Standardisation and implementation of an optimised method for lipid profiling of microbial communities

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Commit everything you do to the Lord. Trust Him, and He will help you.

Psalm 37:5 – New Living Translation

Table of contents

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Preface

The research discussed in this thesis was conducted during the period of January 2013 to November 2015 in the Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa.

The research presented in this thesis represents original work undertaken by the author and has not been submitted for degree purposes to any other university. Where use was made of the work of other researchers, appropriate acknowledgements have been added in the text. The referencing style used in this thesis is in accordance with the requirements of the *Journal of Applied Microbiology*.

Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

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Summary

Although the importance of phospholipid fatty acid (PLFA) analysis to characterise microbial communities has been recognised over the years, the vast number of modifications to this traditional method as well as discrepancies regarding its data interpretation have given rise to scepticism about the accuracy of PLFA analysis in environmental studies. Therefore, comprehensive literature-based studies were necessary to investigate these uncertainties. Additionally, new and modernised high-throughput approaches, such as metabolomics, holds promise for investigating microbial communities and there was a need to evaluate such an approach against traditional methods for characterisation of fatty acid methyl esters (FAMEs). The literature reviews conducted for this study evaluated i) different methods and their modifications in terms of the main steps of PLFA analysis, namely extraction, fractionation, derivatisation and quantification; and ii) recent applications of PLFA analysis in environmental studies with specific reference to data interpretation. Both reviews made significant contributions to summarise and clarify a vast body of literature on the topics and were published in the *Journal of Applied Microbiology*. During the experimental work, targeted analysis of selected FAMEs to characterise both homogenised soil and pure bacterial cultures were evaluated by comparing three methods, namely fractionated PLFA, total lipid extract (TLE) and metabolomics analysis. As an additional outcome, untargeted analysis was also investigated. The results showed that the extraction method and different derivatisation techniques had an effect on FAME concentrations and on repeatability between sample replicates. Furthermore, extraction method had a greater influence when analysing pure culture samples, while derivatisation technique was more important when analysing soil samples. The most appropriate derivatisation technique for fractionated PLFA analysis proved to be mild alkaline methanolysis, while methanolysis with oximation and silylation provided the best results for TLE analysis. The metabolomics-based approach benefited from the inclusion of an oximation step for derivatisation of a complete metabolite profile. An assessment of the applicability of the different methods to distinguish between soil microbial communities exposed to various soil fumigant treatments in a greenhouse study, showed that even though TLE analysis gave higher FAME yields than PLFA analysis, its discrimination potential between treatments were much lower. Therefore, PLFA analysis was recommended for FAME characterisation in microbial communities. The untargeted metabolomics analysis has potential in differentiating between different treatments, despite representing the larger soil community and not microbial communities *per se*. Based on all the obtained results, a standard operating procedure for the targeted analysis of FAMEs and the untargeted analysis of all metabolites from soil is proposed.

Keywords: fatty acid methyl ester; metabolomics; microbial community; phospholipid fatty acid; pure culture; soil; total lipid extract

List of abbreviations

Measuring units

cm	centimetre
cmol kg^{-1}	centimole per kilogram
°C	degrees Celsius
E	East
eV	electron volt
g	gram(s)
g kg^{-1}	gram per kilogram
g L^{-1}	gram per litre
h	hour(s)
<	less than
L	litre
m/z	mass-to-charge ratio
MPa	megapascal
m	metre
µg	microgram
µg ml^{-1}	microgram per millilitre
µl	microlitre
µm	micrometre
µmol	micromole
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
mS cm^{-1}	millisiemens per centimetre
min	minute(s)
M	molar
mol L^{-1}	mole per litre
nm	nanometre
nmol	nanomole
nmol g^{-1}	nanomole per gram
%	percentage
pmol	picomole
pmol ml^{-1}	picomole per millilitre
rpm	revolutions per minute
S	South
v/v	volume to volume

General abbreviations

2-D	two-dimensional
3-D	three-dimensional
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ANOVA	analysis of variance
ASE	accelerated-solvent extraction
BF_3	boron trifluoride
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C	carbon
CEC	cation exchange capacity
$CHCl_3$	chloroform
CH_3NaO	sodium methoxide
$[CH_3]_2S$	dimethyl sulphide
CO_2	carbon dioxide
cy:pre	cyclopropyl fatty acids to their monounsaturated precursors
DNA	deoxyribonucleic acid
EC	electrical conductivity
F	fractionated
FAME	fatty acid methyl ester
F:B	fungal to bacterial
FID	flame ionisation detection
FMASE	focused microwave-assisted Soxhlet extraction
GC	gas chromatography
GC x GC	two-dimensional gas chromatography
GP:GN	Gram-positive to Gram-negative
GP:total	Gram-positive to total
H_2	hydrogen-gas
HCl	hydrochloric acid
HDPE	high density polyethylene
H_2O	water
HPLC	high-performance liquid chromatography
H_2SO_4	sulfuric acid
iPCA	interactive Principal Component Analysis
IS	internal standard
KCl	potassium chloride
K_2HPO_4	dipotassium hydrogen phosphate
KOH	potassium hydroxide
K_2SO_4	potassium sulphate
M	mild alkaline methanolysis
MB	mild alkaline methanolysis with silylation
MBsats	mid-chain branched saturated fatty acids
MeOH	methanol
MeOX	methoxyamination solution
MIDI	Microbial Identification System

MMB	mild alkaline methanolysis with oximation and silylation
mole%	mole percentage
Monos	monounsaturated fatty acids
MPP	Mass Profiler Professional
MS	mass spectrometry
MSTFA	N-methyl-(trimethylsilyl)trifluoroacetamide
MTBE	methyl-*tert*-butyl ether
N	nitrogen
N_2	nitrogen-gas
NaCl	sodium chloride
NaOH	sodium hydroxide
NH_2	aminopropyl
NIST	National Institute of Standards and Technology
NRF	National Research Foundation
Nsats	normal saturated fatty acids
OD	optical density
P	pure culture
PC	principal component
PCA	Principal Component Analysis
PCR	polymerase chain reaction
PLE	pressurised liquid extraction
PLFA	phospholipid fatty acid
Polys	polyunsaturated fatty acids
(Pty) Ltd	property limited
PUFA	polyunsaturated fatty acid
QA/QC	quality assurance or quality control
RNA	ribonucleic acid
S	soil
sat:mono	saturated to monounsaturated
SFE	supercritical fluid extraction
SIP	stable isotope probing
SLB	signature lipid biomarker
SOP	standard operating procedure
SPE	solid-phase extraction
TBsats	terminally branched saturated fatty acids
TL	total lipid
TLC	thin-layer chromatography
TLE	total lipid extract
TMAH	tetramethylammonium hydroxide
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TMS	trimethylsulfonium
TMSH	trimethylsulfonium hydroxide
TSB	tryptic soy broth
UK	United Kingdom

USA	United States of America
US EPA	United States Environmental Protection Agency

List of figures

Chapter 4

Chapter *5*

List of tables

Chapter 2

Chapter 3

Chapter 4

Chapter *5*

Appendix B

Chapter 1
Introduction

1 Background

1.1 Microbial communities as environmental indicators

Based on the importance of microbial communities in ecosystems, a considerable number of studies have focused on microbial communities as indicators of environmental changes or disturbances (Ben-David *et al*. 2004; Moscatelli *et al*. 2005; Córdova-Kreylos *et al*. 2006; Romero-Viana *et al*. 2012; Sun *et al*. 2012). Soil microorganisms in particular, are well-known as the driving force of biogeochemical transformations occurring in soil. Contaminant bioavailability (Duquesne *et al*. 2003; Byss *et al*. 2008), mineral dissolution rate (Boyle *et al*. 2008; Högberg *et al*. 2013), soil structural formation (Huang and Bollag 1998) and organic matter decomposition (Baumann *et al*. 2009; Kuzyakov *et al*. 2014) are all processes mediated by soil microorganisms. Considering the vital part soil microorganisms play in the functioning of soil processes, it is not surprising that soil microbial communities can be used as indicators of soil quality. Furthermore, characteristics such as a high surface-to-volume ratio inducing a close proximity to soil surroundings and a short generation time contribute to their sensitivity to environmental fluctuations (Winding *et al*. 2005). Therefore, the monitoring of changes in the biomass, diversity, functionality and structure of microbial communities can provide early signs of environmental deterioration and stress (Pankhurst *et al*. 1995). Högberg *et al.* (2013) identified soil microbial community indices that can be used as indicators of nitrogen leaching from forests. In general, fungal dominance was associated with lower nitrogen leaching, as was also observed in grasslands with contrasting nitrogen applications (De Vries *et al.* 2006). Changes in the soil microbial community composition have been shown to discriminate between heavy metal polluted sites at an abandoned mine (Ben-David *et al.* 2004), as well as indicate the occurrence of petroleum hydrocarbon biodegradation at a fuel dispensing facility (Ringelberg *et al.* 2008).

Several methods exist to accurately measure the above mentioned microbial community attributes, based on the direct extraction of specific microbial cellular components from environmental samples (Nannipieri *et al.* 2003; Joergensen and Wichern 2008; Hirsch *et al*. 2010). Nucleic acid based techniques involves the extraction of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) from an environmental sample, followed by further investigations necessary for microbial community characterisation. In polymerase chain reaction (PCR)-based approaches, target sequences are

amplified, cloned and sequenced to identify specific microbial species present in the microbial community providing an indication of microbial diversity (Pace 1999; Torsvik *et al.* 2002). However, prior knowledge of target sequences is required for primer development (Feinstein *et al.* 2009). Moreover, PCR-based approaches cannot distinguish among different treatment impacts, as PCR patterns provide a limited amount of statistical data (Ramsey *et al.* 2006). The measurement of soil enzymatic activity can be used as an index of microbial functionality and as an indicator of soil quality (Dick 1994; Nannipieri *et al.* 2002). Extracellular enzymes excreted by soil microorganisms are involved in the turnover of organic matter in soil (Tabatabai 1994), the decomposition of organic soil pollutants (Moreno *et al.* 2003) and the stabilisation of soil aggregates (Dick *et al.* 1994). Examples of soil enzymes include dehydrogenase, phosphatase, ß-glucosidase, urease, chitinase and cellulase (Dick and Tabatabai 1992; Deng and Tabatabai 1994; Makoi and Ndakidemi 2008). Even though enzyme activities provide useful information on the total organic carbon, total nitrogen and microbial oxidative capacity of the soil, enzymatic results are highly variable under differing soil moisture and temperature conditions (Steinweg *et al.* 2012). Therefore, caution must be taken in interpreting enzyme activities as a response to varying environmental factors, when soil moisture is limiting.

Soil microorganisms rarely occur in isolation and continuous interactions among microbial communities lead to changes in soil microbial community structure and biomass. Thus, it is more meaningful to study the physiological status and functional diversity of microbial communities and how the microbial community structure and biomass changes over time, than focusing solely on species identities (Bengtsson 1998; McGill *et al.* 2006). Signature lipid biomarker (SLB) analysis provides insight on the general structural composition of a microbial community (White and Ringelberg 1997; Niemann and Elvert 2008) and is a valuable technique that remains relevant in environmental studies. Especially when combined with other techniques, it can also provide information on physiological and functional status.

1.2 Profiling microbial communities by means of signature lipid biomarker analysis

Signature lipid biomarker analysis is used to characterise the overall lipid composition of cellular membranes in microbial cells. Christie (1989) defined lipids as "...fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds." Fatty acids are synthesised during the condensation of malonyl coenzyme A molecules with a fatty acid synthase complex. Fatty acids mostly consist of a number of carbon atoms in a straight chain, with a carboxylic acid group at one end and a methyl group at the other. The presence of double bonds between the carbon atoms determines the level of saturation, where one or two double bonds indicate unsaturation. Microbial lipids are classified according to their function and structure into apolar and polar lipids. Apolar lipids function as storage bodies for carbon or energy preserves (Athenstaedt and Daum 2006)

and include respiratory quinones (Saitou *et al*. 1999), polyhydroxyalkanoates (Findlay and White 1983), sterols (Newell 1992) and triacylglycerols (Gehron and White 1983). The polar lipids form part of the structure of microbial cell membranes and include amino lipids (Knoche and Shively 1972), glycolipids (Lederer 1967), phospholipids (White *et al.* 1979), sphingolipids (White and Tucker 1970), ladderane lipids (Sinninghe Damsté *et al*. 2002) and ether lipids (De Rosa *et al.* 1982). As a response to irregularities in their immediate environment, microorganisms can modify their membrane lipid properties to maintain membrane integrity and fluidity during compromised conditions (Ingram 1977; Weber and De Bont 1996; Heipieper *et al*. 2003). Such membrane lipid configurations can include higher unsaturation, branched and hydroxylation levels of fatty acid chains (Sinensky 1974; Heipieper *et al.* 2003; Koga 2012), substitution of phospholipids with phosphorous-free lipids under phosphorous-limiting conditions (López-Lara *et al*. 2005) and the methylation and cyclisation of tetraether lipids (Weijers *et al*. 2007). Consequently, monitoring of changes in the ratios of lipid configurations can provide an indication of the physiological state of microbial communities during environmental disturbances. These microbial lipids and their configurations are representative of certain taxonomic structural groups and the physiological status of microbial communities (White and Ringelberg 1997) and can be quantified with SLB analysis.

The key steps necessary for SLB analysis include the appropriate preparation and handling of the samples, the complete extraction of the total lipids directly from the sample matrix with relevant solvent mixtures, conversion of the lipid extracts into appropriate derivatives for quantification and the characterisation and quantification of these derivatives by means of analytical instruments (White and Ringelberg 1997). A comprehensive lipid profile, containing all the detected lipids in the sample, can be compiled from the generated data. Certain lipids are unique to specific microbial groups and act as signature biomarkers for that particular group. Also, by comparing lipid profiles from different environmental sites or treatments, distinct responses of microbial communities to changing conditions can be identified. Ratios of specific SLBs have been associated with environmental conditions and the physiological state of microbial communities (White and Ringelberg 1997). For example, changes in the ratio of methylated branched tetraethers to cyclic branched tetraethers have been associated with changes in soil pH and annual mean air temperature (Weijers *et al*. 2007). Unbalanced microbial growth induced by nutrient stress can be indicated by a high polyhydroxyalkanoate to phospholipid fatty acid (PLFA) ratio (White *et al*. 1996), whereas the respiratory activity of microbial communities can be portrayed using the ratio of total respiratory quinones to total PLFAs (Villanueva *et al*. 2007). The effects of flooding (Ayari *et al*. 2013), altitudinal differences (Sinninghe Damsté *et al*. 2008) and soil temperatures (Peterse *et al*. 2009) on microbial communities have also been assessed using SLBs. More examples of SLB analysis clarifying the diversity and composition of microbial communities, include those present in different layers of laminated microbial sediments from a supratidal sandy

beach (Bühring *et al*. 2014), terrestrial mud volcanoes (Heller *et al*. 2012), dolomite concretions (Hoffmann-Sell *et al*. 2011) and meltwater ponds in Antarctica (Jungblut *et al*. 2009).

Although SLB analysis provides wide-ranging applications for the profiling of microbial lipids in environmental samples, not all studies require such comprehensive characterisation. Indeed, certain investigations only call for differentiation among treatments or soil sites (Bühring *et al*. 2012; Dangi *et al*. 2015), whereas other assessments focus on the living microbial fraction of a microbial community (Khomutova *et al*. 2011). In addition, not all the previously mentioned microbial lipids are present in every environmental sample and their respective extraction procedures are therefore not always required. For instance, agricultural soil samples do not possess high concentrations of ether lipids indicative of archaea (Esperschütz *et al*. 2007), whereas microbial mats in extreme environments do (Yoshinaga *et al*. 2011). This is where the utilisation of more specific SLB analyses is of value in soil microbial research – for example, PLFA analysis.

1.3 Phospholipid fatty acid analysis to characterise microbial communities

Phospholipid fatty acid analysis is a type of SLB analysis that examines only the fatty acids of phospholipids present in all microbial cell membranes (White *et al.* 1979; Ringelberg *et al.* 1989). The phospholipids are rapidly degraded upon cell-death by phospholipases into neutral lipid diglycerides, which are not commonly found in viable cells. Therefore, PLFA analysis provides a practical measure of the total viable microbial biomass (White *et al*. 1979). Some of the earliest studies using PLFA analysis were conducted on pure cultures (Dowling *et al.* 1986; Guckert *et al.* 1986; Kieft *et al.* 1994; Macnaughton *et al.* 1997) and the majority of PLFA databases are derived from the fatty acids of microorganisms grown under controlled conditions (Bossio and Scow 1998). However, the true value of PLFA analysis lies in its application to environmental samples to assess microbial communities in their natural environment. Soil microbial communities affected by anthropogenic practices (Acosta-Martínez *et al*. 2010; Herold *et al*. 2012; Lagerlöf *et al*. 2014; Zhang *et al*. 2014a; Fanin *et al*. 2015), vegetation diversity and distributions (Mitchell *et al*. 2010; Lozano *et al*. 2014; Bragazza *et al*. 2015), chemical compounds (Butler *et al*. 2012; Cycoń *et al*. 2012, 2013) and varying soil physical and chemical properties (Mitchell *et al*. 2010; Bi *et al*. 2011) have all been characterised using PLFA analysis.

