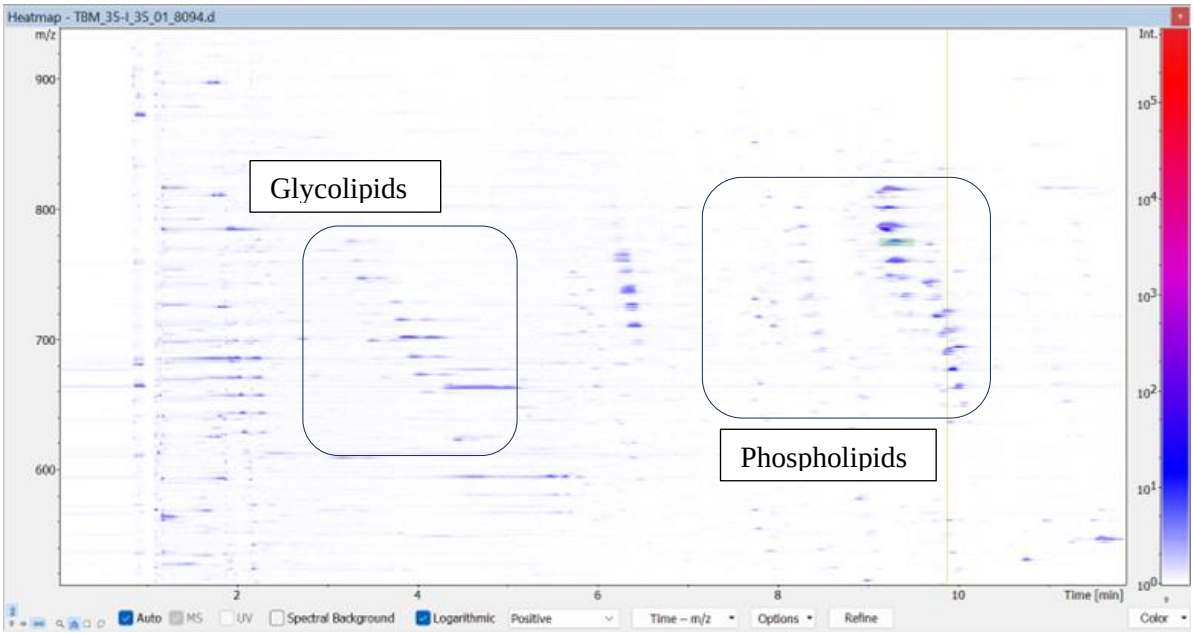

Figure 9. Image of the heatmap (density map) from the Data analysis software used for the LC-MS data analysis. The pattern helping us to understand the elution pattern of the lipids at different retention times.

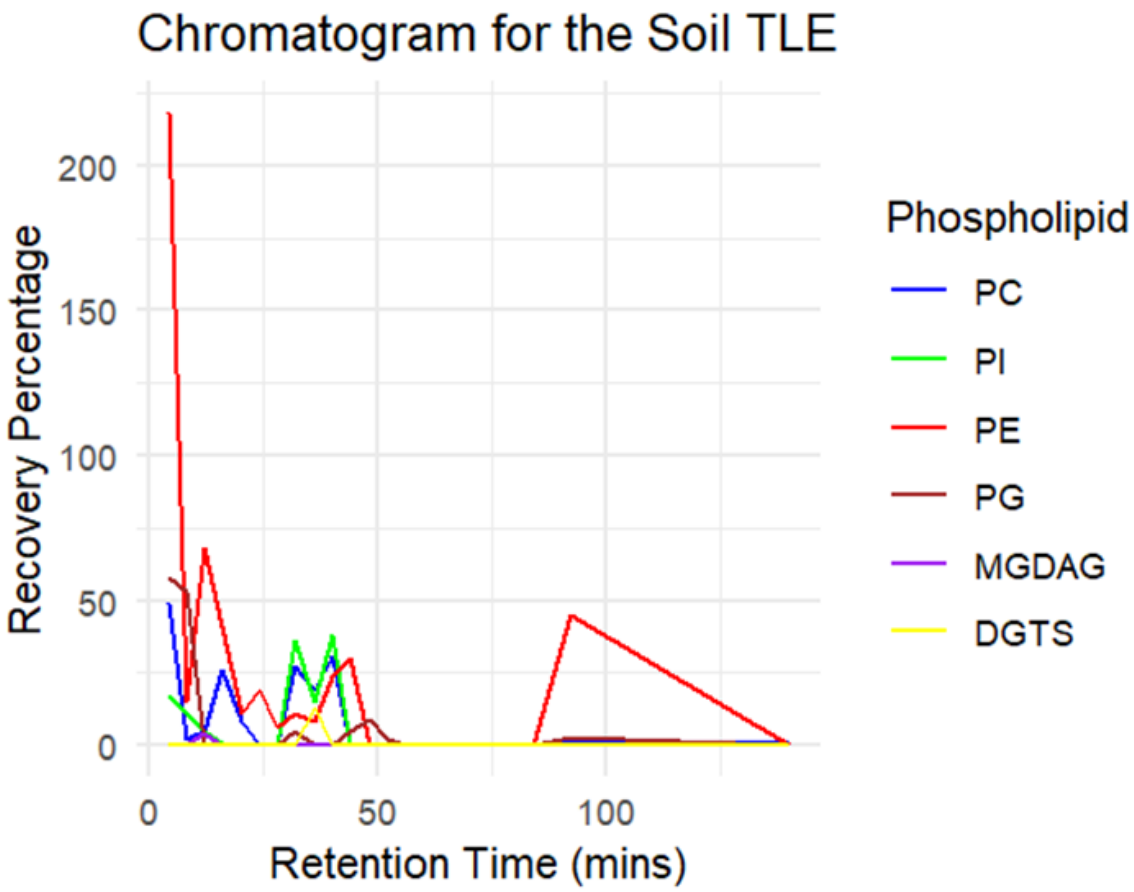

Figure 10. LC-MS data chromatogram of the phospholipids: PC n=5, PE n=4, PI n=3, PG n=6, and betadine lipids such as MG-DAG n=2, and DGTS n=1 (Grouping of the EIC into the categories as listed in the Table 5.) recovery from the fractions collected from the CCC both mobile and stationary phase obtained from the injected soil TLE sample.

EIC Trace	Retention time min	Type of lipid	Recovery %
786.5941 ± 0.01	9.2	PC	9
814.6256 ± 0.01	9.2	PI	39
760.5728± 0.01	9.3	PI	87
736.6012± 0.01	6.3	PG	22
800.6105 ± 0.01	9.2	PC	4
774.5949 ± 0.01	9.3	PC	19
710.5876 ± 0.01	6.4	PG	37
676.4886 ± 0.01	9.9	PE	10
693.6075 ± 0.01	10	PG	65
774.7271 ± 0.3205	9.3	PI	2
758.5644 ± 0.01	9.3	PC	29
662.5031 ± 0.01	10	DGTS	13
746.5717 ± 0.01	3.4	MG-DAG	5
690.5066 ± 0.01	9.9	PE	4
744.5489 ± 0.01	9.7	PE	67
782.5619 ± 0.01	9.2	PC	81
698.6242 ± 0.01	3.5	MG-DAG	0
718.5266 ±0.01	9.7	PE	418
748.6163 ± 0.01	9.2	PC	30
724.6014 ± 0.01	6.4	PG	12
764.6311 ± 0.01	6.3	PG	1

Table 5. List of the EIC Trace detected in the injected sample of the soil TLE sample with the identification of the type of lipid headgroup and their recovery percentage in the collected fractions from the CCC.

3.3.3 Recovery of the Fungal TLE sample

The cordyceps fungal TLE which was used to inject onto the CCC (13.66% each replicate, and 1% onto the LC-MS analysis) showed a combined average recovery of (79/1300) ~6% for the three phospholipids (PC, PE, PI), which comprised~ 95% of the relative abundance of the lipids. The individual recoveries of the phospholipids: PE, PC and PI were 31%, 46% and 2% respectively. The grouping of the phospholipid headgroups into PC, PE and PI was done based on the understanding of the lipid pattern in the heat density map (Figure 12.), retention time and the fractionating pattern of the lipids and the individual recoveries of each lipid (EIC trace) detected in the injected sample is listed in the (Table 6). The chromatogram in the (Figure 11.) shows the elution pattern of the different lipids with their recovery%.

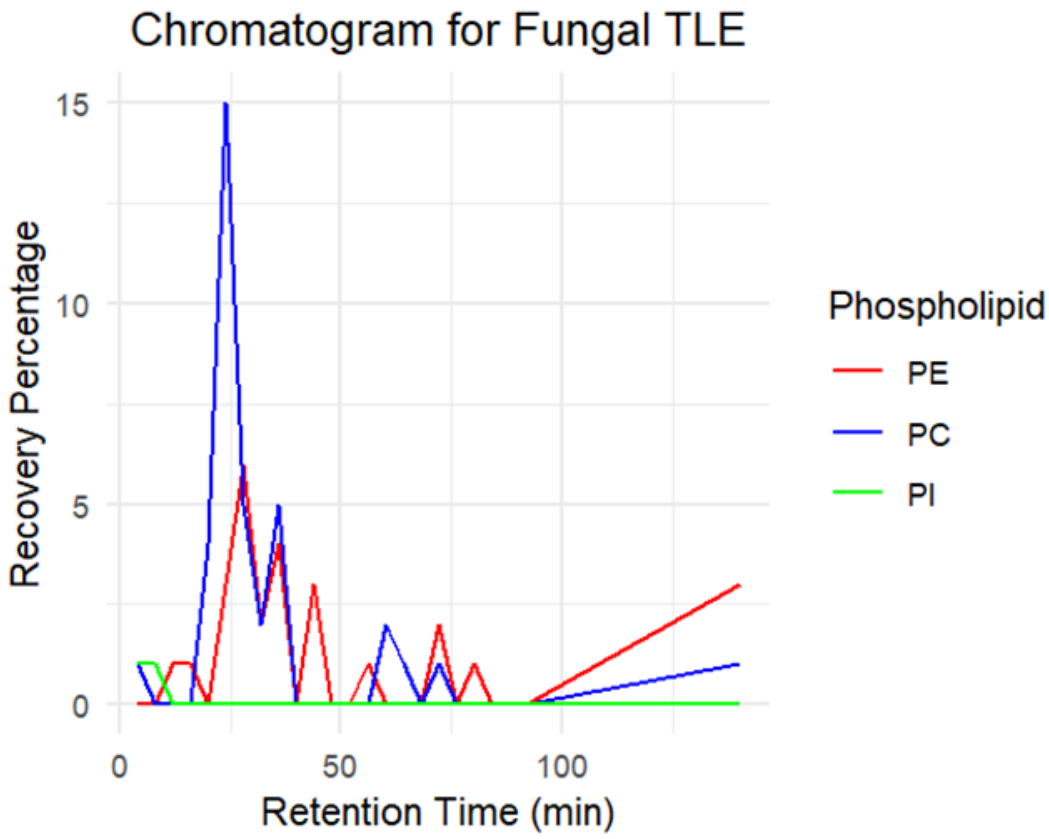

Figure 11. LC-MS data chromatogram of the phospholipids: PC n=7, PE n=5, and PI n=1 (Grouping of the EIC into the categories as listed in the Table 6.) recovery from the fractions collected from the CCC both mobile and stationary phase obtained from the injected soil TLE sample.

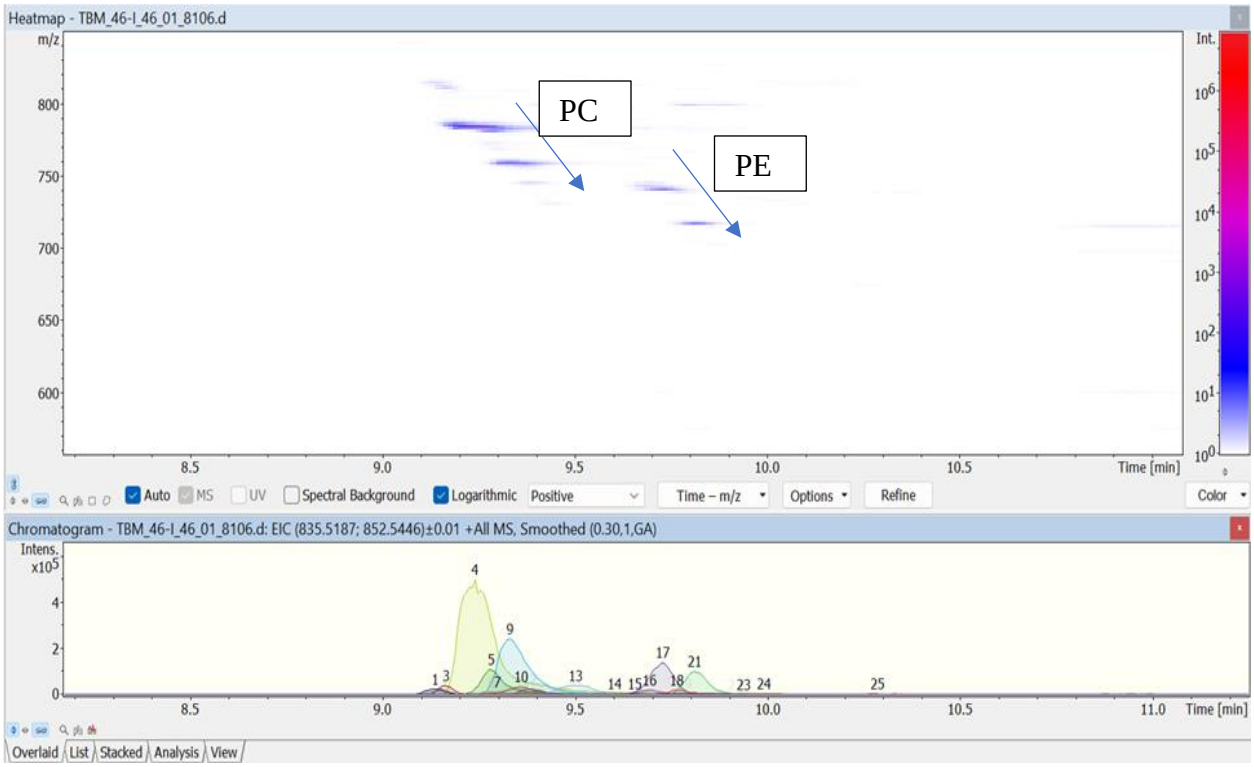

Figure 12. Image of the heatmap (density map) and compound chromatogram from the Data analysis software used for the LC-MS data analysis. The pattern helping us to understand the elution pattern of the lipids at different retention times.