The experimental procedures performed during PLFA analysis from soil samples consist of similar steps to SLB analysis. Basically, the total lipid extract is separated from the soil through the use of organic solvents, the extracted total lipids are fractionated by silicic acid column chromatography into neutral, glyco- and phospholipids, followed by the transesterification of the phospholipid fractions into their respective fatty acid methyl esters (FAMEs) by mild alkaline methanolysis and the quantification

of these FAMEs by gas chromatography-mass spectrometry (GC/MS) (White *et al*. 1979; Guckert *et al*. 1985). Several modifications of each procedure exist (as discussed in Chapter 2) to increase the yield and separation efficiency of the FAMEs detected.

The quantity of total PLFAs detected in a sample, measured in pmol or nmol per gram dry weight of soil, gives an estimate of the total viable microbial biomass (Frostegård *et al*. 1991). Furthermore, the presence of distinctive individual PLFAs or PLFA patterns in a lipid profile can be interpreted as biomarkers for specific structural microbial groups (Guckert *et al*. 1985; White *et al*. 1996). The mole percentage fraction of the following PLFA structural groups is indicative of specific microbial groups (Frostegård and Bååth 1996): methyl or mid-chain branched saturated fatty acids represent Actinomycetes; terminally branched saturated fatty acids indicate Gram-positive bacteria, monounsaturated fatty acids, Gram-negative bacteria; and polyunsaturated fatty acids, fungi. In addition, several ratios calculated from these PLFA structural groups can provide insight into the dominant microbial groups in an environment. These ratios include Gram-positive to Gram-negative (GP:GN) bacterial fatty acids, fungal to bacterial (F:B) biomass and Gram-positive to total (GP:total) PLFAs (Frostegård and Bååth 1996; McKinley *et al*. 2005). The specific fatty acids within each structural group, their associated microbial designations and the different ratios are discussed in Chapter 3.

Fatty acids are designated based on the total number of carbon atoms in the chain, followed by the number of double bonds indicating the unsaturation level. The position of the double bond nearest to the methyl (ω) part of the molecule is added at the end, with the suffixes "c" and "t" representing *cis* and *trans* configuration of monounsaturated fatty acids, respectively. These fatty acids refer to all fatty acids with a double bond between two carbon atoms (monounsaturated), where the hydrogen atoms adjacent to the double bond are either positioned in the same stearic direction (*cis* configuration) or in opposite directions (*trans* configuration) (Heipieper *et al*. 2003). The prefixes "a", "i", "cy" and "br" refer to *anteiso-*, *iso-*, cyclopropyl and unknown branching in branched fatty acids, whereas "OH" and "Me" refer to a hydroxyl and a methyl group, respectively. The position of the particular group from the carboxyl end of the molecule is indicated by a preceding number (Guckert *et al*. 1985; Ringelberg *et al*. 1989; Zelles 1999). During the exposure of microbial communities to environmental changes such as temperature fluctuations, nutrient deficiencies and high contaminant levels, microbial membrane integrity can be compromised by increased membrane fluidity. A survival mechanism namely homeoviscous adaptation, can be implemented by microbial communities through the alteration of fatty acid configurations. The enzymatic conversion of *cis*-monounsaturated fatty acids (16:1ω7c and 18:1ω7c) to their cyclic-derivatives (cy17:0 and cy19:0, respectively) and the formation of *trans*-monounsaturated PLFAs (16:1ω7t and 18:1ω7t), regulate membrane fluidity (Diefenbach *et al*. 1992; Morita *et al*. 1993; Los and Murata 2004). Microbial communities permanently modify their

membranes to an acceptable physiological state to survive and adapt to changing environmental conditions. Thus, the relationship between the *trans* and *cis* monounsaturated fatty acids (*trans*:*cis*), and between the cyclopropyl fatty acids and their monounsaturated precursors (cy:pre) can be used as microbial stress and physiological state assessment tools in environmental monitoring practices (Guckert *et al*. 1986; Kieft *et al*. 1994). Despite the considerable number of useful microbial community characteristics obtained by applying PLFA analysis to environmental samples, attempts have been made to simplify lipid analysis along with acquiring more information of the microbial community at hand (Akoto *et al.* 2008; Gómez-Brandón *et al.* 2008, 2010; Matyash *et al.* 2008). While PLFA analysis of soil samples concentrates solely on phospholipids as biomarkers for microbial community characterisation (White *et al*. 1979), the application of an advanced analytical approach, such as metabolomics, may extend the concept of biomarkers to all metabolites responsive to an exposure (Halter *et al*. 2012; Beale *et al*. 2014; Hernandez-Soriano and Jimenez-Lopez 2014).

1.4 Investigating microbial communities through metabolomics

Metabolomics is a functional-genomic technique used to characterise the changes occurring within the complete range of low molecular weight metabolites (the metabolome) of a cell or tissues, as a response to specific conditions (Fiehn 2002). The metabolome has been found to consist of fatty acids, amino acids, organic acids, sugars and nucleotides (Halter *et al.* 2012; Brown *et al*. 2014; Swenson *et al.* 2015). Given that intra- and extracellular metabolites represent the closest biochemical level to the biological functioning of a cell, metabolomics reflects the phenotypic characteristics or physiological state of a cell at that specific point in time (Van der Werf *et al*. 2005; Nagrath *et al.* 2011). As metabolites are products and substrates of cellular reactions, a metabolic profile can link several cellular processes and biochemical pathways functioning in a viable cell. Such cellular processes include homeostasis, nutrient cycling, redox state and cell to cell signalling (Mosier *et al*. 2013), whereas examples of biochemical pathways are amino acid synthesis, fatty acid oxidation and energy metabolism (D'Alessandro *et al.* 2013; Ribeiro *et al.* 2014; Sasaki *et al.* 2014).

A range of metabolomics approaches exist based on the specific sample matrices analysed. These include clinical (Newgard *et al.* 2009; Zhang *et al*. 2014b), microbial (Winder *et al.* 2008; Tremaroli *et al.* 2009; Vincent *et al*. 2014), environmental (Mishra *et al.* 2014; Swenson *et al.* 2015) and plant metabolomics (Petersen *et al.* 2011; Ribeiro *et al.* 2014; Scalabrin *et al.* 2015). Furthermore, these metabolomics approaches can have different focal points for the analysis of the metabolites present in a biological sample. Referred to as targeted analysis, the detection and characterisation of predefined metabolite classes can provide an indication of the functioning of important metabolic processes under ideal or unfavourable conditions (Evans *et al*. 2014). In contrast, untargeted analysis detects all possible metabolites in an attempt to identify signature patterns or specific biomarker metabolites

indicative of differences among samples (Petersen *et al*. 2011), stress exposures (D'Alessandro *et al*. 2013; Campillo *et al.* 2015) or disease occurrences (Zhang *et al*. 2014b). The concept of environmental metabolomics entails that an organism is exposed to an external stressor. The organism may represent a single individual (plant, human, fish or microbial cell) or a multi-organism system (soil microbial community or biofilm). An external stressor may be any change in the organism's immediate environment such as temperature or nutrient fluctuations, disease outbreaks or contaminant exposures (Lankadurai *et al*. 2013). Environmental metabolomics has proved to be a valuable platform in modelling the metabolites responsible for the adaptation and resistance mechanisms implemented by microorganisms under stress conditions (Halter *et al.* 2012; Beale *et al.* 2014). Changes in their metabolic compositions have been associated with metal or acid tolerance (Tremaroli *et al*. 2009; Mosier *et al*. 2013; Beale *et al.* 2014), as well as coping mechanisms against nutrient deprivation, oxygen limitations and temperature fluctuations (Fleury *et al.* 2009; Liebeke and Lalk 2014). Several studies have reported the potential of metabolomics techniques to categorise indicators of defined stress responses and distinguish between various types of exposures (Beale *et al.* 2014; Brown *et al.* 2014; Jones *et al.* 2014; Campillo *et al.* 2015). Therefore, the use of metabolomics in routine environmental monitoring practices may serve as an early warning indicator of deteriorating environmental health.

Consider that SLB analysis also provide early signs of environmental changes (Peterse *et al*. 2009; Ayari *et al*. 2013) and that the fatty acids detected with PLFA analysis form part of the metabolome identified by metabolomics (Halter *et al.* 2012). It therefore follows that metabolomics may also provide valuable information on the response of soil microbial communities to environmental changes. However, this approach has been largely unexplored, with very few studies addressing the topic. Kakumanu *et al*. (2013) used a metabolomics-based approach in conjunction with PLFA analysis to analyse the changes in microbial communities as a response to soil desiccation; while Jones *et al.* (2014) used metabolomics to show changes in the biochemical profiles of soil communities living in contaminated sites. Thus far, target metabolites specifically associated with microbial communities have not been identified. Given the complex nature of the soil matrix, (Baldock and Skjemstad 2000; Salvia *et al.* 2013), challenges arise in identifying such a large number of metabolites simultaneously without interferences from soil constituents (Swenson *et al.* 2015). Furthermore, the use of cruder sample extracts in metabolomics can cause higher matrix effects of co-eluting analytes masking the true abundance of target metabolites (Wood 2014; Garg *et al.* 2015). This substantiates why several environmental studies isolate and sub-culture the microbial communities under controlled laboratory conditions on artificial media (Giagnoni *et al.* 2011; Halter *et al.* 2012; Beale *et al.* 2014). Even though this type of approach provides an accurate metabolic profile of the cultured microbial community under controlled laboratory conditions, it may only be interpreted as a model or simulation of that microbial community's metabolic response in their natural environment (Halter *et al.* 2012).

Consequently, the model may show discrepancies when the microbial community is studied in the environment. In spite of the current obstacles of using metabolomics-based approaches to assess soil microbial communities, there may be potential in investigating these approaches to characterise soil microbial communities in their natural environment, without prior cultivation. Rather than identifying specific signature or target metabolites, the value of the approach may lie in the identification of signature patterns of change where shifts in the comprehensive metabolic profile will help to clarify the changes in the microbial community when combined with other approaches.

2 Perspective, aim and chapter layout

2.1 Perspective of the current study

Phospholipid fatty acid analysis has been used extensively in soil studies throughout the past 40 years to characterise microbial community structure and it is still a valuable technique. A search (October 2015) on Scopus for "soil AND plfa" in the title, abstract and keywords for the period 2012 to 2015 showed 495 hits. Of these, 258 papers were for the period 2014-2015. This clearly shows that the PLFA method is still widely used in soil investigations. At the North-West University, South Africa, PLFA analysis has been used to study, amongst others, the effect of rehabilitation on different types of post-mining sites (Claassens *et al.* 2006a, 2006b; Maboeta *et al.* 2006; Claassens *et al.* 2008; Claassens *et al.* 2012), the efficiency of various commercial products on the bioremediation of hydrocarbon contaminated soil (Claassens *et al.* 2006c); the influence of take-all disease of wheat on native microbial communities (Habig 2003) and the effect of soil fumigants on soil microbial communities (Potgieter *et al.* 2013; Fouché 2014). Although this technique has proved its worth, the considerable number of studies attempting to improve PLFA yield efficiency by modifying original steps of the method (Pinkart *et al.* 1998; Gómez-Brandón *et al.* 2008, 2010; Matyash *et al.* 2008) or by developing new methods (Akoto *et al.* 2008; White *et al.* 2009; Hanif *et al.* 2012), contributes to the uncertainty of which method is the most appropriate. To add to this, various interpretations are attributed to similar sets of PLFA data (Frostegård *et al.* 1991; McKinley *et al.* 2005; Frostegård *et al.* 2011). In this regard, several discrepancies can be identified that add to the scepticism regarding the accuracy of PLFA analysis in characterising microbial communities in environmental samples. Therefore, the need for comprehensive literature-based evaluations of (i) the various methods and their modified versions (if applicable) available for PLFA analysis from environmental samples, and (ii) the numerous ways in which PLFA analysis is applied and data interpreted, was identified.

In addition to evaluating existing approaches, there is a need to explore new and modernised high-throughput technologies. Since metabolomics shows the potential to indicate microbial responses to environmental changes (Tremaroli *et al*. 2009; Beale *et al.* 2014), the application of a metabolomics-based approach to investigate PLFAs in soil microbiology studies warrants investigation. The use of a

metabolomics-based approach may provide more biological information on a particular microbial community than traditional PLFA analysis or it may serve as a more rapid method to characterise the same fatty acids. Consequently, it is of value to evaluate the performance of a metabolomics-based approach against traditional PLFA analysis with regards to data generation, biological interpretation and characterisation of microbial FAMEs.

The current study assessed the quantitative and qualitative data generation potential of a metabolomics-based approach to characterise the FAMEs of soil microbial communities, in comparison to traditional PLFA/FAME analyses. Firstly, literature-based comparative investigations were done on the various modifications available on PLFA experimental procedures and the different ways in which PLFA data obtained from different environmental matrices can be interpreted. The experimental work compared PLFA (fractionated) and total lipid extract (TLE) analyses with a metabolomics-based approach to quantify FAMEs. Different derivatisation procedures were evaluated to determine the most appropriate procedure for each method. Furthermore, the applicability of the different approaches to distinguish between soil microbial communities exposed to different soil fumigant treatments were compared in a greenhouse study. Considering the results obtained from the complete study, a standard operating procedure for targeted FAMEs and proposed untargeted metabolomics is provided. Additionally, metabolite profiles obtained from the metabolomics analysis were considered for application value to distinguish between fumigant treatments and some recommendations for future studies in soil metabolomics are made.

2.2 Aim and objectives

The aim of this study was the standardisation and implementation of an optimised method for lipid profiling of microbial communities.

Specific objectives included:

- A literature-based comparison of methods available for PLFA analysis on environmental samples focusing on method origin, modifications made to original methods and the advantages and limitations of each;
- A literature-based comparison of the current interpretations of generated PLFA data as applied in environmental studies;
- An evaluation of the targeted analysis of selected FAMEs to characterise both homogenised soil and pure bacterial cultures as obtained from fractionated PLFA, TLE and metabolomics analysis respectively;

- An evaluation to determine the most appropriate derivatisation technique for each of the three methods. These procedures included:
  - alkaline methanolysis,
  - a combination of alkaline methanolysis and silylation,
  - alkaline methanolysis combined with oximation and silylation;
  - and oximation followed by silylation;
- An evaluation of the efficiency of fractionated PLFA, TLE and metabolomics analysis respectively, to distinguish between soil microbial communities exposed to different fumigants in a greenhouse experiment;
- The formulation of a standard operating procedure for optimised lipid profiling of microbial communities and the implementation of this method in the Soil Microbiology Laboratory at the North-West University, Potchefstroom Campus.

2.3 Chapter layout

This thesis represents a compilation of published and unpublished manuscripts, where each chapter is an individual entity. Therefore, some repetition between chapters has been unavoidable.

Chapter 1 is the current chapter and provides an introduction to the study which describes the use of microbial communities as environmental indicators, as well as the profiling of microbial community structure. This chapter also includes the perspective, aim, specific objectives and outline of the thesis chapters.

Chapter 2 reviews the literature on the various methods and modifications to methods available for microbial signature lipid biomarker analysis. This chapter has been published in the *Journal of Applied Microbiology* under the title "Microbial signature lipid biomarker analysis – an approach that is still preferred, even amid various method modifications" (full reference is provided in the chapter).

Chapter 3 reviews the literature on the various interpretations of PLFA results and the different applications thereof in environmental studies. This chapter has been published in the *Journal of Applied Microbiology* under the title "Phospholipid fatty acid profiling of microbial communities – a review of interpretations and recent applications" (full reference is provided in the chapter).

Chapter 4 describes the comparative investigation between the fractionated PLFA, TLE and metabolomics-based methods when applied to homogeneous soil and pure bacterial culture samples. This chapter contains a short motivation, followed by the materials and methods, results and discussion for this chapter.

Chapter 5 describes the application of the fractionated PLFA and metabolomics-based methods to distinguish between soil microbial communities exposed to different fumigant treatments in a

greenhouse experiment. Again, a motivation, materials and methods, results and discussion are provided.

Chapter 6 provides a general discussion and conclusion of all the stated objectives.

References are provided at the end of each chapter.

Appendix A includes the standard operating procedure for targeted FAMEs and proposed untargeted metabolomics, as well as work instructions for the various protocols.

Appendix B provides supplementary information for Chapter 4, including the repeatability of the homogenisation of the soil samples and a motivation for the aqueous phase volume used.

Appendix C contains the title pages of the published articles included in Chapters 2 and 3.

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Chapter 2
Methods for microbial signature lipid biomarker analysis

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Summary

The lipid composition of microbial communities can indicate their response to changes in the surrounding environment induced by anthropogenic practices, chemical contamination or climatic conditions. A considerable number of analytical techniques exist for the examination of microbial lipids. This article reviews a selection of methods available for environmental samples as applied for lipid extraction, fractionation, derivatisation and quantification. The discussion focuses on the origin of the standard methods, the different modified versions developed for investigation of microbial lipids, as well as the advantages and limitations of each. Current modifications to standard methods show a number of improvements for each of the different steps associated with analysis. The advantages and disadvantages of lipid analysis compared to other popular techniques are clarified. Accordingly, the preferential utilisation of signature lipid biomarker analysis in current research is considered. It is clear from recent literature that this technique stays relevant - mainly for the variety of microbial properties that can be determined in a single analysis.