EIC Trace	Retention time min	Type of lipid	Recovery %
814.6198 ± 0.05	9.1	PC	10
810.5903 ± 0.05	9.2	PC	11
782.5573 ± 0.05	9.3	PC	9
780.5420 ± 0.05	9.3	PC	6
758.5574 ± 0.05	9.4	PC	4
756.5411 ± 0.05	9.4	PC	4
744.5428 ± 0.05	9.5	PE	5
796.5377 ± 0.05	9.7	PC	2

744.5428 ± 0.05	9.7	PE	6
740.5125 ± 0.05	9.7	PE	6
738.4969 ± 0.05	9.8	PE	6
716.5121 ± 0.05	9.8	PE	7
760.5023 ± 0.05	15.3	PI	2

Table 6. List of the EIC trace detected in the injected sample of the soil TLE sample with the identification of the type of lipid headgroup and their recovery percentage in the collected fractions from the CCC.

4. Discussion

The separation and purification of natural/biomolecules and synthetic compounds from complex mixtures can easily be performed using counter-current chromatography (CCC). Counter current chromatography is a unique type of liquid-liquid partition chromatography with two-phases - mobile and stationary solvent phase involving a pair of mutually immiscible solvents (Berthod et al., 2009). On the contrary, solid phase extraction (SPE) has a solid support, an adsorbent such as silica and silica gel, activated carbon or glass beads (Rao and Biju, 2005), through which the liquid sample is passed and the analytes retained by the sorbent material is then eluted using an appropriate solvent (Badawy et al., 2022). Unlike SPE, where there is possible complication and limitation of the solid-phase binding and release of analyte, CCC confers the advantage of selection of a solvent system from a broad range of polar and non-polar solvents (Ito, 2005). In addition, CCC technique enables larger loading capacity (~100 mg - ~10 mg) and phase reversibility (Vetter et al., 2017). Further, in the CCC technique, a single sample or multiple samples can be injected. The dual model allows enrichment of minor target components from a multicomponent complex mixture (Kostanyan et al., 2020).

In the present study, two techniques including SPE and CCC were used to extract phospholipids (PL) from phospholipid standards and environmental samples including soil total lipid extract (TLE) and fungi TLE. The highlight of the study is (i) the use of CCC method for purification of PL from soil and fungi TLE; (ii) low combined PL recovery percentage of 45% from the soil TLE and 6% PL recovery from the fungi TLE by CCC method but higher individual lipid recovery indicating the pure enhanced recovery of individual lipids from the sample as mentioned in the results section 3.3.2 and 3.3.3.

Studies report that extraction parameters such as the choice of organic solvent for the extraction of phospholipid fatty acid (PLFA) from soil samples significantly influences fatty acid profiles of PLFA (Papadopoulou et al., 2011). In connection with this, for the application of CCC technique in PL extraction and purification from environmental samples, the solvent system hexane: ethyl acetate: methanol: water (HEMW - 3:1:3:1) was selected based on the distribution constant/coefficient (K) value of 0.96 (Table 2.). Further, the compound K values ranging between 0.4 and 2.5 are the optimal CCC performance indicators. As shown in the LC-MS data, the average compound K value for three categories of PL, namely, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) was 0.83, 1.42 and 0.49, respectively within $0.4 < K < 2.5$ suggestive of optimum recovery (Friesen and Pauli, 2015).

In the present study, the recovery percentage of phospholipids by SPE was ~10% for soil TLE and by CCC was 45% and 50% estimated by Mo-Blue assay and LC-MS, respectively. The data clearly indicates loss of analytes during the SPE process, contrary to the CCC method where recovery rate was higher. In support of this, studies indicate that CCC technique has increased recovery rate (Berthod et al., 2009). On the contrary, in SPE, the possibility of extraction is limited to the available solid phase (Berthod et al., 2009). In addition, the use of solid-supporting materials/matrix has certain complications such as deactivation, denaturation, loss of analysts, contamination from solid matrix and irreversible absorption and this could explain the low recovery rate of soil samples from SPE (Sethi et al., 2009). The higher yet purified PL recovery using CCC suggests that this method may provide an analytical advantage to improve accuracy in the quantification of (all) PL in soils and thereby advance the exploration of lipid diversity and microbial ecology.

In the present study, the observed low recovery percentage of phospholipids by SPE could be due to repeated elution of the cartridge with methanol, which can lead to washing away or loss of some of the important phospholipids. There is also a possibility of interference of organic biomass with the PL molecules in the cartridge of SPE thereby leading to low recovery of PL. The contamination from solid phases of the cartridge is higher than compared to the solvents in CCC. Additionally compared to the solvents, the sorbents in SPE have limited sorption capability which can affect the recovery and purity of elutes (Poole and Poole, 2012). In support of this, eluted PL from soil extract using SPE with methanol was shown to be contaminated with 3-48% betaine lipid indicating that the so-called PL fraction from SPE has low purity due to contamination of PL with other polar lipids (Warren, 2019). Additionally, in a previous study reduced PL recovery of phosphoethanolamine (PE) and phosphocholine (PC) from different commercial silica columns has been attributed to lack of preconditioning of silica from methanol (Mills and Goldhaber, 2010). Further, there are certain limitations of the SPE method. For instance, for samples from SPE, the retention time of 9-10 min for phospholipids is very low compared to retention time of 15- 40 min for PL in CCC.

The methodological limitations of SPE can be overcome by using CCC to purify PL which confers multiple advantages over solid-support matrix or SPE method. A higher retention of a stationary phase, broad range of selection of solvent system, dissolution of sample in stationary phase volume, change of phase role during the run and more solvent saving, yet choice of composition of solvent system for mobile and stationary phase should be made with caution (Sethi et al., 2009). In the case of soil or fungi TLE sample

purification via CCC, PL recovery percentage was high. In the CCC method, the injected samples are collected as different fractions in both mobile (methanol: water) and stationary phase (hexane: ethyl acetate), thus, the sample gets purified with the fractionation technique of the CCC leading to higher and reliable recovery. A larger volume of samples is retained in the liquid stationary phase providing higher resolution power to separate the constituents from a complex mixture (Berthod and Faure, 2015). Thus, using CCC techniques, the soil and fungi TLE samples are fractionated into pure phospholipids and glycolipids fractions with different retention times.