Keywords

derivatisation; environmental samples; fatty acid methyl ester; fractionation; lipid extraction; lipid quantification; microbial lipid analysis

1 Introduction

In the past 40-50 years, the importance of microorganisms as a biomonitoring tool for environmental changes has been recognised. This can be ascribed to their wide-spread distribution, rapid reproduction rate and high susceptibility to environmental disturbances (Fang and Findlay 1996; Ruess and Chamberlain 2010). Considering the role of microorganisms in the biogeochemical cycling of

nutrients and the degradation of organic contaminants, it is evident that the characterisation of the microbial community can provide insight into the functionality of an environment (Mummey *et al.* 2002). Seeing that <1% of all naturally occurring microorganisms can be cultivated on synthetic media (Vestal and White 1989; Hill *et al.* 2000; Gans *et al.* 2005), culture-independent techniques such as signature lipid biomarker analyses, are widely applied (Kaur *et al.* 2005; Joergensen and Emmerling 2006).

Lipids in microbial cells function as the main constituents of membranes, act as carbon storage bodies, facilitate signal transduction and can be used to identify bacteria on species level (Ishida *et al.* 2006). The quantification and identification of these lipids contribute to an understanding of the functioning of microbial metabolic genes and pathways (Basconcillo and McCarry 2008; Bühring *et al.* 2012), can be applied in the screening of pathogenic bacteria (Müller *et al.* 1998; Kellogg *et al.* 2001) and the measurement of microbial community structure and diversity (White *et al.* 1996; Ringelberg *et al.* 1997; Jungblut *et al.* 2009; Naeher *et al.* 2012). For the accurate profiling of microbial lipids from various sample matrices, several important processes are required (Huang *et al.* 2002; Poerschmann and Carlson 2006), including: (i) the complete removal or extraction of the lipids from the samples without any damage; (ii) the fractionation of the extracted whole lipids into various lipid classes; (iii) the conversion of the lipids into their respective methyl derivatives through methylation or derivatisation; and (iv) the quantification and characterisation of the methyl esters. No single experiment can comprehensively analyse the different lipids obtained from the endless possibilities in chain length, branching, unsaturation level, double bond positions and the presence of other functional groups (Härtig 2008). Therefore, numerous methods and modifications to the methods exist for faster and simplified approaches, with the goal of higher throughput analysis and full automation. The present review provides a broad outline of a selection of different techniques currently available for microbial lipid analyses from environmental samples, based on extraction, fractionation, derivatisation and quantification. The original procedures of each methodology are discussed, with clarity given on the specific solvent systems used, modifications in recent developments for applications on microbial lipids, as well as existing advantages and shortcomings regarding each. In conclusion, a summary of the selected methods is provided, with an evaluation on the current use of phospholipid fatty acid (PLFA) based methods against novel advances, for the successful characterisation of microbial lipids from environmental matrices.

2 Lipid extraction procedures

Microbial lipid analysis can be conducted on a range of sample matrices; however, this review focuses primarily on the analysis from environmental matrices. Of the four processes required for microbial lipid analysis, extraction is generally regarded as the most challenging. This process involves the

addition of various organic solvents to the sample, followed by filtration, gravitation or centrifugation to distinguish between different phases. The extracted lipids are situated in the organic layer. Difficulties concerning extraction are based on the extensive extraction time required, the loss or contamination of target lipids during collection and the choice of an appropriate extraction solvent (Smedes and Askland 1999; Ruiz-Gutiérrez and Pérez-Camino 2000; Cescut *et al.* 2011). A selection of extraction techniques that are often used for microbial lipid profiling (Table 2.1) include the methods developed by Folch (Folch *et al.* 1957), Bligh and Dyer (Bligh and Dyer 1959) and Soxhlet (Soxhlet 1879). More recent techniques make use of the solvent methyl-*tert*-butyl ether (MTBE) (Matyash *et al.* 2008), automated accelerated-solvent extraction (ASE) and supercritical fluid extraction (SFE) for adequate extraction.

The original Folch method (Folch *et al.* 1957) was developed to extract lipids from animal tissues such as brain matter, liver or muscle. It is based on the homogenisation of the tissues in a 2:1 chloroform/methanol ($CHCl_3$/MeOH) *(v/v)* mixture, followed by washing with water (H_2O) or a salt buffer. After phase separation the lipids will reside in the organic phase, whereas the nonlipid compounds are found in the aqueous phase. A single washing procedure with H_2O ensured a minimal lipid loss of 0.3-0.6%, while the addition of mineral salts significantly reduced the loss of acidic lipids into the aqueous phase. Although this method is considered as the 'gold standard' for lipid extraction, with its high extraction efficiency (Matyash *et al.* 2008; Sheng *et al.* 2011) and reduced interference of nonlipid compounds due to the presence of polar solvents (Sheng *et al.* 2011), several of its limitations provided starting points for method improvement. First, the need for high volumes of extraction solvents (Folch *et al.* 1957) were reduced without hampering lipid recovery from bacterial cultures (Basconcillo and McCarry 2008; Matyash *et al.* 2008) and photosynthetic cyanobacteria (Sheng *et al.* 2011). Exclusion of the washing process during PLFA analysis from soil, animal manures and compost, showed no restriction on microbial lipid yield (Gómez-Brandón *et al.* 2008, 2010). On the contrary, this modified Folch method, without the washing step, gave the highest total PLFA and specific biomarker recovery compared to a modified Bligh and Dyer method (White *et al.* 1979) and microwave-assisted extraction (López-Ávila *et al.* 1994). This could be ascribed to the longer incubation period and higher volumes of solvent used during Folch extraction. Even though attempts have been made to shorten Folch extraction time, findings confirmed that the conventional solvent exposure time of 24 h is the most favourable period for yielding the highest fatty acid concentrations (Taha *et al.* 2012).

Table 2.1: Methods for microbial lipid extraction from environmental sample matrices

Methods	Sample matrices	Solvent systems	Advantages	Limitations	References
Folch	Algae; bacterial cultures; cattle manure; compost; cyanobacteria; pig slurry; soil; vermicompost	Chloroform:methanol (2:1; *v/v*), followed by washing with water/ 0.88% KCl/NaCl *Modifications* Removal of washing procedure	• Regarded as 'golden standard' for lipid extraction • Chloroform:methanol combination successfully disrupts cell membranes, extracting microbial lipids • Use of polar solvents decrease interference of nonlipid compounds	• Requires high volumes of solvents • Long incubation periods • Chloroform is carcinogenic	Basconcillo and McCarry 2008; Folch *et al.* 1957; Gómez-Brandón *et al.* 2008, 2010; Matyash *et al.* 2008; Meier *et al.* 2006; Sheng *et al.* 2011
Bligh and Dyer	Activated sludge; air; algae; bacterial cultures; biofilms; cattle manure; compost; cyanobacteria; marine and estuarine sediments; microbial mats; pig slurry; soil; vermicompost; water; yeast	Chloroform:methanol:water (1:2:0.8; *v/v/v*) *Modifications* Substitution of water with citrate buffer, phosphate buffer, 1% trichloro-acetic acid	• Efficiently extracts polar lipids • Nonlipid compounds do not interfere, due to strong polar solvents used • Buffered solvent system prevents ionic salt adsorption effects	• Polar lipids have been found in aqueous phase • Chloroform is carcinogenic • Time-consuming • Difficulty in extracting chloroform organic layer without contamination occurring	Allen *et al.* 2010; Bligh and Dyer 1959; Cescut *et al.* 2011; Chowdhury and Dick 2012; Conrad *et al.* 2003; Frostegård *et al.* 1991; Gómez-Brandón *et al.* 2008; Guckert *et al.* 1985; Hanif *et al.* 2010; Kumari *et al.* 2011; Liu *et al.* 2012; Macnaughton *et al.* 1997; Sheng *et al.* 2011; Vinten *et al.* 2011; White *et al.* 1979
MTBE	Bacterial cultures; cyanobacteria	MTBE:methanol (1.5:5; *v/v*) *Modifications* MTBE:methanol:water (20:6:7; *v/v/v*)	• Lipids in upper MTBE phase are easier to collect (simplified) • Fast with high precision • Lipid loss reduced • MTBE is environmentally friendly, chemically stable and does not alter lipids during storage • Suitable for automation	• No specific limitations	Matyash *et al.* 2008; Sheng *et al.* 2011
Soxhlet	Cold-seep carbonates; cyanobacteria; green algae; soil; yeast	Hexane:acetone (1:1; *v/v*) Acetone:hexane (10:7; *v/v*) Dichloromethane:methanol Isopropanol Petroleum ether *Modifications* FMASE	• Sample continuously in contact with fresh solvent • Not matrix dependent • No specialised personnel required • No filtration required after leaching step • Extract more sample mass than other methods	• Long extraction time • Generates high volumes of waste solvent • Thermal degradation of analytes due to long exposures to high temperatures • Evaporation-concentration step required • Restricted to solvent selectivity	Cescut *et al.* 2011; Guan *et al.* 2013; Guckert *et al.* 1985; Luque de Castro and Garcia-Ayuso 1998; Sheng *et al.* 2011; Soxhlet 1879
ASE (PLE)	Air; bacterial cultures; coal; lake sediments; peat bogs; soil; solid waste; water; yeast	Dichloromethane:methanol (9:1; *v/v*) Hexane:acetone (1:1; *v/v*) Methanol:chloroform:disodium citrate buffer (2:1:0.8; *v/v/v*) Chloroform:methanol (1:2; *v/v*) Methanol:chloroform:phosphate buffer (2:1:0.8; *v/v/v*) Dichloromethane:methanol (2:1; *v/v*)	• Rapid analysis • Reduced solvent volumes required • No filtration required • Can be left unattended (automated) • Rinse and purge of sample containers with solvent, results in quantitative transfer	• Functions under extreme temperatures and pressures to keep extraction solvent above boiling point, while still in liquid phase	Cescut *et al.* 2011; Ezzell *et al.* 1995; Macnaughton *et al.* 1997; Peterse *et al.* 2009; Poerschmann and Carlson 2006; Richter *et al.* 1996; Sorho *et al.* 2006; Weijers *et al.* 2011; White *et al.* 2009
SFE	Activated sludge; archaeal and bacterial cultures	CO_2 gas *Modifications* 10% methanol as modifier	• Rapid analysis • Coupled to detectors or chromatographs • Reduced solvent volumes required • CO_2 gas is nontoxic and have low critical values	• Addition of CO_2 is not polar enough to separate PLFAs from sludge • Optimisation of SFE conditions needed for specific matrix • Matrix dependent	Cescut *et al.* 2011; Gharaibeh and Voorhees 1996; Hanif *et al.* 2010, 2012; Hawthorne *et al.* 1992; Hedrick *et al.* 1991

In 1959, Bligh and Dyer published a similar method (Bligh and Dyer 1959) to the original Folch procedure (Folch *et al.* 1957). However, this technique was originally optimised for fish muscle phospholipid extraction by using a 1:2 $CHCl_3$/MeOH *(v/v)* solution, with the addition of H_2O to produce an initial solvent ratio of 1:2:0.8 (*v/v/v*). Despite the use of a smaller amount of solvent and sample than the Folch method, more than 95% of the total polar lipids were extracted by this technique. Since then, various modifications have been made to the Bligh and Dyer method for successful microbial lipid extraction from a diverse range of sample matrices. The earliest modifications with the widest recent applications are the substitution of the H_2O fraction in the extraction mixture with either phosphate buffer (White *et al.* 1979) or citrate buffer (Frostegård *et al.* 1991). Both these approaches are used to assess the soil microbial community biomass and structure through PLFA analysis. It has been suggested that the presence of a buffer in the solvent mixture reduces the ionisation of phospholipids (Christie 1993). This ionisation leads to the increased loss of phospholipids into the aqueous phase, inducing a lower lipid yield in the organic phase. Other studies, utilising buffered solvent systems for microbial lipid extraction, have been conducted on macroalgae (Kumari *et al.* 2011); soil (Kehrmeyer *et al.* 1996; Gómez-Brandón *et al.* 2008, 2010; Chowdhury and Dick 2012; Ayari *et al.* 2013); activated sludge (Conrad *et al.* 2003; Hanif *et al.* 2010, 2012); estuarine sediments (Guckert *et al.* 1985), biofilms (Vinten *et al.* 2011; Liu *et al.* 2012) and pure microbial cultures (Macnaughton *et al.* 1997). Consistent with the fact that microbial mats may contain Archaea, the use of 1% trichloroacetic acid instead of H_2O, has been shown to successfully extract archaeal lipids from these matrices (Nishihara and Koga 1987; Allen *et al.* 2010). In addition to the hazardous nature of $CHCl_3$ and the long extraction time required (Macnaughton *et al.* 1997; Greim and Reuter 2001; Sheng *et al.* 2011), the potential for target lipid contamination, occurring during lower organic phase collection, is extremely high in the Bligh and Dyer method (Bligh and Dyer 1959).

A recent study by Matyash *et al.* (2008) investigated the use of MTBE/MeOH (1.5:5; *v/v*) for microbial lipid extraction, where $CHCl_3$ is substituted with MTBE. The hazardous nature of $CHCl_3$ (Nagano *et al.* 2006), as well as the possible formation of phosgene and hydrochloric acid during $CHCl_3$ decomposition, which results in lipid modifications (Schmid *et al.* 1973), necessitated this alternative approach. In comparison to $CHCl_3$, MTBE is chemically stable; does not induce lipid changes during storage and is less toxic (Greim and Reuter 2001; Hamid and Ali 2004). Owing to its low density, MTBE forms the upper phase of a two-phase (organic/aqueous) separation. Therefore, collection of the organic phase containing the lipids is simplified; reducing lipid loss during extraction. This approach recovered microbial lipids within a yield range of 90-98%. It was faster, ensured higher recovery precision and was more appropriate for the direct sample injection into a mass spectrometer, than the conventional Bligh and Dyer method (Matyash *et al.* 2008). Lipid extraction from cyanobacterial samples showed a yield of 93% when a mixture of MTBE/MeOH (1.5:5; *v/v*) was used (Sheng *et al.* 2011).

The introduction of the Soxhlet extraction apparatus in 1879 by Frans von Soxhlet, enabled the successful determination of the amount of lipids in milk (Jensen 2007). Basically, the sample is placed in a porous thimble-holder and extraction of the target analytes accomplished by condensed solvent overflow onto the sample. Soxhlet extraction is straightforward as it requires no specialised personnel, is not dependent on specific sample matrices and no filtration is needed afterwards (US EPA Method 1995). However, target analyte extraction is restricted to extraction solvent selectivity. For instance, the use of apolar solvents (dichloromethane, isopropanol or hexane-acetone) resulted in the increased extraction of nonpolar lipids and nonlipid compounds from green algae (Guckert *et al.* 1988), cyanobacteria (Sheng *et al.* 2011) and yeast (Cescut *et al.* 2011). In recent years, conventional Soxhlet extraction was modified to improve the limitations experienced and to automate the method for a higher throughput. Various new extraction techniques with improved efficiency, less solvent consumption, reduced thermal degradation of analytes; faster leaching time and multi-sample automation have been developed. Such techniques include focused microwave-assisted Soxhlet extraction (FMASE) and automated Soxtec® Systems (Ndiomu and Simpson 1988; Luque García and Luque de Castro 2004). Microbial lipid biomarkers have been successfully extracted from cold-seep carbonates, sampled at the seafloor, by a Soxtec® System with dichloromethane and MeOH (9:1; *v/v*) as solvent mixture (Guan *et al.* 2013).

The first applications of ASE, also known as pressurised liquid extraction, were used for the removal of organic pollutants from waste samples (Ezzell *et al.* 1995; Richter *et al.* 1996). Dionex Incorporated (Sunnyvale, CA) commercialised automated ASE systems with ovens as heating sources. This technique applies elevated temperature and pressure to keep the extractant above its boiling point, while the solvent is still in a liquid phase at pressures higher than 200 bar. As these systems have characteristic advantages over the conventional Folch, Bligh and Dyer and Soxhlet methods; a considerable number of recent case studies include ASE in their experimental design. Such advantages include reduced solvent volumes, faster extraction, full automation, no filtration and qualitative transfer due to the rinse and purge of sample containers with solvent. Various studies have used ASE for microbial lipid extraction from pure bacterial cultures, water and air samples (Macnaughton *et al.* 1997), peat bogs, coal (Weijers *et al.* 2011) and soil (Sorho *et al.* 2006; Peterse *et al.* 2009). It has been shown that ASE is 5-fold faster and uses 20-fold less solvent than the Soxhlet or Bligh and Dyer extraction methods during extraction of yeast lipids (Cescut *et al.* 2011). Furthermore, it extracted higher concentrations of PLFAs and neutral lipids from soil samples, compared to the traditional Bligh and Dyer reagent mixture (White *et al.* 2009). Considering these studies and the different solvent combinations used, it should be highlighted that appropriate extraction conditions should be optimised for each matrix type before loading multiple samples on an ASE system.