In CCC technique, the injected sample is recovered in fraction and in purified form. Unlike SPE, where once the sample is loaded, there is a risk that it may not be retained or recovered from the cartridge, in the CCC technique there is no loss of injected sample or solvent eluents. Phospholipids (PL) are also referred to as microbial life markers due to their larger presence in the structural components such as the cell membranes of the microbes and their involvement in different biological processes including storage, signal transduction and adaptation to the changing environment (Mangelsdorf et al., 2020; Wang et al., 2019). Based on the head groups, phospholipids are classified into four types, namely, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Nagle and Tristram-Nagle, 2000). In the present study, the recovery of PL including PC, PE and PI in the fungi TLE was higher than in the soil TLE extracted by SPE method. Although the average recovery of the lipid from the CCC is low but its higher in terms of individual lipid recovery in the sample which indicates a possibility of handling issues due to multiple transfers of the sample or the collected fraction into different vials for measurements which led to an overall loss of the lipids due to quick evaporation of the solvents. In the experimental set up, the PL are assessed in various matrices to quantify the life, to identify the spatial distribution and taxonomic information on the microorganisms. For instance, the presence of PE indicates the presence of living microorganisms in the matrices such as soil (Mangelsdorf et al., 2020). Further, PE and PC are involved in vegetative growth, mycotoxin production and virulence in pathogenic fungi such as Fusarium (Wang et al., 2019). Studies suggest that the synthesis of PL in fungi happens in multiple ways. For instance, zonal differences in gene expression and composition of PL, especially PE and PC, in mycelial fungi have been reported (Senik et al., 2023). In other microbes such as bacteria, PS and PE act as a modulator of virulence (Cassilly and Reynolds, 2018). Hence, the lipid headgroup analysis indicates that based on lipid profiles, the composition of the microbial community in any sample including soil

can be achieved (Ding et al., 2020). In addition, the turnover of PL has the potential to define the growth rates of the bacterial community (Schmidt et al., 2011).

The present study did not experiment on the identification of the type of bacteria in the soil TLE extract. However, the PL can indicate bacterial diversity and based on the presence of PL the bacterial community can be identified (Sohlenkamp and Geiger, 2015). For instance, in marine heterotrophic bacteria, phospholipids PG and PE are predominant, however, bacteria such as cyanobacteria thriving in low-phosphate conditions will have lower levels of PG (Popendorf et al., 2020). To date there is little if any information on the distribution of phosphatidyl headgroups, or their relationship to microbial diversity in soils. Higher soil biodiversity can lead to significant organic matter decomposition and higher nutrient availability (Maron et al., 2018). The obtained data has implications. The PL headgroup separation by the CCC technique can help with the diversity indices of the different bacterial and fungi groups in the sample which can be beneficial to identify the potential drug targets and the data can also be used to understand the nutrient turnover activities.

5. Conclusion

- Purification of PL from soil organic matter or other matrices using SPE may disregard a major portion of PL that are poorly recovered by this method.

- Traditional SPE methods used to purify PL may disproportionately alter the PL composition of the sample.

- Although the CCC method is time consuming, but its promising for advancing studies of microbial lipids extracted from soil and other matrices.

- CCC complimented with LC-MS is a useful and quantitative technique to explore lipid diversity, including headgroup and fatty acid distribution, which should be applied more extensively in the study of soil microbial biomass and diversity.

- The developed CCC method is reliable and with little optimization can give higher recoveries of the phospholipids from the samples due to its highly efficient fractionating technique.

6. Future directions

The results for the recovery of the intact polar lipids from the environmental samples, suggest that the developed method needs to be further optimized, in terms of improving the recovery %. The Counter Current Chromatography provides more reliable results in terms of the lipid recovery unlike the other traditional methods.

With further advancements in the optimization of the CCC method for the recovery of polar lipids from the environmental samples, the obtained results can be used to calculate the microbial diversity in the soil, understand the soil microbial community which is now studied by the classic DNA and PLFA methods.

In this study, while developing the method for the CCC, only a single coil was used for the separation of the polar lipids in the collected fractions, but for future if one wishes to optimize the method, they should try using both the coils for enhanced recovery of the phospholipids.

In future, the CCC method could help in separating a lot of complex organic compounds such as ketones and other complex biomolecules from the various matrices.

7. References

Abhilash, P.C., Dubey, R.K., Tripathi, V., Srivastava, P., Verma, J.P., Singh, H.B., 2013. Remediation and management of POPs-contaminated soils in a warming climate: Challenges and perspectives. Environ. Sci. Pollut. Res. 20, 5879–5885. https://doi.org/10.1007/s11356-013-1808-5

Adu, J.K., Oades, J.M., 1978. Utilization of organic materials in soil aggregates by bacteria and fungi. Soil Biol. Biochem. 10, 117–122.

Al-kaisi, M.M., Lal, R., Olson, K.R., Lowery, B., 2017. Fundamentals and Functions of Soil Environment 1–23. https://doi.org/10.1016/B978-0-12-805317-1.00001-4

Ali, O., Szabó, A., 2023. Review of Eukaryote Cellular Membrane Lipid Composition, with Special Attention to the Fatty Acids. Int. J. Mol. Sci. 24, 15693.

Badawy, M.E.I., El-Nouby, M.A.M., Kimani, P.K., Lim, L.W., Rabea, E.I., 2022. A review of the modern principles and applications of solid-phase extraction techniques in chromatographic analysis, Analytical Sciences. Springer Nature Singapore. https://doi.org/10.1007/s44211-022-00190-8

Banwart, S.A., Nikolaidis, N.P., Zhu, Y., Peacock, C.L., Sparks, D.L., 2019. Soil Functions : Connecting Earth ' s Critical Zone.

Bardgett, R.D., Freeman, C., Ostle, N.J., 2008. Microbial contributions to climate change through carbon cycle feedbacks. ISME J. 2, 805–814.

Barnes, M.A., Turner, C.R., 2016. The ecology of environmental DNA and implications for conservation genetics. Conserv. Genet. 17, 1–17. https://doi.org/10.1007/s10592-015-0775-4

Berthod, A., Faure, K., 2015. Separations with a liquid stationary phase: countercurrent chromatography or centrifugal partition chromatography. Anal. Sep. Sci. 1177–1206. https://doi.org/10.1002/chin.199126329

Berthod, A., Maryutina, T., Spivakov, B., Shpigun, O., Sutherland, I.A., 2009. Countercurrent chromatography in analytical chemistry (IUPAC technical report). Pure Appl. Chem. 81, 355–387. https://doi.org/10.1351/PAC-REP-08-06-05

Bhat, A.K., 2013. Review Article Preserving Microbial diversity of Soil ecosystem : A key to sustainable productivity 2, 85–101.

Bhattacharyya, S.S., Ros, G.H., Furtak, K., Iqbal, H.M.N., Parra-Saldívar, R., 2022. Soil carbon sequestration – An interplay between soil microbial community and soil

organic matter dynamics. Sci. Total Environ. 815. https://doi.org/10.1016/j.scitotenv.2022.152928

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bojko, O., Kabala, C., 2017. Organic carbon pools in mountain soils — Sources of variability and predicted changes in relation to climate and land use changes. Catena 149, 209–220. https://doi.org/10.1016/j.catena.2016.09.022

Brady, N.C., Weil, R.R., 2002. Organisms and Ecology of the Soil., in: In Nature and Properties of Soils. Prentice Hall: New Jersey, 2002, pp. 449–497.

Brant, J.B., Myrold, D.D., Sulzman, E.W., 2006. Root controls on soil microbial community structure in forest soils. Oecologia 148, 650–659. https://doi.org/10.1007/s00442-006-0402-7

Brent Friesen, J., Pauli, G.F., 2005. GUESS—a generally useful estimate of solvent systems for CCC. J. Liq. Chromatogr. Relat. Technol. 28, 2777–2806.

Brewer, T.E., Aronson, E.L., Arogyaswamy, K., Billings, S.A., Botthoff, J.K., Campbell, A.N., Dove, N.C., Fairbanks, D., Gallery, R.E., Hart, S.C., Kaye, J., King, G., Logan, G., Lohse, K.A., Maltz, M.R., Mayorga, E., O'Neill, C., Owens, S.M., Packman, A., Pett-Ridge, J., Plante, A.F., Richter, D.D., Silver, W.L., Yang, W.H., Fierer, N., 2019. Ecological and Genomic Attributes of Novel Bacterial Taxa That Thrive in Subsurface Soil Horizons. MBio 10. https://doi.org/10.1128/mBio.01318-19

Bünemann, E.K., 2015. Assessment of gross and net mineralization rates of soil organic phosphorus - A review. Soil Biol. Biochem. 89, 82–98. https://doi.org/10.1016/j.soilbio.2015.06.026

Bünemann, E.K., Oberson, A., Liebisch, F., Keller, F., Annaheim, K.E., Huguenin-Elie, O., Frossard, E., 2012. Rapid microbial phosphorus immobilization dominates gross phosphorus fluxes in a grassland soil with low inorganic phosphorus availability. Soil Biol. Biochem. 51, 84–95. https://doi.org/10.1016/j.soilbio.2012.04.012

Cassilly, C.D., Reynolds, T.B., 2018. PS, it's complicated: The roles of phosphatidylserine and phosphatidylethanolamine in the pathogenesis of Candida albicans and other microbial pathogens. J. Fungi 4. https://doi.org/10.3390/jof4010028

Chen, J., Seven, J., Zilla, T., Dippold, M.A., Blagodatskaya, E., Kuzyakov, Y., 2019. Microbial C:N:P stoichiometry and turnover depend on nutrients availability in soil: A 14 C, 15 N and 33 P triple labelling study. Soil Biol. Biochem. 131, 206–216.

https://doi.org/10.1016/j.soilbio.2019.01.017

Compant, S., Van Der Heijden, M.G.A., Sessitsch, A., 2010. Climate change effects on beneficial plant-microorganism interactions. FEMS Microbiol. Ecol. 73, 197–214. https://doi.org/10.1111/j.1574-6941.2010.00900.x

Condron, L., Stark, C., O'Callaghan, M., Clinton, P., Huang, Z., 2010. The role of microbial communities in the formation and decomposition of soil organic matter. Soil Microbiol. Sustain. Crop Prod. 81–118.

Ding, S., Lange, M., Lipp, J., Schwab, V.F., Chowdhury, S., Pollierer, M.M., Krause, K., Li, D., Kothe, E., Scheu, S., Welti, R., Hinrichs, K.U., Gleixner, G., 2020. Characteristics and origin of intact polar lipids in soil organic matter. Soil Biol. Biochem. 151, 108045. https://doi.org/10.1016/j.soilbio.2020.108045

Doran, J.W., Werner, M.R., 1990. Management and soil biology, in: Francis C A, Flora C B, and K.L.D. (Ed.), Sustainable Agriculture in Temperate Zones. pp. 205–230.

Dove, N.C., Arogyaswamy, K., Billings, S.A., Botthoff, J.K., Carey, C.J., Cisco, C., Deforest, J.L., Fairbanks, D., Fierer, N., Gallery, R.E., Kaye, J.P., Lohse, K.A., Maltz, M.R., Mayorga, E., Pett-Ridge, J., Yang, W.H., Hart, S.C., Aronson, E.L., 2020. Continental-scale patterns of extracellular enzyme activity in the subsoil: An overlooked reservoir of microbial activity. Environ. Res. Lett. 15. https://doi.org/10.1088/1748-9326/abb0b3

Dove, N.C., Barnes, M.E., Moreland, K., Graham, R.C., Berhe, A.A., Hart, S.C., 2021. Depth dependence of climatic controls on soil microbial community activity and composition. ISME Commun. 1. https://doi.org/10.1038/s43705-021-00081-5

Drenovsky, R.E., Vo, D., Graham, K.J., Scow, K.M., 2004. Soil water content and organic carbon availability are major determinants of soil microbial community composition. Microb. Ecol. 48, 424–430. https://doi.org/10.1007/s00248-003-1063-2

Dubey, A., Ahmad, M., Farhat, M., Kanika, K., 2019a. Soil microbiome : a key player for conservation of soil health under changing climate. Biodivers. Conserv. 28, 2405–2429. https://doi.org/10.1007/s10531-019-01760-5

Dubey, A., Malla, M.A., Khan, F., Chowdhary, K., Yadav, S., Kumar, A., Sharma, S., Khare, P.K., Khan, M.L., 2019b. Soil microbiome: a key player for conservation of soil health under changing climate. Biodivers. Conserv. 28, 2405–2429. https://doi.org/10.1007/s10531-019-01760-5

Fierer, N., Schimel, J.P., Holden, P.A., 2003. Variations in microbial community

composition through two soil depth profiles. Soil Biol. Biochem. 35, 167–176. https://doi.org/10.1016/S0038-0717(02)00251-1

Friedlingstein, P., Betts, R., Bopp, L., Bloh, W. Von, Brovkin, V., Doney, S., Eby, M., Fung, I., Govindasamy, B., John, J., Jones, C., Joos, F., Kato, T., Kawamiya, M., Knorr, W., Lindsay, K., Matthews, H.D., Raddatz, T., Rayner, P., Reick, C., Roeckner, E., Schnitzler, K.G., Schnurr, R., Strassmann, K., Thompson, S., J Weaver, A., Yoshikawa, C., Zeng, N., 2006. Climate –carbon cycle feedback analysis, results from the C4MIP model intercomparison. J. Clim. 19, 3337–3353.

Friesen, B., Pauli, G., 2015. Solvent Selection in Countercurrent Chromatography (CCC). Countercurrent.Org 1–11.

Frostegård, Å., Tunlid, A., Bååth, E., 2011. Use and misuse of PLFA measurements in soils. Soil Biol. Biochem. 43, 1621–1625. https://doi.org/10.1016/j.soilbio.2010.11.021

Gayan, A., Borah, P., Nath, D., Kataki, R., 2023. Soil microbial diversity, soil health and agricultural sustainability., in: Sustainable Agriculture and the Environment. Academic Press, pp. 107–126.

Griffiths, B.S., Hallett, P.D., Kuan, H.L., Gregory, A.S., Watts, C.W., Whitmore, A.P., 2008. Functional resilience of soil microbial communities depends on both soil structure and microbial community composition. Biol. Fertil. Soils 44, 745–754. https://doi.org/10.1007/s00374-007-0257-z

Han, T., Cao, X., Pei, H., Fan, C., 2023. Overview of Solvent System Selection Strategies for Countercurrent Chromatography. Sep. Purif. Rev. 52, 305–325.

Hubert, J., Plé, K., Hamzaoui, M., Nuissier, G., Hadef, I., Reynaud, R., Guilleret, A., Renault, J.H., 2012. New perspectives for microbial glycolipid fractionation and purification processes. Comptes Rendus Chim. 15, 18–28. https://doi.org/10.1016/j.crci.2011.11.002

Islam, R.K., Wright, S.R., 2003. “Soil microbial communities,” in: Encyclopedia of Soil. Marcel Dekker, Inc., New York.