The application of a supercritical fluid as the extraction solvent is a nontoxic, rapid extraction method for microbial lipids with the capacity to be linked to chromatographs or detectors (Hedrick *et al.* 1991; Hawthorne *et al.* 1992). A supercritical fluid is a liquid with solubility features similar to a liquid, but with diffusivity and viscosity similar to a gas (Hawthorne *et al.* 1992). Several supercritical fluids are used as extractants, namely ammonia, freons (chlorofluorocarbons) and carbon dioxide (CO_2). The use of CO_2 gas as extraction fluid in SFE has been shown to be effective (Cescut *et al.* 2011). CO_2 has low critical values (31.1°C and 7.4 MPa), is nontoxic and does not give rise to environmental and health risks (Hanif *et al.* 2010). Hanif *et al.* (2012) used supercritical CO_2 extraction to simultaneously extract microbial respiratory quinones, PLFAs and phospholipid ether lipids from anaerobically digested sludge. For improved SFE polarity and solvent strength, a modifier such as 10% MeOH was added to the supercritical solvent, as the polarity of CO_2 alone is not high enough for PLFA separation from sludge. It was evident that optimisation of the SFE conditions was needed for a specific matrix (Hanif *et al.* 2012). SFE has also been applied on activated sludge (Hanif *et al.* 2010), bacterial cultures (Gharaibeh and Voorhees 1996) and archaeal cultures (Hedrick *et al.* 1991).

The selection of extraction techniques discussed in this section, produce a whole-lipid fraction from the sample matrix. Assessment of the microbial community structure based on lipid composition requires the specific classification of the lipids into separate groups.

3 Lipid fractionation procedures

The whole microbial lipid fractions obtained from environmental samples, consist of an assortment of lipid classes. These classes are categorised, according to polarity, into neutral, glyco- and polar lipids (Kates 1986; Hammond 1993). The polarity influences the fractionation efficiency in certain organic solvents. Various studies have found that the fatty acid composition of microbial neutral lipids is an indication of energy consumption, whereas the polar lipids provide information on the membrane characteristics (Wältermann *et al.* 2005; Athenstaedt and Daum 2006). The most frequently used techniques for microbial lipid fractionation (Table 2.2) are based on liquid chromatography. Compounds are separated according to their polarity into a liquid mobile phase or a stationary phase (Ettre 1993). If an analyte has a higher affinity for the mobile phase, it will be eluted faster, in comparison to one with a higher affinity for the specific stationary phase (McNair and Miller 2009). Liquid chromatography can be conducted in columnar mode, with packed columns or prepacked solid-phase extraction (SPE) cartridges, or in planar mode, with thin-layer chromatography (TLC) plates.

Table 2.2: Methods for microbial lipid fractionation from environmental sample matrices

Methods	Sample matrices	Solvent systems	Advantages	Limitations	References
Packed columns	Activated sludge; estuarine sediments	Neutral lipids – chloroform Glycolipids – acetone Polar lipids – methanol	• Inexpensive	• Time-consuming • Require sterilisation before use • Not suited for large number of samples	Dobbs and Findlay 1993; Guckert *et al.* 1985; Pernet *et al.* 2006; Russell and Werne 2007; White 1988
SPE cartridges	Cyanobacteria; soil	Neutral lipids – chloroform:2-propanol (2:1; *v/v*) Glycolipids – acetone Polar lipids – methanol Cholesteryl esters – hexane Triglycerides – 1% diethyl ether, 10% methylene chloride in hexane Cholesterol – 5% ethyl acetate in hexane Diglycerides – 15% ethyl acetate in hexane Monoglycerides – chloroform:methanol (2:1; *v/v*)	• Simple and rapid • Uses less solvent • Increased sample analysis in shorter time • Packing weight of sorbent is controlled, to ensure reproducibility • Homogeneous bedding • Sterile packaging	• HDPE columns can contaminate fatty acids • Cross contamination of polar lipids can occur	Dobbs and Findlay 1993; Guckert *et al.* 1985; Hanif *et al.* 2010; Kaluzny *et al.* 1985; Kates 1986; Pernet *et al.* 2006; Pinkart *et al.* 1998; Poerschmann and Carlson 2006; Ruiz-Gutiérrez and Pérez-Camino 2000; Russell and Werne 2007; Rychlik *et al.* 2006; White *et al.* 1979
TLC	Cyanobacteria; estuarine sediments; yeast	Mobile phase Hexane:diethyl ether (1:1; *v/v*) Hexane:diethyl ether:acetic acid (88:15:1; *v/v/v*) Hexane:diethyl ether:acetic acid (70:30:1; *v/v/v*) Hexane:MTBE:acetic acid (70:30:0.2; *v/v/v*)	• Expensive instrumentation not required • Not affected by impurities • No contamination, new stationary phase used each time	• Specific dyes required • Additional detection needed • High exposure to light & air leading to hydrolysis of lipids • Time-consuming • Requires large volumes of solvents	Cescut *et al.* 2011; Fuchs 2012; Guckert *et al.* 1985

Columns can be packed manually with silicic acid and a cotton wool frit (White 1988); however, this process is tedious, not suitable for a large number of samples and requires sterilisation before use. After the whole-lipid sample is dissolved in an appropriate solvent, it is loaded onto the column and the different lipid groups are sequentially eluted from the column with specific solvents (Dobbs and Findlay 1993; Pernet *et al.* 2006; Russell and Werne 2007).

Commercially prepacked disposable SPE cartridges may provide advantages over manually packed columns such as saving on packing time, controlled packing weight of the sorbents, possible higher reproducibility; and lower solvent volumes used with smaller columns (Ruiz-Gutiérrez and Pérez-Camino 2000; Rychlik *et al.* 2006). However, the use of high density polyethylene barrels (columns) used for packing the aminopropyl-bonded silica (NH_2) SPE columns, has been reported to contaminate polar lipid fractions (Russell and Werne 2007). NH_2 SPE columns provide the best results for fractionation of both neutral and polar lipids from standard lipid mixtures (Kaluzny *et al.* 1985; Russell and Werne 2007). Pinkart *et al.* (1998) separated microbial lipids with NH_2 SPE columns by modifying the original method applied on bovine adipose tissue (Kaluzny *et al.* 1985). Solvent mixtures used for the elution of specific lipid classes included: $CHCl_3$ for neutral lipids, acetone for polyhydroxyalkanoate, MeOH:$CHCl_3$ (6:1, *v/v*) and 0.05 mol L^{-1} sodium acetate in MeOH:$CHCl_3$ (6:1, *v/v*) for polar lipids. The neutral lipid fraction was further separated by elution with hexane into steryl esters, hexane:methylene chloride:$CHCl_3$ (88:10:2, *v/v*) into triacylglycerols, hexane: ethyl acetate (5:95, *v/v*) into sterols, hexane: ethyl acetate (15:85, *v/v*) into diacylglycerols and $CHCl_3$: MeOH (2:1, *v/v*) into monoacylglycerols.

During TLC, the lower end of a TLC plate is placed in an appropriate organic solvent (mobile phase) and capillary rise of the mobile phase (with target analytes) through the fixed stationary phase takes place. The result is the separation of the target analytes based on their chromatographic mobility. After separation, the plate is sprayed with a dye to visualise the lipid bands. The use of TLC to fractionate and purify microbial lipids has been applied to estuarine sediments (Guckert *et al.* 1985), cyanobacteria (Sampels and Pickova 2011) and yeast (Cescut *et al.* 2011). Across a diverse range of sample matrices, three distinct advantages are consistent, namely that TLC does not require expensive instrumentation; it is not impaired by impurities and has a low contamination rate as a new stationary phase is used with each analysis (Hahn-Deinstrop 2006). Nonetheless, this method is time-consuming; requires large volumes of solvents, specific dyes and additional detection techniques; and the increased exposure to light and air leads to the oxidation of lipids on the plate (Hahn-Deinstrop 2006; Fuchs 2012).

All the methods reported above produce fractionated lipid classes. Each of these groups or classes should be analysed for characterisation purposes. For the analysis by gas chromatography (GC), these compounds of interest should be volatile.

4 Lipid derivatisation procedures

The main objectives of derivatisation are to increase the volatility of analytes for better separation, to improve thermal stability and to decrease the interaction of the sample with the GC column (Halket 1993; Poole 1997). Various types of derivatisation reactions exist namely silylation; acylation and methylation. In the majority of studies utilising derivatisation reagents for microbial lipid analysis, silylating and methylating reagents are preferred (Table 2.3). Examples include N-methyl-trimethylsilyltrifluoroacetamide (Basconcillo and McCarry 2008) or N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Coucheney *et al.* 2008). Silylation substitutes an active hydrogen atom with a trimethylsilyl group (Halket and Zaikin 2003). Despite their high sensitivity to moisture, these reagents are highly volatile and react fast and completely. The addition of trimethylchlorosilane increases the reactivity of BSTFA in forming volatile by-products that do not interfere with other peaks. Derivatisation reagents convert all possible compounds in a sample, generating an assortment of target and unwanted analytes. For this reason, microbial lipid research is usually based on the profiling of fatty acid methyl esters (FAMEs) only. Another derivatisation agent, trimethylsulfonium hydroxide (TMSH), was found to deprotonate fatty acids with the formation of trimethylsulfonium (TMS) salts. After thermal decomposition, FAMEs and dimethyl sulphide ($[CH_3]_2S$) are produced (Yamauchi *et al.* 1979). The by-products, H_2O and $[CH_3]_2S$, do not cause any chromatographic interference, as they elute as part of the solvent peak during the delay period. According to Gómez-Brandón and co-workers, a combination of a modified Folch method with TMSH as derivatisation agent was the most appropriate option for total microbial fatty acid estimation from environmental samples (soil, animal manure, compost and vermicompost) (Gómez-Brandón *et al.* 2008). This finding is consistent with other microbial studies conducting PLFA analysis on solid organic environmental samples (Gómez-Brandón *et al.* 2010; Lores *et al.* 2010).

The conversion of fatty acids into their methyl ester derivatives is known as methylation, methanolysis or transesterification (Chowdhury and Dick 2012). A mixture of excess MeOH and a catalyst is added to convert the microbial lipids into their respective less polar and more volatile, methyl ester derivatives (FAMEs) (Meier *et al.* 2006). Two types of methylation reactions can be used, namely base-catalysed or acid-catalysed methanolysis. Base-catalysed reactions are performed with alkaline catalysts that transesterify lipids at a faster rate and at lower temperatures, than acidic catalysts. These catalysts should be freshly prepared as they are hygroscopic and function only under anhydrous conditions (Meier *et al.* 2006).

Table 2.3: Methods for microbial lipid derivatisation from environmental sample matrices

Methods	Sample matrices	Solvent systems	Advantages	Limitations	References
Base catalysts	Bacterial cultures; cattle manure; compost; estuarine sediments; pig slurry; soil; vermicompost	0.2 M methanolic KOH 0.01 M methanolic NaOH 0.2 M KOH + toluene:methanol (1:1; *v/v*)	• Reacts faster than acid-catalysed reactions • Mild temperature conditions	• Prepare fresh as KOH is hygroscopic	Basconcillo and McCarry 2008; Chowdhury and Dick 2012; Gómez-Brandón *et al.* 2008; Guckert *et al.* 1985; Kumari *et al.* 2011; White *et al.* 1979
Acid catalysts	Activated sludge; cattle manure; compost; pig slurry; soil; vermicompost; yeast	5% methanolic HCl 20% methanolic BF_3 3% methanolic H_2SO_4	• Esterification of all lipid classes	• Cannot detect PLFAs with methyl groups • Require heating • Artefact formation • BF_3 potentially harmful • Restricted by drainage laws • Limited shelf life • Unstable, decrease during storage • Slow reaction rate • Neutralisation of sample required before injection into capillary column, as stationary phase degradation can occur	Carrapiso and Garcia 2000; Cescut *et al.* 2011; Chowdhury and Dick 2012; Christie 2003; Gómez-Brandón *et al.* 2008; Kishimoto and Radin 1959; Kumari *et al.* 2011; Lepage and Roy 1986; Meier *et al.* 2006; Müller *et al.* 1998
Other reagents	Animal manure; bacterial cultures; compost; mud volcanoes; soil; vermicompost; yeast	TMSH + methanol + MTBE MSTFA + pyridine BSTFA + TMCS	• Simple • Not time-consuming • Performed at room temperature • By-products elute with solvent peak • MSTFA is the most volatile TMS-amide available • BSTFA acts fast and completely • TMCS increases reactivity of BSTFA	• Sensitive to moisture • TMS derivatives should be analysed on silicon stationary phase, as they are sensitive to H_2 atoms	Basconcillo and McCarry 2008; Coucheney *et al.* 2008; Gómez-Brandón *et al.* 2008, 2010
Direct methylation	Algae; bacterial cultures; organic samples; soil	0.25 M TMSH 2.5 M HCl in methanol 2.2 M TMAH 75% 2.5 M methanolic HCl + 25% toluene Acetyl chloride:methanol (5:100 or 1:19; *v/v*)	• Rapid extraction time • Reduced solvent volumes • Sufficiently extract polar lipids • One-step reaction at room temperature • By-products do not cause interference • Small sample sizes • No pre-extraction required • TMSH reduces isomerisation / degradation	• TMAH causes degradation of polyunsaturated fatty acids, resulting in low detection • TMSH and TMAH have strong alkalinity • H_2O hampers reaction • Do not provide extensive studies on lipid classes, as extraction and methylation is done in single step	Akoto *et al.* 2008; Blokker *et al.* 2002; Estévez and Helleur 2005; Gómez-Brandón *et al.* 2010; Kumari *et al.* 2011; Lepage and Roy 1984, 1986; Meier *et al.* 2006; Nakanishi *et al.* 2003; Sekino *et al.* 1997

Examples of alkaline catalysts include methanolic potassium hydroxide (KOH) and methanolic sodium methoxide (CH_3NaO). Acid-catalysed methylation esterifies all lipid classes. However, considerable limitations are associated with the use of acidic catalysts such as methanolic hydrochloric acid (HCl), methanolic sulphuric acid (H_2SO_4) and boron trifluoride (BF_3) in MeOH (Ackman 1998; Christie 2003). Acidic catalysts require longer reaction time and heating; they have a limited shelf life; waste disposal is costly as these catalysts are potentially harmful; and neutralisation of the sample is required to prevent capillary column degradation (Kishimoto and Radin 1959; Carrapiso and Garcia 2000; Christie 2003). Chowdhury and Dick (2012) assessed the effect of different methylation methods on the microbial PLFA profiles of soil samples. It was found that acid-catalysed methylation with methanolic HCl, resulted in higher methylation efficiency and lower analytical variability than base-catalysed methylation with methanolic KOH. On the other hand, PLFAs with methyl groups were not detected when methanolic HCl was used for methylation. Therefore, it was recommended that the methanolic KOH-catalysed methylation method is used for microbial PLFA profiling in environmental samples. When considering various processes for the formation of FAMEs from pure bacterial cultures, a combination of methanolic KOH and methanolic HCl provided the most detailed fatty acid profiles (Basconcillo and McCarry 2008). This is in accordance with results from another study using a combination of CH_3NaO (basic) and BF_3 (acidic) as methylation agent (Griffiths *et al.* 2010). Overall, there is consensus among authors that the concentration yield and composition of fatty acids are influenced by the methylation method used.

As lipid extraction is widely reported as the timeous and error-prone step in lipid analysis, development of a direct methylation procedure (without pretreatment steps required) was proposed by Lepage and Roy (1984, 1986). This method is based on a one-step reaction with a rapid reaction rate, using small sample sizes with reduced solvent volumes. The first methylation reagent used successfully for direct methylation was acetyl chloride dissolved in MeOH applied on biological tissues (Lepage and Roy 1984, 1986). Lipids in zooplankton have been characterised by means of thermally assisted hydrolysis and methylation, using tetramethylammonium hydroxide (TMAH) as reagent (Sekino *et al.* 1997; Ishida *et al.* 1998). However, it was found that the strong alkalinity of TMAH resulted in the degradation of the polyunsaturated fatty acids (PUFAs) through extensive isomerisation of double bonds. This suggests a low detection of PUFAs, as also reported in Nakanishi *et al.* (2003). An alternative, TMSH, provides a higher sensitivity for PUFA components with less degradation occurring (Jun-Kai *et al.* 1997; Blokker *et al.* 2002; Estévez and Helleur 2005). This may be ascribed to the decomposition of TMS salts at lower temperatures than derivatisation products containing hydroxides (TMAH) (Akoto *et al.* 2008). In addition, TMSH by-products do not interfere with chromatographic separation and have been shown to sufficiently extract polar lipids from soil, compost, animal manures, vermicompost (Gómez-Brandón *et al.* 2010), whole bacterial cells (Müller

et al. 1998), algae, cyanobacteria (Blokker *et al.* 2002; Akoto *et al.* 2008) and yeast samples (Cescut *et al.* 2011).

The fatty acid composition of whole lipids or specific lipid classes, obtained by the previously described methods, is generally quantified by gas chromatography with flame ionisation detection (GC-FID) and/or gas chromatography-mass spectrometry (GC-MS).

5 Lipid quantification procedures

Since the 1980s, GC has been the principal analytical technique for lipid analysis and quantification (Bobbie and White 1980). It serves as a fast and sensitive method requiring small sample sizes to provide accurate, high resolution quantitative data (Ettre 1993). Gas chromatography is restricted to volatile samples that are not thermally labile and makes use of a gaseous mobile phase and a liquid stationary phase. Various types of detectors can be coupled to a gas chromatograph, but the common one in lipid research is FID (Table 2.4). It uses an oxygen-hydrogen flame to ionise the analytes. These ions form a current producing a signal. It is responsive to all organic compounds capable of burning in the flame (McNair and Miller 2009). Since GC-FID is adaptable to all column sizes; is highly sensitive with excellent linearity and reliable peak recognition, it is widely applied in microbial lipid studies (Guckert *et al.* 1985; Coucheney *et al.* 2008). The choice of capillary column has a tremendous impact on the separation efficiency of the target analytes, as the sample partitions between the mobile phase and stationary phase are based on the level of polarity (solubility). Microbial lipids from environmental samples have been separated with various nonpolar columns including 5% Phenyl-methylpolysiloxane or 100% Dimethylpolysiloxane (White *et al.* 2009; Yang *et al.* 2011; Yao *et al.* 2012). On the other hand, polar columns with 50% Cyanopropyl-methylpolysiloxane or 88% Cyanopropyl-aryl-polysiloxane, have also been used for microbial lipid analysis (Gómez-Brandón *et al.* 2008, 2010; Lores *et al.* 2010). Härtig (2008) combined retention time and mass spectra from capillary columns with different polarities to identify fatty acids. 5% Phenyl-methylpolysiloxane was used as the nonpolar column, 50% Cyanopropyl-methylpolysiloxane as the mid-polar and 88% Cyanopropyl-aryl-polysiloxane as the polar column. If the stationary phase polarity is changed, the FAME elution order may change. On polar columns the saturated FAMEs elute before unsaturated FAMEs and the *trans* configurations before the *cis*. This elution order is reversed on nonpolar columns. The polyunsaturated ω6 FAME always elute before its ω3 analogue and *iso*-branched FAMEs before *anteiso*-branched ones, no matter which column is used. Retention time may be unreliable for identification, as it may change as a response to minor chromatographic condition modifications which include temperature, pressure fluctuations, varied inlets and detectors, types of injections and column changes (Härtig 2008).

Table 2.4: Methods available for microbial lipid quantification from environmental sample matrices

Methods	Sample matrices	Column phases	Advantages	Limitations	References
GC-FID	Bacterial cultures; estuarine sediments; soil	Polyethylene glycol 100% Dimethylpolysiloxane 5% Phenyl-methylpolysiloxane 50% Cyanopropyl-methylpolysiloxane 88% Cyanopropyl-aryl-polysiloxane	• Reliable peak recognition • High sensitivity • Adaptable to all column sizes • Can function at very high temperatures • Excellent linearity	• Derivatisation of analytes required • Does not provide structural information • Identifies solely on retention time • Nonspecific detector	Akoto *et al.* 2008; Bobbie and White 1980; Cescut *et al.* 2011; Coucheney *et al.* 2008; Guckert *et al.* 1985; Härtig 2008; Meier *et al.* 2006; Pernet *et al.* 2006; Sheng *et al.* 2011; White *et al.* 2009
GC-MS	Activated sludge; air; bacterial cultures; estuarine sediments; soil; water	5% Phenyl-methylpolysiloxane 50% Cyanopropyl-methylpolysiloxane 88% Cyanopropyl-aryl-polysiloxane 100% Dimethylpolysiloxane 50% Phenyl-methylpolysiloxane	• Distinguish between isomeric and isobaric lipids • Matrix effect of sample reduced • Identification / confirmation of compounds • Structural information provided	• No specific limitations	Basconcillo and McCarry 2008; Coucheney *et al.* 2008; Fang and Findlay 1996; Gómez-Brandón *et al.* 2008, 2010; Guckert *et al.* 1985; Härtig 2008; Macnaughton *et al.* 1997
GC x GC MS	Algae; cyanobacteria	First dimension column HP-5 5% Phenyl-methylpolysiloxane Second dimension column BPX50 50% Phenyl-methylpolysiloxane	• Detectability of low abundance FAMEs • Enhanced resolution • Provide target analyte dimensions • Elucidate clusters of co-eluting peaks • Signal-to-noise ratio improved	• No specific limitations	Akoto *et al.* 2008; De Geus *et al.* 2001
HPLC	Bacterial cultures; dolomite concretions; soil; well-head fluid; yeast	**Solvent systems** A – hexane B – hexane/isopropanol (9/1;v/v) A – acetonitrile B – water + trifluoroacetic acid C – hexane/isopropanol (4/5, v/v)	• Identification / quantification of lipids in low concentrations • Lipid class separation • Constant solvent flow rate • Derivatisation is not required	• Time-consuming • High solvent volumes required • Expensive • Cross-contamination may occur • Clogging of HPLC columns can occur	Ayari *et al.* 2013; Bühring *et al.* 2012; Cescut *et al.* 2011; Kellogg *et al.* 2001; Oldenburg *et al.* 2009; Peterse *et al.* 2009

As a GC-FID identifies analytes solely on retention time and cannot provide structural information, the additional confirmation of compound identity is needed.

During mass spectrometric analysis, compounds undergo ionisation to form molecular ions which are fragmented in the mass spectrometer (De Hoffmann and Stroobant 2007). During GC-MS, the lipids are separated with GC and transferred to the mass spectrometer for quantification (Sommer *et al.* 2006). The structural information of the target analytes that is available from this analysis, makes GC-MS suitable for confirmation purposes (Macnaughton *et al.* 1997; Basconcillo and McCarry 2008; Coucheney *et al.* 2008). To enhance the resolution of GC, a second capillary column can be coupled to form multidimensional GC or two-dimensional GC (GC x GC) (Zakaria *et al.* 1983). As a result, only a small portion of analytes eluted from the first column are selected for separation on the second column. It has several advantages, namely the enhancement of chromatographic resolution, additional second dimension target analyte information given and the improvement of signal-to-noise ratios (De Geus *et al.* 2001). The coupling of GC x GC to mass spectrometers has been shown to improve detectability. In a study conducted on green algae and cyanobacteria to explore the use of GC x GC-MS for FAME identification, the detectability of low abundance FAMEs was enhanced (Akoto *et al.* 2008).

Even though GC is the most suitable method for lipid analysis, high-performance liquid chromatography (HPLC) plays a role in applications for unusual samples, heat sensitive functional groups and also for preparative or semi-preparative applications. HPLC has been applied to collect fractions for analysis by other techniques such as MS or nuclear magnetic resonance spectroscopy (Arsenault and McDonald 2007) and to quantify the alpha-branched, beta-hydroxy fatty acids and mycolic acids found in *Mycobacterium* species (Butler *et al.* 1991). Environmental samples from which microbial lipids have been identified by means of HPLC include disturbed soil (Peterse *et al.* 2009; Ayari *et al.* 2013), biofilms and sediments under acidic conditions (Bühring *et al.* 2012), yeast (Cescut *et al.* 2011) and biodegraded oil reservoirs (Oldenburg *et al.* 2009).

6 The standing of lipid analysis in current environmental research

The analysis of signature lipid biomarkers depicts the actual microbial community status within an immediate functioning ecosystem without the bias associated with cultivation (Hill *et al.* 2000). It is also a rapid and inexpensive technique to determine biomass and community structure (Frostegård *et al*. 2011). This is the major reason PLFA analysis became so popular during the time before molecular methods. In recent years, methods based on nucleic acid extraction and analyses have often been preferred to PLFA analysis for studying microbial communities in environmental samples. However, PLFA still holds several advantages above other methods and may even be more sensitive in

detecting shifts in the microbial community structure when compared to nucleic acid based methods (Ramsey *et al*. 2006).

Molecular methods based on deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) extraction and quantification are sensitive, possess a high genetic specificity (Šnajdr *et al*. 2011) and can identify individual microbial biomarkers at species level (Wallander *et al.* 2013). Despite these strong points, polymerase chain reaction (PCR)-based methods are not capable of accurately distinguishing among various treatment effects, due to limited statistical data available from PCR patterns (Ramsey *et al.* 2006). Furthermore, DNA has a longer persistence after cell-death than phospholipids and therefore indicates nonviable biomass. The development of specific primers and probes for the PCR amplification process requires knowledge of the particular target sequence beforehand (Feinstein *et al*. 2009).

Phospholipids are essential constituents of microbial cell membranes which are rapidly degraded upon cell-death and are not found in storage bodies (White *et al*. 1979). Therefore, analysis of PLFAs is an accurate measure of the viable biomass of a microbial community (White *et al.* 1979). Specific PLFAs have been assigned as signature biomarkers of certain microbial groups (Frostegård and Bååth 1996). These include terminally branched saturated fatty acids (Gram-positive bacteria), monounsaturated fatty acids (Gram-negative bacteria), mid-branched saturated fatty acids (actinomycetes), and PUFAs (fungi). For characterisation of Archaea, phospholipid etherlipids are used, since the standard PLFA protocols do not hydrolyse archaeal ether lipids (Gattinger *et al*. 2003; Elhottová *et al*. 2012). Other chemical markers present in microbial cell membranes, such as chitin and ergosterol can also be used as accurate measures of microbial biomass and structure (Baldrian *et al.* 2013). Chitin analysis has a restricted ability to distinguish between different fungal groups or between living and dead fungal biomass, whereas ergosterol analysis can make these distinctions (Wallander *et al.* 2013). However, both these markers are solely for fungal estimations, whereas PLFA analysis simultaneously characterises bacteria and fungi (Frostegård *et al.* 2011). The presence of PLFAs in the microbial cell membrane means that the PLFA content can vary in response to both intracellular and extracellular environmental changes. This is another characteristic that makes PLFA analysis useful as it enables the determination of physiological changes related to function. Thus, PLFA analysis provides information on the phenotype and activity level of a microbial community, rather than on the genotype and relative species level obtained from molecular methods (Ramsey *et al.* 2006; Frostegård *et al.* 2011). Baldrian *et al.* (2013) reported PLFA analysis to be more closely related to soil processes and the physiological abilities of soil microbial communities, than the number of internal transcribed spacer copies quantified with quantitative PCR.

Soil microbial communities are notoriously difficult to study and literature often refers to the soil microbial 'black box' (Tiedje *et al*. 1999; Shade *et al*. 2009; Yao *et al*. 2014). The combination of lipid analysis with other techniques holds great promise to increase our understanding of soil microbiology. By combining PLFA analysis with isotopic labelling, the identity of metabolically active microbial groups can be linked with specific biogeochemical cycling processes (Boschker and Middelburg 2002). In plant-soil ecosystems, $^{13}CO_2$-labelling provides insight into the microbial communities responsible for the active assimilation of plant-derived carbon (Yao *et al.* 2014). PLFA-stable isotope probing (SIP) can provide substantial information on the microbial utilisation of soil organic matter mineralisation products (Garcia-Pausas and Paterson 2011; Paterson *et al.* 2011; Dungait *et al.* 2013); differentiate between the microbial groups responsible for contaminant biodegradation (Jakobs-Schönwandt *et al.* 2010; Mahmoudi *et al.* 2013) and determine the seasonal variations occurring in soil microbial communities (Högberg *et al.* 2010; Andresen *et al.* 2014). The disadvantage is that ^{13}C PLFA analysis cannot provide information on species composition or phylogenetic resolution. However, when used with DNA/RNA-SIP methods, the two approaches can complement each other to give insight into both functional and structural aspects of soil microbial communities.

7 Conclusions

Microbial lipid research has been an important focus area since the 1970s, with the continuous use of original and modified reference methods in environmental studies. A number of improvements on these traditional methods have been made to broaden the application possibilities and enhance characterisation efficiency. Regarding extraction techniques, the exclusion of the washing step in the Folch method, shortens the extraction time without influencing the lipid yield. The substitution of the H_2O fraction with a buffer, in the Bligh and Dyer process, enables the wide application of the method on environmental samples. The solvent MTBE in a solvent mixture can be used as an alternative for $CHCl_3$. New techniques such as FMASE, ASE and SFE lead the way to higher throughput analyses with lower waste solvent generation and faster extraction. The development of commercially available SPE cartridges for lipid fractionation has relegated TLC as a lengthy, non-specific technique with excessive hazardous solvent consumption. Advances with the derivatisation reagent, TMSH, provide the ability to directly methylate a sample, without the need for pre-extraction. Identification and quantification techniques, such as GC-FID and GC-MS, are predominantly used for microbial lipid profiling from environmental samples, as high sensitivity and reliable peak recognition are key features. More recent techniques, including GC x GC-MS focus on enhanced resolution. Overall, the described methods share common objectives: to analyse more samples with a higher accuracy, in a shorter period of time, with reduced solvent volumes required. Furthermore, the utilisation of conventional methods for the extraction, fractionation, methylation and quantification of microbial

lipids is not inferior to newly developed techniques. On the contrary, conventional techniques are nowadays applied as reference methods against which modified versions or innovations are tested. Additionally, numerous current studies still prefer traditional protocols for microbial lipid profiling, which may be ascribed to the preceding reputation of these methods; as well as the expenses concerning new apparatuses. The significance of uncultivable microorganisms in the upholding of the ecosystem has received considerable recognition over the years. Microbial lipid research is known as a culture-independent technique to accurately characterise such microbial communities from environmental samples. Given that microbial diversity in environmental studies is complex, no single experiment can successfully analyse the vast number of lipid profiles. Therefore, future advances and improvements on conventional lipid research will continue to be an important aim for several researchers. Use of the techniques discussed here will provide a better understanding of the microbial ecology during stress conditions; will indicate early signs of pollution and degradation of an environment by means of microbial biomarkers, will lead to improved disease diagnosis and control and lastly, provide a profile of soil quality for sustainable agriculture and increased food production.

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Chapter 3

Interpretations and recent applications of phospholipid fatty acid profiling of microbial communities

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Summary

Profiling of microbial communities in environmental samples often utilises phospholipid fatty acid (PLFA) analysis. This method has been used for more than 35 years and is still popular as a means to characterise microbial communities in a diverse range of environmental matrices. This review examines the various recent applications of PLFA analysis in environmental studies with specific reference to the interpretation of the PLFA results. It is evident that interpretations of PLFA results do not always correlate between different investigations. These discrepancies in interpretation and their subsequent applications to environmental studies are discussed. However, in spite of limitations to the manner in which PLFA data are applied, the approach remains one with great potential for improving our understanding of the relationship between microbial populations and the environment. This review highlights the caveats and provides suggestions towards the practicable application of PLFA data interpretation.

Keywords

fungal to bacterial ratio, microbial activity, microbial biomass, microbial community structure, phospholipid fatty acid, physiological stress ratio

1 Introduction

Microbial ecologists have long been interested in the relationship between microbial communities and their environments. The development of culture-independent techniques, such as phospholipid fatty acid (PLFA) analysis lead to major changes in the manner in which microbial communities were studied. David C. White and colleagues, at the Florida State University, Florida, USA and later at the University of

Tennessee, Knoxville, USA, initiated the use of phospholipid biomarkers for microbial community characterisation. The first report of the use of PLFA biomarker analysis dates back to 1979 when it was used to estimate microbial biomass from marine and estuarine sediments (White *et al*. 1979b). This approach was a modification of an original procedure to extract fish tissue lipids (Bligh and Dyer 1959) and pioneered the use of microbial lipids as biomarkers for microbial community structure and metabolic activity in subsequent environmental studies (Bobbie and White 1980; Guckert *et al*. 1985; Findlay and White 1987; Findlay *et al*. 1990; Tunlid and White 1992; Zelles *et al*. 1994; Frostegård and Bååth 1996). These early studies were associated with the need to find an appropriate indicator of biomass (Frostegård *et al*. 2011), as defined by Jenkinson and Ladd (1981): (i) the measured component should be present in all portions of the environmental biomass in known concentrations; (ii) the component should only be present in viable organisms; (iii) it should be quantitatively extractable from the environment and (iv) an accurate technique should be available for estimating the component concentration in the environmental samples. Phospholipid fatty acid analysis proved to be highly successful as such an indicator and has been popular in environmental studies for determining microbial community structure ever since. This is evident by the large number of recent publications (since 2011) applying this approach. However, there are numerous limitations associated with the method that relate to specific extraction, fractionation and analysis methods and have been reviewed recently (Watzinger 2015; Willers *et al*. 2015). In addition to these limitations, there are also certain caveats associated with the interpretation of PLFA data and the application thereof in environmental studies. This review examines the various applications of PLFA analysis in such studies. Attention is given to the interpretation of PLFA results and how this is applied in recent research compared to when it was first proposed. It is evident that interpretations of PLFA results do not always correlate between different investigations. The continued relevance of applying this approach to detect changes in microbial communities in environmental samples is also discussed.