Ito, Y., 2005. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. J. Chromatogr. A 1065, 145–168. https://doi.org/10.1016/j.chroma.2004.12.044

Ito, Y., Bowman, R.L., 1970. Countercurrent chromatography: liquid–liquid partitionchromatography without solid support. Science (80-.). 167, 281–283.

Jansson, J.K., 2019. Soil microbiomes and climate change. Nat. Rev. Microbiol. 18, 35–46. https://doi.org/10.1038/s41579-019-0265-7

Jensen, L.S., Magid, J., 2009. Nutrient Turnover In Soil After The Addition of Organic Matter. Appl. Plant Nutr. 1–33.

Joergensen, R.G., 2022. Phospholipid fatty acids in soil—drawbacks and future prospects. Biol. Fertil. Soils 58, 1–6. https://doi.org/10.1007/s00374-021-01613-w

Johnston, A.S.A., Sibly, R.M., 2018. The influence of soil communities on the temperature sensitivity of soil respiration. Nat. Ecol. Evol. 2, 1597–1602. https://doi.org/10.1038/s41559-018-0648-6

Katan, M., Cockcroft, S., 2020. Phosphatidylinositol (4, 5) bisphosphate: diverse functions at the plasma membrane. Essays Biochem. 64, 513–531.

Kostanyan, A.A., Voshkin, A.A., Belova, V. V., 2020. Analytical, Preparative, and Industrial-Scale Separation of Substances by Methods of Countercurrent Liquid-Liquid Chromatography. Molecules 25, 26–44. https://doi.org/10.3390/MOLECULES25246020

Leive, L., Cullinane, L.M., Ito, Y., Bramblett, G.T., 1984. Countercurrent chromatographic separation of bacteria with known differences in surface lipopolysaccharide. J. Liq. Chromatogr. 7, 403–418. https://doi.org/10.1080/01483918408073975

Liu, Y., Friesen, J.B., McAlpine, J.B., Lankin, D.C., Chen, S.N., Pauli, G.F., 2018. Natural Deep Eutectic Solvents: Properties, Applications, and Perspectives. J. Nat. Prod. 81, 679–690. https://doi.org/10.1021/acs.jnatprod.7b00945

Lladó, S., López-Mondéjar, R., Baldrian, P., 2017. Forest soil bacteria: diversity, involvement in ecosystem processes, and response to global change. Microbiol. Mol. Biol. Rev. 81, 10–1128.

Lloyd, J., Taylor, J.A., 1994. On the Temperature Dependence of Soil Respiration. Funct. Ecol. 8, 315–323.

Mackey, K.R.M., Paytan, A., 2009. Phosphorus Cycle. Encycl. Microbiol. Third Ed. 322–334. https://doi.org/10.1016/B978-012373944-5.00056-0

Madsen, E.L., 2011. Microorganisms and their roles in fundamental biogeochemical cycles. Curr. Opin. Biotechnol. 22, 456–464. https://doi.org/10.1016/j.copbio.2011.01.008

Mangelsdorf, K., Karger, C., Zink, K.G., 2020. Phospholipids as life markers in geological

habitats., in: Handbook of Hydrocarbon and Lipid Microbiology. Springer, Cham., pp. 445–473.

Maron, P.A., Sarr, A., Kaisermann, A., Lévêque, J., Mathieu, O., Guigue, J., Ranjard, L., 2018. High microbial diversity promotes soil ecosystem functioning. Appl. Environ. Microbiol. 84, 1–13.

Meador, T.B., Goldenstein, N.I., Gogou, A., Herut, B., Psarra, S., Tsagaraki, T.M., Hinrichs, K.-U., 2017. Planktonic lipidome responses to aeolian dust input in low-biomass oligotrophic marine mesocosms. Front. Mar. Sci. 4, 113.

Mekala, S., Polepongu, S., 2019. Plant biotic interactions: State of the art, Plant Biotic Interactions: State of the Art. https://doi.org/10.1007/978-3-030-26657-8

Merchant, S.S., Helmann, J.D., 2012. Elemental Economy. Microbial Strategies for Optimizing Growth in the Face of Nutrient Limitation, 1st ed, Advances in Microbial Physiology. Elsevier Ltd. https://doi.org/10.1016/B978-0-12-398264-3.00002-4

Mills, C.T., Goldhaber, M.B., 2010. On silica-based solid phase extraction techniques for isolating microbial membrane phospholipids: Ensuring quantitative recovery of phosphatidylcholine-derived fatty acids. Soil Biol. Biochem. 42, 1179–1182.

Morita, S., Ikeda, Y., 2022. Regulation of membrane phospholipid biosynthesis in mammalian cells. Biochem. Pharmacol. 206, 115296.

Müller, A.K., Westergaard, K., Christensen, S., Sørensen, S.J., 2002. The diversity and function of soil microbial communities exposed to different disturbances. Microb. Ecol. 44, 49–58. https://doi.org/10.1007/s00248-001-0042-8

Nagle, J.F., Tristram-Nagle, S., 2000. Structure of lipid bilayers. Biochim. Biophys. Acta (BBA)-Reviews Biomembr. 1469, 159–195. https://doi.org/10.1016/j.surfcoat.2014.12.042

Nannipieri, P., Ascher, J., Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions. Eur. J. Soil Sci. 54, 655–670. https://doi.org/10.1046/j.1351-0754.2003.0556.x

Naylor, D., McClure, R., Jansson, J., 2022. Trends in Microbial Community Composition and Function by Soil Depth. Microorganisms 10. https://doi.org/10.3390/microorganisms10030540

Osburn, E.D., Badgley, B.D., Strahm, B.D., Aylward, F.O., Barrett, J.E., 2021. Emergent properties of microbial communities drive accelerated biogeochemical cycling in

disturbed temperate forests. Ecology 102, 0–2. https://doi.org/10.1002/ecy.3553

Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J.R., Tsai, C.S., Park, W., Jeon, C., Madsen, E.L., 2003. Respiration of 13C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of 13C-labeled soil DNA. Appl. Environ. Microbiol. 69, 1614–1622. https://doi.org/10.1128/AEM.69.3.1614-1622.2003

Pajares, S., Bohannan, B.J.M., 2016. Ecology of nitrogen fixing, nitrifying, and denitrifying microorganisms in tropical forest soils. Front. Microbiol. 7, 1–20. https://doi.org/10.3389/fmicb.2016.01045

Papadopoulou, E.S., Karpouzas, D.G., Menkissoglu-Spiroudi, U., 2011. Extraction Parameters Significantly Influence the Quantity and the Profile of PLFAs Extracted from Soils. Microb. Ecol. 62, 704–714. https://doi.org/10.1007/s00248-011-9863-2

Perez-Quezada, J.F., Pérez, C.A., Brito, C.E., Fuentes, J.P., Gaxiola, A., Aguilera-Riquelme, D., Lopatin, J., 2021. Biotic and abiotic drivers of carbon, nitrogen and phosphorus stocks in a temperate rainforest. For. Ecol. Manage. 494. https://doi.org/10.1016/j.foreco.2021.119341

Picariello, E., Baldantoni, D., De Nicola, F., 2023. Annual dynamics of indigenous microbial communities of forest soils after severe PAH contamination. Appl. Soil Ecol. 186, 104812.

Piotrowska-Seget, Z., Mrozik, A., 2003. Signature Lipid Biomarker (SLB) analysis in determining changes in community structure of soil microorganisms. Polish J. Environ. Stud. 12, 669–675.