2 How PLFAs are used

Analysis of PLFAs provides a quantitative description of the microbial community in the particular environment sampled at a given time. A total or representative extraction of fatty acids from environmental samples is performed with organic solvents. The microbial lipid extract is then fractionated into neutral, glyco- and phospholipids and the latter fraction is subjected to mild alkaline methanolysis to produce fatty acid methyl esters (FAMEs). The FAMEs are quantitatively analysed by gas chromatography-mass spectrometry (Guckert *et al*. 1985; White and Ringelberg 1998). A sample profile represents the abundance of each of the extracted PLFAs and is based on the variability in fatty acids present in the cell membranes of different microorganisms. The composition of these PLFA profiles is

determined by fatty acids of varying chain length, saturation and branching and can therefore be used as 'fingerprints' of the microbial community (Steer and Harris 2000; Leckie 2005). In addition, PLFA profiles are affected by the metabolic state of the organism, environmental factors and exposure to toxic substances (Guckert *et al*. 1986; Kieft *et al*. 1994; Mahmoudi *et al*. 2013; Reinsch *et al*. 2014). Subsequently, PLFA analysis can be applied for different reasons: to determine microbial biomass; as biomarkers of community structure; and to give insight into the functional status of the microbial community. Each of these applications requires the interpretation of the data obtained from the analysis and it is this interpretation that is frequently disputed (Watzinger 2015) and can lead to variable conclusions in similar studies.

3 Applications and interpretations of PLFA data

3.1 Microbial biomass

Originally, extractable microbial lipid phosphate was measured colorimetrically after a modified Bligh and Dyer (1959) solvent extraction, as an indicator of microbial biomass in marine and estuarine sediments (White *et al*. 1979b). However, with a moderate sensitivity of approx. 10^{-9} mole phosphate and detection of roughly 10^{8} bacterial cells the size of *Escherichia coli*, it was not adequate for complex environmental matrices (White 1983). A few years later, Gehron and White (1983) established the use of glycerol phosphate as an accurate measure of phospholipid concentration and microbial biomass. This complicated approach involved the acid hydrolysis of phosphate from lipid glycerol and the analysis of labile glycerol by gas chromatography (GC). With an increased sensitivity of about 10^{-11} mole glycerol and detection of approx. 10^{12} bacterial cells equivalent to the size of *E. coli*, this technique proved to be more efficient than extractable lipid phosphate for microbial biomass estimation but was never widely applied.

The use of PLFAs as a measure of viable microbial biomass was widely accepted based on studies that reported the rapid degradation of PLFAs after cell-death (White *et al*. 1979b; Zelles *et al*. 1992; Janzen *et al*. 1994; Drenovsky *et al*. 2004). An approach introduced by Frostegård *et al*. (1991) used the total amount of PLFAs detected in a sample to determine the total viable microbial biomass. The results are measured as the quantity of PLFAs (expressed in pmol or nmol per gram soil) and correlated with other measures of microbial biomass such as chloroform-fumigation extraction (Bailey *et al*. 2002a; Leckie *et al*. 2004), substrate-induced respiration (Johansen and Olsson 2005) and respiratory quinone concentrations (Kunihiro *et al*. 2014). However, Frostegård *et al*. (2011) advised that environmental conditions determine the turnover rate of PLFAs and therefore the degradation of dead bacteria and their PLFAs may proceed at different rates under certain conditions. Nevertheless, this approach remains

widely applied for the estimation of microbial biomass in current environmental studies (Cycoń *et al*. 2013; Fichtner *et al*. 2014; Lange *et al*. 2014; Sasaki *et al*. 2014; García-Orenes *et al*. 2015).

Other authors proposed the use of conversion factors to interpret biomass as cell abundance (cells $pmol^{-1}$ PLFA). Problematically, there are a range of different conversion factors available without consensus among authors as to which is more appropriate. The majority of these conversion factors are based on the amount of PLFAs g^{-1} (dry weight) of bacteria, which was estimated by White *et al*. (1979a) as 100 μmol lipid phosphate g^{-1} dry weight. Conversion factors derived from this estimation include 5.9×10^4 cells $pmol^{-1}$ PLFA (Kieft *et al*. 1994) and 2.0×10^4 cells $pmol^{-1}$ PLFA (Balkwill *et al*. 1988; Scow and Green 2000). Franzmann *et al*. (1996) expressed microbial biomass as 2.4×10^4 stationary phase *E. coli* equivalent cells $pmol^{-1}$ PLFA. All these studies focused on pure culture enumerations, whereas the use of a complex microbial sample would be more representative of environmental matrices. In addition, the biomass conversion factors obtained from complex samples may differ from pure cultures given that a tremendous amount of microbial cells of different shapes and sizes exist in complex samples. For example, Findlay *et al*. (1989) generated a conversion factor of 3.4×10^4 cells $pmol^{-1}$ PLFA from a mixed microbial sample naturally occurring in the environment. Discrepancies such as these explain the limited number of recent investigations portraying microbial biomass as cell abundance. A few recent studies all apply the conversion factors based on the study of White *et al*. (1979a) (Ringelberg *et al*. 2008; Carr *et al*. 2013; Mahmoudi *et al*. 2013).

Another way of expressing microbial biomass is as microbial biomass carbon (C) (Jenkinson and Powlson 1976; Bailey *et al*. 2002a). The theory behind this interpretation is that the rate of carbon dioxide (CO_2) production during microbial respiration is comparable to the biomass of the microorganisms (Jenkinson and Powlson 1976). Both the chloroform-fumigation extraction and substrate-induced respiration methods are commonly used to measure microbial biomass C. During the chloroform-fumigation extraction process, soil samples are fumigated with chloroform and extracted with potassium sulphate (K_2SO_4), followed by quantification of the extractable C (Vance *et al*. 1987). By dividing the difference between the C extracted from the fumigated and nonfumigated soil with a conversion factor, the total microbial biomass C can be calculated (Joergensen and Mueller 1996). Substrate-induced respiration is based on the stimulated response of the microbial community to the addition of an easily degradable C substrate, such as glucose (Lin and Brookes 1999). The production rate of the respiratory CO_2, excreted during the stimulated metabolic reaction, is a measure of the metabolically active microbial biomass. The microbial biomass C is calculated with the regression equation (Anderson and Domsch 1978): μg biomass C g^{-1} soil = 40.04 x CO_2 (μl h^{-1} g^{-1} soil) + 0.37. The analysis of PLFAs differs from these methods by indicating

viable microbial biomass, rather than microbial biomass C. However, different conversion factors also exist to calculate a relationship between PLFA concentrations and specific microbial biomass C values. Such factors include (i) 1 nmol microbial PLFAs = 3.2 μg microbial biomass C (Leckie *et al.* 2004); (ii) 11.8 μmol 18:2ω6,9 PLFAs = 1 g microbial biomass C (Klamer and Bååth 2004); (iii) 38 nmol 16:1ω5 PLFA = 1 mg arbuscular mycorrhizal fungal biomass C (Olsson *et al.* 1995); and (iv) 1 nmol microbial PLFAs = 2.4 μg microbial biomass C (Bailey *et al.* 2002a). Only a few current studies have reported PLFA values as microbial biomass C. In two of these studies (Seifert *et al*. 2013; Williams *et al*. 2014), the common conversion factor used was 11.8 μmol 18:2ω6,9 PLFAs = 1 g microbial biomass C. Given the number of proposed conversion factors for microbial biomass estimation and the lack of consensus as to their application, it is proposed that microbial biomass be expressed only quantitatively as the total amount of extractable PLFAs (nmol or pmol) per weight of the sample (McKinley *et al*. 2005). Furthermore, care should be taken to explicitly refer to these estimations as 'viable' biomass due to the uncertainty associated with turnover rates.

Determination of microbial biomass by means of PLFA analysis in environmental studies provides insight into the distribution of microbial communities as a response to nutrient availability. This approach was applied by Zhao *et al*. (2014) to show changes in biofilm communities associated with vermifiltration. More often, the approach is found in studies on soils or sediments. Generally, microbial biomass increases with higher organic matter content in soil (Lagerlöf *et al*. 2014), marine sediments (Carr *et al*. 2013; Kunihiro *et al*. 2014) and aquatic reservoirs (Ertefai *et al*. 2008). This can be attributed to the stimulation of microbial growth by the available carbon sources. Moreover, the secretion of carbon-rich root exudates by above-ground plant species also favours the below-ground microbial biomass (Bertin *et al*. 2003; Carrasco *et al*. 2010; Yevdokimov *et al*. 2013). Land-use practices such as conventional farming with tillage practices and biocide applications (Bailey *et al*. 2002b; Helgason *et al*. 2010; Montecchia *et al*. 2011) and forest clear-cutting (Moore-Kucera and Dick 2008; Churchland *et al*. 2013), often have a suppressive influence on the viable microbial biomass. In these soils, the reduction in microbial biomass can be associated with decreased labile carbon supply, removal of vegetation cover or the physical disturbance of the soil aggregates.

3.2 Biomarkers

Although PLFA biomarkers have a low taxonomic resolution and microbial identification cannot be conducted on species-level, several microbial taxa can be identified using whole biomarker patterns instead of single biomarkers (Zelles 1999; Dijkman and Kromkamp 2006; De Carvalho and Caramujo

2014). Overall, the traditional biomarkers assigned to specific microbial groups are still used in recent interpretations (Table 3.1), but care should be taken in interpreting certain fatty acids as sole biomarkers of microbial groups. The interpretation of certain PLFAs as biomarkers for different structural and functional groups within a microbial community can be attributed to the presence of unique individual lipids or unique lipid distributions in a lipid profile (Guckert *et al*. 1985; Ringelberg *et al*. 1989; White *et al*. 1996). However, several problems arise for this approach. Given that the majority of biomarkers were obtained from pure culture studies, inconsistencies may be found in complex environmental samples. Furthermore, not all microbial species and their fatty acids are known (Watzinger 2015). Also, changes in fatty acid composition may not necessarily denote changes in community structure, but may be influenced by environmental conditions and cell activity (Leckie 2005; Wixon and Balser 2013). The same PLFA, considered a biomarker for a specific microbial group, has been found in different microbial groups (White *et al*. 1996; Olsson 1999; Ruess and Chamberlain 2010). Review and research articles often list the latter as one of the main reasons why the use of PLFA biomarkers are unreliable. Even so, Table 3.1 shows that of the 67 PLFA biomarkers commonly used, only eight have more than one designation. Of these different designations, several can be excluded when considered in the context of the relevant investigation. For example, the marker 16:1ω7c is designated to Gram-negative bacteria and to cyanobacteria and diatoms. Here, the origin of the samples should be considered for clarity; the designation for Gram-negative bacteria was originally applied to pure cultures and soil samples, whereas the designation to cyanobacteria and/or diatoms will be more suited if a water sample is analysed.

Generally, the PLFAs 3OH 12:0, i13:0, a13:0, 2OH 14:0, i14:0, i15:0, a15:0, 15:0, cy15:1, 2OH 16:0, i16:0, 16:0, 16:1ω7c, 16:1ω9, i17:0, a17:0, cy17:0, 17:0, 17:1ω6, i17:1ω7, 18:1ω7c, 18:0, cy19:0, 19:1ω9c and 20:0 are used to assess the bacterial fraction of microbial communities (Taylor and Parkes 1983; Nichols *et al*. 1985; Dowling *et al*. 1986; Ringelberg *et al*. 1989). However, the presence of 16:0 and 18:0 fatty acids have been found in plants (Millar *et al*. 2000; Welti *et al*. 2002). When the focus is mainly on bacteria, these fatty acids should either be completely omitted from the collection of PLFAs used or the successful homogeneity of the samples should be ensured by removing all possible plant residues (Mitchell *et al*. 2010; Zheng *et al*. 2013).

Table 3.1: Phospholipid fatty acids used as signature biomarkers

PLFA biomarkers	Designations	Sample origin	Original citations	Recent studies using traditional designations
STRAIGHT-CHAIN SATURATED FATTY ACIDS 14:0; 15:0; 16:0; 17:0; 18:0	General bacterial marker	Pure cultures; soil	Zelles (1997)	Balasooriya *et al*. (2014); Lange *et al*. (2014)
METHYL-BRANCHED FATTY ACIDS 10Me16:0; 10Me17:0; 10Me18:0	Actinomycetes (Actinobacteria)	Pure cultures; soil	Kroppenstedt (1985); Vestal and White (1989)	Högberg *et al*. (2013); Breulmann *et al*. (2014); Dong *et al*. (2014); Fichtner *et al*. (2014); Mechri *et al*. (2014); Reinsch *et al*. (2014)
MONOUNSATURATED FATTY ACIDS 14:1ω5c; 15:1; 15:1ω6c; 16:1ω7t; 16:1ω9c; 16:1ω11c; 17:1; 18:1ω5c; 19:1ω9c; 19:1ω12c; 20:1ω9c; 20:1ω9t; 22:1ω9c; 22:1ω9t	Gram-negative bacteria	Pit mud; pure cultures; soil	Wilkinson (1988); Zelles (1997)	Buckeridge *et al*. (2013); Djukic *et al*. (2013); Zheng *et al*. (2013); Banks *et al*. (2014); Lange *et al*. (2014); Zhang *et al*. (2014)
16:1ω5c	Arbuscular mycorrhizal fungi	Plant roots; soil	Pacovsky and Fuller (1988); Olsson *et al*. (1995)	Djukic *et al*. (2013); Tavi *et al*. (2013); Balasooriya *et al*. (2014); Banks *et al*. (2014); Mechri *et al*. (2014); Zhang *et al*. (2014)
16:1ω7c	Gram-negative bacteria	Pure cultures; soil	Wilkinson (1988)	Tavi *et al*. (2013); Reinsch *et al*. (2014)
	Cyanobacteria; diatoms	Freshwater microalgae; microbial mats; pond water	Ahlgren *et al*. (1992)	Dijkman *et al*. (2010); De Carvalho and Caramujo (2014)
16:1ω5t; 16:1ω8c	Type I methanotrophs (*Gammaproteobacteria*) *Methylococcaceae*	Pure cultures; soil	Nichols *et al*. (1985); Bowman *et al*. (1991)	Knoblauch *et al*. (2008); Bodelier *et al*. (2009)
17:1ω8; 17:1ω5	Sulfate-reducing bacteria	Microbial mats	Kaneda (1991)	Bühring *et al*. (2014)
18:1ω7c	Cyanobacteria; diatoms	Freshwater microalgae; microbial mats; pond water	Ahlgren *et al*. (1992)	Dijkman *et al*. (2010); De Carvalho and Caramujo (2014)
	Gram-negative bacteria	Pure cultures; soil	Wilkinson (1988)	Tavi *et al*. (2013); Mechri *et al*. (2014)
18:1ω7t	Gram-negative bacteria	Pure cultures; soil	Zelles (1997)	Mechri *et al*. (2014); García-Orenes *et al*. (2015)
18:1ω8c	Type II methanotrophs (*Alphaproteobacteria*) *Methylocystaceae* & *Beijerinckiaceae*	Pure cultures; soil	Nichols *et al*. (1985); Bowman *et al*. (1991)	Knoblauch *et al*. (2008); Bodelier *et al*. (2009)
HYDROXY-SUBSTITUTED FATTY ACIDS 2OH 12:0; 3OH 12:0; 2OH 14:0; 3OH 14:0; 2OH 16:0; 2OH 18:0	Gram-negative bacteria	Soil	Parker *et al*. (1982)	Buckeridge *et al*. (2013); Fichtner *et al*. (2014); Wei *et al*. (2014)

(continued)

Table 3.1 *(Continued)*

PLFA biomarkers	Designations	Sample origin	Original citations	Recent studies using traditional designations
CYCLOPROPYL SATURATED FATTY ACIDS				
cy17:0; cy19:0	Gram-negative bacteria	Pure cultures; soil	Wilkinson (1988)	Breulmann et al. (2014); Lange et al. (2014); Mechri et al. (2014); Reinsch et al. (2014)
	Anaerobic bacteria	Soil	Guckert et al. (1985)	Dong et al. (2014); Fichtner et al. (2014)
TERMINALLY BRANCHED FATTY ACIDS				
a13:0; i13:0; i14:0; i15:0; a15:0; i16:0; a17:0; i17:0; a18:0; i18:0	Gram-positive bacteria	Pit mud; pure cultures; soil	O'Leary and Wilkinson (1988); Vestal and White (1989)	Velasco *et al*. (2010); Tavi *et al*. (2013); Zheng *et al*. (2013); Breulmann *et al*. (2014); Dong *et al*. (2014); Fichtner *et al*. (2014); Mechri *et al*. (2014); Reinsch *et al*. (2014)
POLYUNSATURATED FATTY ACIDS				
18:2ω6c; 18:3ω6c	Saprotrophic fungi	Pure cultures; soil	Federle (1986); Stahl and Klug (1996)	Buckeridge *et al*. (2013); Högberg *et al.* (2013); Banks *et al*. (2014); García-Orenes *et al*. (2015)
	Cyanobacteria; diatoms	Freshwater microalgae; microbial mats; pond water	Ahlgren *et al*. (1992)	Jungblut *et al*. (2009); Dijkman *et al*. (2010); De Carvalho and Caramujo (2014)
18:3ω3	Fungi	Biofilms; pure cultures; soil	Zelles (1997)	Banks *et al*. (2014); Zhao *et al*. (2014)
	Cyanobacteria	Freshwater microalgae; microbial mats	Ahlgren *et al*. (1992)	Jungblut *et al*. (2009); Dijkman *et al*. (2010)
18:2ω9c	Saprotrophic fungi	Soil	Federle (1986)	Velasco *et al*. (2010); Buckeridge *et al*. (2013); Wei *et al*. (2014); Zhang *et al*. (2014); García-Orenes *et al*. (2015)
16:2ω4; 16:2ω6; 16:2ω7; 16:3ω3; 16:3ω4; 16:4ω3; 16:4ω1; 18:4ω3; 18:5ω3; 20:4ω6; 20:5ω3; 22:5ω3; 22:6ω3	Cyanobacteria; diatoms; green algae	Freshwater microalgae; microbial mats	Volkman *et al*. (1989); Ahlgren *et al*. (1992)	Dijkman *et al*. (2010); Bühring *et al*. (2014)

Bold lettering indicates the same phospholipid fatty acid (PLFA) biomarker used for different designations.

Such discrepancies are also evident in the range of PLFA biomarkers (18:1ω9c, 18:2ω6,9c, 18:3ω3 and 18:3ω6c) used to indicate saprotrophic and ectomycorrhizal fungi (Federle 1986; Frostegård and Bååth 1996). Strong correlations between these PLFAs and the DNA sequences of ectomycorrhizal fungi (Högberg *et al*. 2011), as well as ectomycorrhizal root colonization (Kaiser *et al*. 2010) have been reported. Again, the obstacle for interpretation is that the PLFAs 18:1ω9c and 18:2ω6,9c are also common in plants (Zelles *et al*. 1997; Napier *et al*. 2014). Both the neutral and phospholipid 16:1ω5 are considered representative of arbuscular mycorrhizal fungi (Pacovsky and Fuller 1988; Olsson *et al*. 1995). After observing 16:1ω5 PLFA in bacteria, Butler *et al*. (2003) recommended the use of this PLFA only in

samples with low bacterial abundance. If this is not feasible, the use of the neutral lipid fatty acid form should rather be applied as biomarker for arbuscular mycorrhizal fungi.

PLFA analysis is often used in soil studies to differentiate between bacterial and fungal biomass. By dividing the sum of the mole percentage values of the fungal fatty acid markers (saprotrophic and mycorrhizal) by the sum of the mole percentage values of the bacterial fatty acid markers, a fungal to bacterial (F:B) ratio can be determined (Frostegård and Bååth 1996). An increase in the F:B ratio indicates the rise in fungal concentrations in the microbial community. Fluctuations in this ratio have been associated with environmental processes such as nutrient cycling, organic matter decomposition and carbon sequestration (Romaniuk *et al*. 2011; Högberg *et al*. 2013; Bragazza *et al*. 2015) and is often used to compare the effect of agricultural practices on soil microbial communities (Bailey *et al*. 2002b; De Vries *et al*. 2006; Romaniuk *et al*. 2011; Zhang *et al*. 2012). In a similar manner, other markers are used in the calculation of ratios to indicate shifts in the microbial community. Terminally branched saturated fatty acids such as i13:0, a13:0, i14:0, i15:0, a15:0, i16:0, i17:0, a17:0, i18:0 and a18:0 are mainly indicative of the presence of Gram-positive bacteria (O'Leary and Wilkinson 1988; Frostegård and Bååth 1996), whilst Gram-negative bacteria are represented by the monounsaturated fatty acids and hydroxyl containing biomarkers 2OH 12:0, 14:1, 2OH 14:0, 3OH 14:0, 15:1ω6c, 16:1ω9c, 16:1ω7c, 16:1ω7t, cy17:0, 17:1ω7,8,9,11c, 2OH 16:0, 18:1ω12c, 18:1ω9t, 18:1ω7c, 18:1ω5c, 2OH 18:0, cy19:0 and 19:1ω9c (Wilkinson 1988; Frostegård and Bååth 1996). The ratio of Gram-positive to Gram-negative bacterial lipids (GP:GN) provides an indication of the relative dominance of these bacterial groups in an environment and some studies associate an increase of Gram-negative PLFAs with stress conditions (Frostegård *et al*. 1993; Zelles *et al*. 1994). However, care should be taken to interpret disturbance or recovery of soils merely based on a shift from Gram-positive to Gram-negative bacteria or *vice versa* as contradictory results have also been reported (Kaur *et al*. 2005, McKinley *et al*. 2005; Bertram *et al*. 2012). Other ratios that are used to interpret microbial community shifts in soil investigations include bacterial to total PLFA and Gram-positive to total PLFA (McKinley *et al*. 2005; Bertram *et al*. 2012).

3.3 Physiological status and metabolic activity

Early reports on lipid studies demonstrated that the composition of PLFA markers change due to nutritional fluctuations (Findlay and White 1983; Guckert *et al*. 1986) and other environmental conditions. The composition of fatty acids can subsequently be used to indicate stress in the microbial community (Guckert *et al*. 1986; Kieft *et al*. 1994; Keweloh and Heipieper 1996; Smith *et al*. 2000). Increased ratios of saturated to monounsaturated fatty acids (sat:mono), *trans*- to *cis* monoenoic fatty acids (*trans*:*cis*) and

cyclopropyl fatty acids to their monoenoic precursors (cy:pre) are used as stress signatures. The amount of poly-β-hydroxybutyrate relative to total PLFA biomass is associated with unbalanced growth and also with nutritional stress (White *et al*. 1996; McKinley *et al*. 2005). A survey of several newly published studies revealed that all of the ratios for physiological stress are still widely applied. These include the effect of soil moisture and bovine urine on microbial stress (Bertram *et al*. 2012); the interaction between soil microbial communities and invading *E. coli* in soils from vegetable fields (Yao *et al*. 2014); the soil microbial community structure in shrub patches in semi-arid and arid landscapes (Ben-David *et al*. 2011); soil microbial community indices identified as predictors for soil solution chemistry and nitrogen leaching from forests (Högberg *et al*. 2013); the impacts of herbicides and surfactants on soil microbial communities (Banks *et al*. 2014) and the use of FAME biomarkers to assess the detrimental effects of petroleum contamination on estuarine microbial biomass (Nilsen *et al*. 2015). Interpretations should be made with caution and the indiscriminate application of stress ratios avoided in environmental studies. Rather than indicating stress, the observed changes may also be due to a shift in the species composition (Frostegård *et al*. 2011) and it should be kept in mind that the original conclusions regarding these ratios were often made from investigations with pure cultures.

Understanding the specific assimilation processes occurring within microbial communities following substrate uptake, is an important part of current phospholipid biomarker research. Advances in this field are based on the stable isotope probing (SIP) of microbial biomarkers (Treonis *et al*. 2004; Denef *et al*. 2007). Given that environmental samples contain a wide variety of signature lipid biomarkers, isotope labelling of PLFAs (PLFA-SIP) is a popular practice and important for functional interpretation (Watzinger 2015). Through isotopic analysis, the rate of specific processes in microbial communities can be measured *in situ* – including nitrogen fixation, denitrification and respiration (Boschker and Middelburg 2002). The decomposition, microbial utilisation and transformation of organic matter (Kuzyakov *et al*. 2014), the specific microbial groups involved in bioremediation (Mahmoudi *et al*. 2013) and the flow of carbon from plants to soil microbes (Tavi *et al*. 2013) can be distinguished. Specifically, ^{13}C PLFA analysis has received a lot of attention in recent years. The application of this technique to soil has been reviewed by Yao *et al*. (2015). The approach is advantageous therein that it can identify living microbial biomass and is more sensitive in detecting shifts in the microbial community compared to DNA/RNA based methods. Conversely, ^{13}C PLFA analysis does not provide detailed species composition or phylogenetic resolution when used on its own.

4 Conclusions

Despite the many controversies surrounding the interpretation of PLFA data, this approach has a considerable number of advantages when applied with caution. No other method provides information on such a variety of microbial characteristics (both functional and structural) in a single analysis. It is fast, simple, sensitive and when used in combination with other techniques such as SIP, can be used to gain insight into the functional component of the microbial community. The main problem with PLFA analysis is not the method(s) used, but rather the manner in which data are interpreted and applied to environmental studies. As reviewed in this paper, there are several caveats and subsequent suggestions to improve the accuracy of PLFA interpretations. Researchers should also focus on determining patterns from literature before interpreting their own investigations. With the numerous studies still using PLFA analysis, there are bound to be trends regarding the structure and function of microbial communities in specific types of environments and for different management practices. Considering these are crucial to correctly applying tried and tested methods and interpreting new information.

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Chapter 4
Comparison of methods for signature lipid biomarker analysis

1 Introduction

Traditional signature lipid biomarker techniques, such as phospholipid fatty acid (PLFA) and total lipid extract (TLE) analyses are well-known for their capacity to characterise microbial communities in response to environmental disturbances (Drenovsky *et al*. 2004; Bühring *et al*. 2012; Chen *et al*. 2013a; Fichtner *et al*. 2014). Phospholipid fatty acid analysis aims to accurately characterise the fatty acid composition of polar lipids only, whereas TLE analysis generates a complete lipid profile of a sample. It should be noted that the term "TLE" is also referred to in some studies as "whole lipid extract" (Cescut *et al.* 2011; Donato *et al.* 2011). During PLFA analysis, the TLE is fractionated into various lipid classes and only the phospholipid fraction is used for further analysis (Guckert *et al.* 1985). If required, the other fractions can also be collected and used to study fungal biomass (neutral lipids) (Olsson *et al.* 1995) or the nutritional status of microbes (glycolipids) (Findlay and White 1983). The PLFA analysis provides an indication of viable microbial biomass and signature fatty acid biomarkers representative of specific microbial groups, while TLE analysis broadens the range of lipids detected to include neutral, glyco- and phospholipids (White *et al*. 1979; Zelles 1999; Drenovsky *et al*. 2004; Kaur *et al*. 2005). For both methods, the lipids are converted to their respective fatty acid methyl esters (FAMEs) through methylation, before being analysed by gas chromatography-mass spectrometry (GC-MS) (Schutter and Dick 2000; Drenovsky *et al*. 2004; Fernandes *et al*. 2013). However, as different derivatisation procedures have been found to influence FAME recovery yield and quality (Basconcillo and McCarry 2008; Gómez-Brandón *et al*. 2010; Chowdhury and Dick 2012), it is necessary to identify the most suitable derivatisation technique for optimal FAME characterisation. In addition, newer approaches such as metabolomics analysis may also prove useful when used to analyse certain target compounds. Considering that a metabolomics-based approach requires less sample clean-up and preparation (Wood 2014; Garg *et al.* 2015), it may provide a higher sample throughput compared to lipid analyses. Yet, despite the increasing utilisation of new metabolomics-based approaches in environmental studies, no clear comparison has been made between conventional signature lipid biomarker techniques and a metabolomics-based approach. Two types of analyses can be used, namely targeted or untargeted. With targeted analysis, a specific class of metabolites are quantified (Shulaev 2006; Creek and Barrett 2014). Untargeted analysis, on the

other hand, provides a total metabolic profile of all the measurable metabolites in a sample (Dunn *et al.* 2013).

The objective of this chapter was to evaluate the targeted analysis of selected FAMEs to characterise both homogenised soil and pure bacterial cultures as obtained from fractionated PLFA, TLE and metabolomics analysis respectively. The use of homogeneous soil and pure bacterial culture samples ensured that the variation obtained could only be ascribed to the different methods and not to sample variability. The effect of different sample matrices could also be demonstrated by using soil and bacterial cultures, respectively. The most appropriate derivatisation technique for each of the three methods was determined by evaluating the derivatives formed during the following procedures: mild alkaline methanolysis, a combination of mild alkaline methanolysis and silylation, mild alkaline methanolysis combined with oximation and silylation, and lastly oximation followed by silylation. In addition, the untargeted analysis obtained from the metabolomics-based approach is presented as a possibility for future investigations of soil metabolomics not restricted to microbial communities.

2 Materials and methods

2.1 Chemicals and glassware

The following high purity solvents (Honeywell Burdick & Jackson®) were purchased from Anatech Instruments (Pty) Ltd. (Olivedale, South Africa): acetone, chloroform, hexane, methanol and water. Potassium hydroxide (KOH) and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck Millipore (Modderfontein, South Africa). Glacial acetic acid; silicic acid; 2,2,4-trimethylpentane (isooctane); N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + TMCS, 99:1), hereafter referred to as BSTFA-TMCS; methoxyamine hydrochloride and pyridine were obtained from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). Internal standards were used for quantification and quality control purposes and also purchased from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). The internal standard (IS) methyl nonadecanoate (C19:0 FAME) consisted of C19:0 FAME dissolved in isooctane (50 pmol ml^{-1}), whereas the IS mix consisted of L-norleucine, 3-phenylbutyric acid, 2-acetamidophenol and nonadecanoic acid, dissolved in methanol (5 µg ml^{-1}). Glassware as required was purchased from Lasec South Africa (Pty) Ltd. (Midrand, South Africa).

All glassware used for the analytical procedures were washed with tap water and phosphate-free detergent (Liqui-Nox®, Alconox Inc., Separations, Johannesburg, South Africa), rinsed thoroughly with tap, deionised and nano-pure water, air-dried and heated in a muffle furnace at 450°C for 4 h to remove any possible lipid contaminants. The Teflon-lined caps were washed with phosphate-free detergent, rinsed with tap, deionised and nano-pure water, air-dried and sonicated in acetone for 2 min.

2.2 Experimental design

The experiment consisted of a laboratory assay in which homogeneous soil and pure bacterial culture samples were used to evaluate the targeted analysis of selected FAMEs as obtained from fractionated PLFA, TLE and metabolomics analysis, respectively. All three methods used a modified Bligh and Dyer (1959) extraction procedure (White *et al*. 1979). For TLE analysis, the complete organic phase (without fractionation) was used for further analysis, whereas during PLFA analysis the organic phase underwent silicic acid column chromatography (fractionation) (Guckert *et al.* 1985) and only the phospholipid fraction was used in further procedures. For the metabolomics-based approach, organic and aqueous phases were obtained and used for further analysis. The organic phase represents a TLE and can be used for both targeted (FAMEs only) and untargeted analysis (FAMEs, other knowns and unknowns). The aqueous phase can also be included as part of the untargeted analysis and would not contain any FAMEs.

Table 4.1: Experimental conditions for each method used in the laboratory assay

Method	**Extraction**	**Fractionation**	**Derivatisation**			**Quantification**
			I	**II**	**III**	
PLFA (Fractionated samples)	Modified Bligh & Dyer (phospholipid fraction)	Silicic acid column chromatography	Methanolic KOH			GC-MS
				Methanolic KOH & BSTFA-TMCS		
					Methanolic KOH, MeOX & BSTFA-TMCS	
TLE (Total lipid extracts)	Modified Bligh & Dyer (total lipid extract)	No fractionation	Methanolic KOH & BSTFA-TMCS			GC-MS
				Methanolic KOH, MeOX & BSTFA-TMCS		
					MeOX & BSTFA-TMCS	
Metabolomics-based approach	Modified Bligh & Dyer (organic & aqueous phases)	No fractionation	Organic phase			GC-MS
			Methanolic KOH & BSTFA-TMCS			
				Methanolic KOH, MeOX & BSTFA-TMCS		
					MeOX & BSTFA-TMCS	
			Aqueous phase – MeOX & BSTFA-TMCS			

Key to abbreviations: BSTFA-TMCS - N,O-bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane; GC-MS – gas chromatography - mass spectrometry; KOH – potassium hydroxide; MeOX – methoxyamination solution; PLFA – phospholipid fatty acid; TLE – total lipid extract.

The most appropriate derivatisation technique for each method was determined by applying the following procedures to each of the three methods: (i) mild alkaline methanolysis (methanolic KOH) for targeted analysis of FAMEs in the fractionated samples, (ii) mild alkaline methanolysis (methanolic KOH) and silylation (BSTFA-TMCS) of the fractionated samples and TLEs, (iii) mild alkaline methanolysis (methanolic KOH) with oximation (methoxyamination solution) and silylation (BSTFA-TMCS) of the fractionated samples and TLEs, and (iv) oximation (methoxyamination solution) with silylation (BSTFA-TMCS) of the TLEs and aqueous phases of the metabolomics-based approach. The experimental conditions for each method are summarised in Table 4.1. All procedures were conducted on six replicates of soil and pure bacterial culture samples.