Poole, C.F., Poole, S.K., 2012. Principles and Practice of Solid-Phase Extraction, in: Principles and Practice of Solid-Phase Extraction. Academic Press, Inc., pp. 273–297.

Popendorf, K.J., Koblížek, M., Van Mooy, B.A.S., 2020. Phospholipid turnover rates suggest that bacterial community growth rates in the open ocean are systematically underestimated, Limnology and Oceanography. https://doi.org/10.1002/lno.11424

Quideau, S.A., McIntosh, A.C.S., Norris, C.E., Lloret, E., Swallow, M.J.B., Hannam, K., 2016. Extraction and analysis of microbial phospholipid fatty acids in soils. JoVE (Journal Vis. Exp. e54360.

Raj, A., Jhariya, M.K., Yadav, D.K., Banerjee, A., Meena, R.S., 2019. Soil for Sustainable Environment and Ecosystems Management.

Rao, T.P., Biju, V.M., 2005. SPECTROPHOTOMETRY | Organic Compounds, in: Encyclopedia of Analytical Science. Elsevier, pp. 358–366.

Russell, E.W., 1973. Soil Conditions and Plant Growth. Longman Group Limited, New York.

Schimel, D.S., 1995. Terrestrial Ecosystems & the Carbon Cycle. Glob. Chang. Biol. 1, 77–91.

Schimel, J.P., Schaeffer, S.M., 2012. Microbial control over carbon cycling in soil. Front. Microbiol. 3, 1–11. https://doi.org/10.3389/fmicb.2012.00348

Schlesinger, W.H., Jeffrey, A.A., 2000. Soil respiration and the global carbon cycle. Biogeochemistry 48, 7–20. https://doi.org/10.2307/40019248

Schmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I.A., Kleber, M., Kögel-Knabner, I., Lehmann, J., Manning, D.A.C., Nannipieri, P., Rasse, D.P., Weiner, S., Trumbore, S.E., 2011. Persistence of soil organic matter as an ecosystem property. Nature 478, 49–56. https://doi.org/10.1038/nature10386

Senik, S. V., Manzhieva, B.S., Maloshenok, L.G., Serebryakov, E.B., Bruskin, S.A., Kotlova, E.R., 2023. Heterogeneous Distribution of Phospholipid Molecular Species in the Surface Culture of Flammulina velutipes: New Facts about Lipids Containing α-Linolenic Fatty Acid. J. Fungi 9. https://doi.org/10.3390/jof9010102

Sergaki, C., Lagunas, B., Lidbury, I., Gifford, M.L., Schäfer, P., 2018. Challenges and approaches in microbiome research: from fundamental to applied. Front. Plant Sci. 9, 1–12. https://doi.org/10.3389/fpls.2018.01205

Sethi, N., Anand, A., Sharma, A., Chandrul, K., Jain, G., Srinivasa, K., 2009. High speed counter current chromatography: A support-free LC technique. J. Pharm. Bioallied Sci. 1, 8. https://doi.org/10.4103/0975-7406.62680

Shulga, Y. V, Topham, M.K., Epand, R.M., 2011. Regulation and functions of diacylglycerol kinases. Chem. Rev. 111, 6186–6208.

Skalicka-Woźniak, K., Garrard, I., 2014. Counter-current chromatography for the separation of terpenoids: A comprehensive review with respect to the solvent systems employed. Phytochem. Rev. 13, 547–572. https://doi.org/10.1007/s11101-014-9348-2

Sohlenkamp, C., Geiger, O., 2015. Bacterial membrane lipids: Diversity in structures and pathways. FEMS Microbiol. Rev. 40, 133–159.

https://doi.org/10.1093/femsre/fuv008

Stein, L.Y., Klotz, M.G., 2016. The nitrogen cycle. Curr. Biol. 26, R94–R98. https://doi.org/10.1016/j.cub.2015.12.021

Sturt, H.F., Summons, R.E., Smith, K., Elvert, M., Hinrichs, K., 2004. Intact polar membrane lipids in prokaryotes and sediments deciphered by high-performance liquid chromatography/electrospray ionization multistage mass spectrometry—new biomarkers for biogeochemistry and microbial ecology. Rapid Commun. mass Spectrom. 18, 617–628.

Tate, R.L.I., 1995. Soil Microbiology. John Wiley & Sons, Inc., New York.

Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J., Locey, K.J., Prill, R.J., Tripathi, A., Gibbons, S.M., Ackermann, G., Navas-Molina, J.A., Janssen, S., Kopylova, E., Vázquez-Baeza, Y., González, A., Morton, J.T., Mirarab, S., Xu, Z.Z., Jiang, L., Haroon, M.F., Kanbar, J., Zhu, Q., Song, S.J., Kosciolek, T., Bokulich, N.A., Lefler, J., Brislawn, C.J., Humphrey, G., Owens, S.M., Hampton-Marcell, J., Berg-Lyons, D., McKenzie, V., Fierer, N., Fuhrman, J.A., Clauset, A., Stevens, R.L., Shade, A., Pollard, K.S., Goodwin, K.D., Jansson, J.K., Gilbert, J.A., Knight, R., Agosto Rivera, J.L., Al-Moosawi, L., Alverdy, J., Amato, K.R., Andras, J., Angenent, L.T., Antonopoulos, D.A., Apprill, A., Armitage, D., Ballantine, K., Bárta, J., Baum, J.K., Berry, A., Bhatnagar, A., Bhatnagar, M., Biddle, J.F., Bittner, L., Boldgiv, B., Bottos, E., Boyer, D.M., Braun, J., Brazelton, W., Brearley, F.Q., Campbell, A.H., Caporaso, J.G., Cardona, C., Carroll, J.L., Cary, S.C., Casper, B.B., Charles, T.C., Chu, H., Claar, D.C., Clark, R.G., Clayton, J.B., Clemente, J.C., Cochran, A., Coleman, M.L., Collins, G., Colwell, R.R., Contreras, M., Crary, B.B., Creer, S., Cristol, D.A., Crump, B.C., Cui, D., Daly, S.E., Davalos, L., Dawson, R.D., Defazio, J., Delsuc, F., Dionisi, H.M., Dominguez-Bello, M.G., Dowell, R., Dubinsky, E.A., Dunn, P.O., Ercolini, D., Espinoza, R.E., Ezenwa, V., Fenner, N., Findlay, H.S., Fleming, I.D., Fogliano, V., Forsman, A., Freeman, C., Friedman, E.S., Galindo, G., Garcia, L., Garcia-Amado, M.A., Garshelis, D., Gasser, R.B., Gerdts, G., Gibson, M.K., Gifford, I., Gill, R.T., Giray, T., Gittel, A., Golyshin, P., Gong, D., Grossart, H.P., Guyton, K., Haig, S.J., Hale, V., Hall, R.S., Hallam, S.J., Handley, K.M., Hasan, N.A., Haydon, S.R., Hickman, J.E., Hidalgo, G., Hofmockel, K.S., Hooker, J., Hulth, S., Hultman, J., Hyde, E., Ibáñez-Álamo, J.D., Jastrow, J.D., Jex, A.R., Johnson, L.S., Johnston, E.R., Joseph, S., Jurburg, S.D., Jurelevicius, D., Karlsson, A., Karlsson, R., Kauppinen, S., Kellogg, C.T.E., Kennedy, S.J., Kerkhof, L.J., King, G.M., Kling,

G.W., Koehler, A. V., Krezalek, M., Kueneman, J., Lamendella, R., Landon, E.M., Lanede Graaf, K., LaRoche, J., Larsen, P., Laverock, B., Lax, S., Lentino, M., Levin, I.I., Liancourt, P., Liang, W., Linz, A.M., Lipson, D.A., Liu, Y., Lladser, M.E., Lozada, M., Spirito, C.M., MacCormack, W.P., MacRae-Crerar, A., Magris, M., Martín-Platero, A.M., Martín-Vivaldi, M., Martínez, L.M., Martínez-Bueno, M., Marzinelli, E.M., Mason, O.U., Mayer, G.D., McDevitt-Irwin, J.M., McDonald, J.E., McGuire, K.L., McMahon, K.D., McMinds, R., Medina, M., Mendelson, J.R., Metcalf, J.L., Meyer, F., Michelangeli, F., Miller, K., Mills, D.A., Minich, J., Mocali, S., Moitinho-Silva, L., Moore, A., Morgan-Kiss, R.M., Munroe, P., Myrold, D., Neufeld, J.D., Ni, Y., Nicol, G.W., Nielsen, S., Nissimov, J.I., Niu, K., Nolan, M.J., Noyce, K., O'Brien, S.L., Okamoto, N., Orlando, L., Castellano, Y.O., Osuolale, O., Oswald, W., Parnell, J., Peralta-Sánchez, J.M., Petraitis, P., Pfister, C., Pilon-Smits, E., Piombino, P., Pointing, S.B., Pollock, F.J., Potter, C., Prithiviraj, B., Quince, C., Rani, A., Ranjan, R., Rao, S., Rees, A.P., Richardson, M., Riebesell, U., Robinson, C., Rockne, K.J., Rodriguezl, S.M., Rohwer, F., Roundstone, W., Safran, R.J., Sangwan, N., Sanz, V., Schrenk, M., Schrenzel, M.D., Scott, N.M., Seger, R.L., Seguinorlando, A., Seldin, L., Seyler, L.M., Shakhsheer, B., Sheets, G.M., Shen, C., Shi, Y., Shin, H., Shogan, B.D., Shutler, D., Siegel, J., Simmons, S., Sjöling, S., Smith, D.P., Soler, J.J., Sperling, M., Steinberg, P.D., Stephens, B., Stevens, M.A., Taghavi, S., Tai, V., Tait, K., Tan, C.L., Taş, N., Taylor, D.L., Thomas, T., Timling, I., Turner, B.L., Urich, T., Ursell, L.K., Van Der Lelie, D., Van Treuren, W., Van Zwieten, L., Vargas-Robles, D., Thurber, R.V., Vitaglione, P., Walker, D.A., Walters, W.A., Wang, S., Wang, T., Weaver, T., Webster, N.S., Wehrle, B., Weisenhorn, P., Weiss, S., Werner, J.J., West, K., Whitehead, A., Whitehead, S.R., Whittingham, L.A., Willerslev, E., Williams, A.E., Wood, S.A., Woodhams, D.C., Yang, Y., Zaneveld, J., Zarraonaindia, I., Zhang, Q., Zhao, H., 2017. A communal catalogue reveals Earth's multiscale microbial diversity. Nature 551, 457–463. https://doi.org/10.1038/nature24621

U.S. Global Change Research Program, 2018. Impacts, Risks, and Adaptation in the United Staes: Fourth National Climate Assessment, Volume II [WWW Document]. Fourth Natl. Clim. Assess.

USDA-NRCS, 2014. SOIL : An Essential Link in the Cycle of Life, Guide for educators.

Vance, J.E., 2015. Phospholipid synthesis and transport in mammalian cells. Traffic 16, 1–18.

Vetter, W., Hammann, S., Müller, M., Englert, M., Huang, Y., 2017. The use of countercurrent chromatography in the separation of nonpolar lipid compounds. J. Chromatogr. A 1501, 51–60. https://doi.org/10.1016/j.chroma.2017.04.035

Wagg, C., Hautier, Y., Pellkofer, S., Banerjee, S., Schmid, B., van der Heijden, M.G.A., 2021. Diversity and asynchrony in soil microbial communities stabilizes ecosystem functioning. Elife 10, 1–19. https://doi.org/10.7554/eLife.62813

Waldrop, M.P., Firestone, M.K., 2006. Response of microbial community composition and function to soil climate change. Microb. Ecol. 52, 716–724. https://doi.org/10.1007/s00248-006-9103-3

Wang, J., Wang, H., Zhang, C., Wu, T., Ma, Z., Chen, Y., 2019. Phospholipid homeostasis plays an important role in fungal development, fungicide resistance and virulence in Fusarium graminearum. Phytopathol. Res. 1, 1–12. https://doi.org/10.1186/s42483-019-0023-9

Wang, K., Peng, C., Zhu, Q., Zhou, X., Wang, M., Zhang, K., Wang, G., 2017. Modeling Global Soil Carbon and Soil Microbial Carbon by Integrating Microbial Processes into the Ecosystem Process Model TRIPLEX-GHG. J. Adv. Model. Earth Syst. 9, 2368–2384. https://doi.org/10.1002/2017MS000920

Warren, C.R., 2019. Does silica solid-phase extraction of soil lipids isolate a pure phospholipid fraction?. Soil Biol. Biochem. 128, 175–178.

Wieder, W.R., Bonan, G.B., Allison, S.D., 2013. Global soil carbon projections are improved by modelling microbial processes. Nat. Clim. Chang. 3, 909–912. https://doi.org/10.1038/nclimate1951

Wörmer, L., Lipp, J.S., Hinrichs, K.-U., 2017. Comprehensive analysis of microbial lipids in environmental samples through HPLC-MS protocols. Hydrocarb. lipid Microbiol. Protoc. Pet. Hydrocarb. lipid Anal. 289–317.

Wörmer, L., Lipp, J.S., Hinrichs, K.-U., 2015. Comprehensive Analysis of Microbial Lipids in Environmental Samples Through HPLC-MS Protocols 289–317. https://doi.org/10.1007/8623_2015_183

Wu, Y., Ding, N., Wang, G., Xu, J., Wu, J., Brookes, P.C., 2009. Effects of different soil weights, storage times and extraction methods on soil phospholipid fatty acid analyses. Geoderma 150, 171–178. https://doi.org/10.1016/j.geoderma.2009.02.003

Xue, P.P., Carrillo, Y., Pino, V., Minasny, B., McBratney, A.B., 2018. Soil Properties Drive Microbial Community Structure in a Large Scale Transect in South Eastern

Australia. Sci. Rep. 8, 1–11. https://doi.org/10.1038/s41598-018-30005-8

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterisation of microbial communities in soil: A review. Biol. Fertil. Soils 29, 111–129. https://doi.org/10.1007/s003740050533

Zelles, L., Bai, Q.Y., 1993. Fractionation of fatty acids derived from soil lipids by solid phase extraction and their quantitative analysis by GC-MS. Soil Biol. Biochem. 25, 495–507. https://doi.org/10.1016/0038-0717(93)90075-M