2.3 Sample preparation

2.3.1 Pure cultures

The pure bacterial culture samples used in the laboratory assay consisted of a mixture of two common soil bacteria namely Gram-positive *Bacillus subtilis* (ATCC® 11774™) and Gram-negative *Pseudomonas aeruginosa* (ATCC® 10845™). Each bacterial species was cultivated in separate suspensions to ensure optimal growth, without one species inducing growth inhibition of the other (Ozawa and Yamaguchi 1979). Starter cultures of the bacteria were prepared by inoculating 10 ml of tryptic soy broth (TSB) medium (Merck Millipore, Modderfontein, South Africa) and incubating overnight at 37°C on a rotary shaker (Labcon, California, USA) with vigorous agitation of 120 rpm. The prepared starter cultures were added in their entirety to 5 L Erlenmeyer flasks containing 1 L TSB medium and incubated at 37°C on the rotary shaker (120 rpm). Cell-growth was monitored by measuring the optical density of the culture suspensions at 600 nm (OD_{600}) with a SPECTRONIC 200™ spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA). Once the cultures reached the exponential growth phase (OD_{600} ~ 0.4-0.9), bacterial cells were harvested from the culture media by centrifugation (Harrier 18/80 centrifuge, MSE (UK) Ltd., London, UK) at 4000 rpm for 10 min at 4°C (Halouska *et al.* 2013). The resulting pellets were washed twice with deionised water and centrifuged at 4°C for 10 min at 4000 rpm, to remove residual medium. After discarding the supernatant, the pellets were flash frozen in liquid nitrogen and lyophilised immediately. The dried pellets were ground into a powder with a sterile mortar and pestle, and stored at -80°C until extraction. The bacterial biomass used in all sample replicates was obtained from a single batch of culture for each bacterial species (Sheng *et al*. 2011). Each sample replicate consisted of approximately 15 mg cell dry mass of *B.subtilis* and 15 mg cell dry mass of *P.aeruginosa* to make up the required mass of ~30 mg cell dry mass for lipid extraction (Basconcillo and McCarry 2008).

2.3.2 Homogenised soil samples

A composite soil sample was obtained from an agricultural site by taking individual samples from the top 0-15 cm soil layer. The soil was mixed thoroughly, homogenised by sieving through a 2 mm sieve to ensure the removal of all visible plant components and divided into smaller subsamples of approximately 50 g each. According to the United States Environmental Protection Agency Quality Assurance / Quality Control (US EPA QA/QC) protocol for soil sampling, it is important to use split samples when assessing sample methodology in order to exclude the inherent variability in the sample due to the heterogeneity of the soil. The soil samples were frozen, lyophilised and stored at -80°C until further use. The homogeneity of the replicate soil samples was confirmed by testing the repeatability with a standard PLFA analysis, to ensure that the samples were representative of a single composite soil sample providing similar results (Table B1, Appendix B, p.136).

2.4 Extraction procedures

Lyophilised soil and pure bacterial culture samples were extracted with the single-phase chloroform-methanol-phosphate buffer solvent mixture of Bligh and Dyer (1959), as modified by White *et al*. (1979). Briefly, whole lipids were extracted from ~5 g soil or ~30 mg dry weight cells with 5 ml chloroform, 10 ml methanol and 4 ml phosphate buffer (50 mM) (1:2:0.8; *v/v/v*). The samples were allowed to extract for 2 h at room temperature before centrifugation for 15 min at 1800 rpm. The supernatants were decanted into clean test tubes. The resulting pellets were re-extracted with 5 ml chloroform and the collected supernatants combined. The addition of 5 ml of water induced phase separation. The tubes were shaken gently and left to separate overnight, at room temperature. The lower organic phase (chloroform) was transferred to a clean test tube, evaporated to dryness at 37°C under a gentle stream of N_2 and stored at -20°C until further use; this represents the TLE. For the metabolomics-based approach, 8 ml of the upper aqueous layers were also collected and evaporated to complete dryness at 37°C. For PLFA analysis, the TLEs obtained from lipid extraction were dissolved in chloroform (3 x 150 µl), loaded onto pre-conditioned glass columns containing 0.5 g activated silicic acid and fractionated into neutral, glyco- and phospholipids, with chloroform (2 x 2.5 ml), acetone (2 x 2.5 ml) and methanol (4 x 2.5 ml), respectively (Guckert *et al*. 1985). For the purpose of this investigation, the neutral and glycolipids were discarded and only the phospholipid fractions were dried down under a gentle stream of N_2 at 37°C and stored at -20°C until further use.

2.5 Derivatisation procedures

The first set of samples (phospholipid fractions) was subjected to mild alkaline methanolysis (methanolic KOH) to form FAMEs (derivatisation I, Table 4.1) (White *et al*. 1979). In short, the dried residues obtained from the extraction procedures were suspended in 0.5 ml chloroform and 0.5 ml methanol, to which 1 ml methanolic KOH (0.2 mol L^{-1}) was added, capped and heated for 30 min at 40°C, followed by cooling to room temperature. The FAMEs were extracted by the addition of 2 ml hexane, 200 µl glacial acetic acid (1 mol L^{-1}), 2 ml nano-pure water and centrifugation for 5 min at 2000 rpm. The upper hexane layers were recovered in clean test tubes and the lower phases washed three times with 2 ml hexane. The pooled hexane layers were evaporated under a gentle stream of N_2, transferred to GC-vials by dissolving in 3 x 100 µl hexane and again dried under N_2 at 37°C. Prior to GC-MS analysis of the fractionated samples, the dried FAMEs were redissolved in 50 µl of C19:0 FAME IS.

The second set of samples, including TLEs (derivatisation I, Table 4.1) and phospholipid fractions (PLFA method – derivatisation II, Table 4.1) were first transesterified by methanolysis to form FAMEs and then derivatised with BSTFA-TMCS to ensure the silylation of compounds not methylated (Lindeque 2011). The majority of compounds containing hydroxyl, carboxylic acid, amine, thiol and phosphate functional groups can be silylated by substituting an acidic hydrogen atom on the compound with a trimethylsilyl (TMS) group (Halket and Zaikin 2003). The dried fractions underwent methanolysis with the addition of methanolic KOH as previously described. To the transferred FAMEs dried in GC-vials, aliquots of 100 µl of C19:0 FAME IS and 50 µl of IS mix were added and evaporated under a gentle stream of N_2 at 37°C. Then, 50 µl BSTFA-TMCS was added to each vial and incubated for 1 h at 60°C (Venter *et al*. 2015), followed by the addition of 50 µl isooctane before GC-MS analysis.

The third set of samples, including TLEs (derivatisation II, Table 4.1) and phospholipid fractions (PLFA method – derivatisation III, Table 4.1) were methylated, followed by oximation and silylation to ensure the formation of TMS-derivatives of the unmethylated compounds (Lindeque 2011). The dry lipid fractions were methylated as previously described and evaporated to dryness in GC-vials. To each vial, 150 µl of C19:0 FAME IS and 50 µl of IS mix were added and the total solution dried under N_2 at 37°C. The dried solutions were resuspended in 50 µl methoxyamination solution (MeOX) prepared from methoxyamine hydrochloride dissolved in anhydrous pyridine (20 mg ml^{-1}) and incubated for 1 h at 60°C. Next, 50 µl BSTFA-TMCS was added to each sample and incubated for 1 h at 60°C (Venter *et al*. 2015). After incubation, 50 µl isooctane was added to each vial and the samples were ready for GC-MS analysis.

Lastly, the dried aqueous phases (8 ml aliquots) and dried TLEs for the metabolomics-based approach (derivatisation III, Table 4.1) were subjected to oximation and silylation. Briefly, the dried aqueous phases were redissolved in 3 x 100 µl methanol:water (1:1; *v/v*), transferred to GC-vials and dried down under N_2 at 37°C. Each vial received 150 µl of C19:0 FAME IS and 50 µl of IS mix; these standard solutions were evaporated under a gentle stream of N_2 at 37°C. Next, 50 µl MeOX was added to each dry sample and kept at 60°C for 1 h (Venter *et al*. 2015). After the addition of 50 µl BSTFA-TMCS, the samples were heated at 60°C for 1 h, followed by the addition of 50 µl isooctane prior to GC-MS analysis.

The 8 ml aliquots of the aqueous phases were based on an evaluation of the number of compounds and the abundance of compounds determined from experimental data to determine the most appropriate sample volume for use (Table B2, Appendix B, p. 136). In current literature for metabolomics, no set standard method for the determination of sample volume exists (Berk *et al*. 2011). This is even more undefined in soil and environmental metabolomics. The only way to estimate sample volume would be to use experimental pilot data on which the actual sample volume selection can be based (Nyamundanda *et al*. 2013). In the protocol of Chen and Chen (2014) polar metabolites and fatty acids from yeast cells and culture supernatant were extracted and the authors standardised on using approximately 50% of the polar (water-methanol) phase for polar metabolite analysis. For this purpose, the present study compared the results from different volumes of the polar water-methanol phase collected from the modified Bligh and Dyer extraction. The total phase had an approximate volume of 19 ml and due to practical considerations and time constraints associated with the drying of the samples; it was not feasible to use the whole phase. Respective volumes of 1, 2, 4 and 8 ml were compared and 8 ml was determined to be the most appropriate.

2.6 Data acquisition

An Agilent GC-MS instrument (Agilent Technologies, Wilmington, Delaware, USA) consisting of a 7890A gas chromatograph with a split/splitless injector (280°C), equipped with a 7683B autosampler coupled to a 5975B inert XL mass selective detector was used for lipid analysis. The gas chromatograph was equipped with a SPB-1 column (60 m x 0.25 mm x 0.250 µm film thickness) (Supelco, Sigma-Aldrich, Johannesburg, South Africa) using helium as the carrier gas at a flow rate of 1.0 ml min^{-1}. The initial oven temperature was 60°C for 2 min, ramping to 150°C at 10°C/min and then to 320°C at 3°C/min, followed by a hold at 320°C for 2 min. The transfer line temperature was set to 280°C, the source temperature at 230°C and the MS Quad at 150°C. The mass analyser performed full scans throughout the run (40-450 m/z) at 175 scans per minute. Electron impact voltage of 70eV with a dwell time of 100 was used. Samples (1 µl) were injected in splitless mode.

2.7 Data analysis

GC-MS spectra were identified by spectral analysis using the Automated Mass Spectral Deconvolution and Identification System (AMDIS V 2.71) available from National Institute of Standards and Technology (NIST) (Max Planck Institute, Golm, Germany), the NIST 2008 mass spectral library and in-house FAME databases. For the identification of a compound, five of its qualifier ions must be matched with ions in the library. A match factor over 60% is preferred (Reinecke *et al.* 2011). The raw data from the GC-MS was processed by AMDIS and resulted in two types of output files that can be used for statistical analysis, namely the elute data file (.elu) and the find compound data file (.fin). The elute data file contains a list of all the peaks found, including ions and abundances. This file contains unidentified compounds not in the database (Stein 1999; Lindeque 2011). The find data file contains a list of all of the compounds found in the database. The resulting data was imported into Agilent's MassHunter Mass Profiler Professional (MPP) (B.02). First, the found peaks in each data file across all files are aligned. Mass Profiler Professional determines which peaks in each chromatographic run are the same compound and which are different. Compound identification prior to peak alignment makes this task much easier for the identified compounds. Prior identification of compounds using AMDIS is thus very helpful in the statistical analysis of data sets. Once all data were aligned, data were imported into MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) for a number of interpretation algorithms to find the compound relationships in regards to the experiment (Xia *et al.* 2009, 2015). Clustering and scores plots obtained by Principal Component Analysis (PCA) were also performed in MetaboAnalyst 3.0. Statistical analyses were performed with Statistica 12.0 (StatSoft, Inc., Tulsa, Oklahoma, USA). One-way analysis of variance (ANOVA) was used, followed by the Tukey's test to determine statistically significant differences ($p<0.05$) between the various methods.

Standard fatty acid nomenclature was used for the representation of all results; this follows the form A: BωC. "A" is the total number of carbon atoms, "B" is the number of double bonds and "C" is the position of the first double bond from the aliphatic (ω) end. The suffixes "c" and "t" indicate *cis* and *trans* conformations. All double bonds are supposedly *cis*. Anteiso- and iso-branching are indicated by the prefixes "a" and "i", "OH" and "Me" refer to a hydroxyl group and a methyl group, respectively, and the preceding number indicates the position from the carboxyl end of the molecule; "cy" shows a cyclopropyl fatty acid (Guckert *et al*. 1985; Ringelberg *et al*. 1989; Zelles 1999).

3 Results

To clarify the following results obtained from the fractionated PLFA, TLE and metabolomics analysis, respectively, the specific fractions analysed by each method are briefly explained. By applying a modified Bligh and Dyer (1959) solvent mixture to the samples, both polar and non-polar metabolites

were extracted into a single-phase (White *et al.* 1979). Through the addition of water to the collected single-phase, separation between the upper aqueous and lower organic phases occurred. After separation, the aqueous phase consists of the water as well as the methanol added during the extraction step. The organic phase contains apolar metabolites such as lipids, whereas the aqueous phase contains polar metabolites (Snyder *et al.* 2013; Tambellini *et al.* 2013). It thus follows that only the organic phase will contain FAMEs. During PLFA analysis, the organic phase was fractionated into several lipid classes, of which only the phospholipid fraction was used to form FAME-derivatives for GC-MS analysis. For TLE analysis, the whole organic phase underwent derivatisation. Considering that the metabolomics analysis focuses on all metabolites present in a sample, analysis of the collected single-phase can be sufficient for metabolite characterisation (Chen *et al.* 2013b). However, as both polar and apolar metabolite groups are present in the single-phase, the accurate analysis of these groups simultaneously may be hampered by their diverse polarities (Ejsing *et al.* 2009). Therefore in this study, the metabolomics-based approach consisted of separate analysis of the organic and aqueous phases. The targeted analysis of selected FAMEs was evaluated first and the aqueous phase was not included in these results. The selected FAMEs shown in the results were those consistently detected in the samples. The metabolomics analysis results for the targeted analysis were the same as the results of the TLE analysis and only the fractionated PLFA and TLE results are therefore shown. For the untargeted analysis, the results of the TLEs with their respective aqueous phases were included.

3.1 Targeted analysis of selected FAMEs to characterise pure culture samples

A total of 16 FAMEs containing between 14 and 19 carbon atoms were detected in all pure culture samples. All of the FAMEs were fatty acids normally found in Gram-positive and Gram-negative bacteria. The total concentration of FAMEs and concentrations of individual FAMEs identified from the pure culture samples differed depending on the method used (Table 4.2). Overall, the concentration of total FAMEs from pure cultures was significantly higher with the TLE method than with fractionated PLFA analysis. The TLE analysis provided significantly higher concentrations for the specific fatty acids a15:0, i16:0, 16:1ω7c, 16:1ω7t, i17:0, 18:1ω7c and 18:1ω7t. The other individual fatty acids showed significant differences based on the derivatisation technique. Figures 4.1A and 4.1B show the two-dimensional (2-D) and three-dimensional (3-D) Principal Component Analysis (PCA) scores plots of the targeted analysis of selected FAMEs from pure culture samples, using fractionated PLFA and TLE analyses. The first two components explained 99.1% of the total variance. The scores plots showed clear separations between the fractionated PLFA and TLE methods along the component axes. Compared to the fractionated PLFA method, the TLE method portrayed a less condensed clustering which indicated higher sample-to-sample variability (Figure 4.1A).

Table 4.2: Concentrations of total and selected FAMEs from pure culture samples using various methods

nmol g^{-1}	Pure culture phospholipid fraction			Pure culture total lipid extract	
	M	**MB**	**MMB**	**MB**	**MMB**
Total FAMEs	8.947 ± 1.888 a	9.515 ± 1.931 a	7.546 ± 0.997 a	34.157 ± 14.844 b	30.187 ± 10.205 b
i14:0	0.198 ± 0.050 ab	0.109 ± 0.032 a	0.085 ± 0.015 a	0.569 ± 0.270 c	0.404 ± 0.113 bc
14:0	0.592 ± 0.149 ab	0.288 ± 0.072 a	0.217 ± 0.041 a	1.290 ± 0.696 c	0.930 ± 0.410 bc
i15:0	1.299 ± 0.222 ab	1.010 ± 0.333 a	0.728 ± 0.063 a	3.670 ± 1.951 c	2.752 ± 0.867 bc
a15:0	1.821 ± 0.385 a	1.366 ± 0.498 a	0.965 ± 0.081 a	5.514 ± 2.534 b	4.220 ± 1.059 b
15:0	0.057 ± 0.015 ab	0.031 ± 0.008 a	0.021 ± 0.004 a	0.134 ± 0.068 c	0.106 ± 0.034 bc
10Me15:0	0.409 ± 0.084 ab	0.298 ± 0.088 a	0.199 ± 0.021 a	1.115 ± 0.612 c	0.882 ± 0.225 bc
i16:0	0.696 ± 0.145 a	0.556 ± 0.184 a	0.371 ± 0.036 a	2.222 ± 1.040 b	1.593 ± 0.379 b
16:1ω7c	0.016 ± 0.009 a	0.013 ± 0.000 a	0.011 ± 0.000 a	2.422 ± 1.884 b	3.749 ± 1.955 b
16:1ω7t	0.013 ± 0.007 a	0.010 ± 0.000 a	0.008 ± 0.000 a	2.803 ± 2.198 b	2.884 ± 1.504 b
16:0	1.257 ± 0.135 a	4.184 ± 0.459 b	3.942 ± 0.879 b	1.221 ± 0.321 a	0.855 ± 0.142 a
i17:0	1.262 ± 0.252 a	1.002 ± 0.321 a	0.685 ± 0.051 a	3.918 ± 1.809 b	2.760 ± 0.639 b
17:0	0.041 ± 0.009 ab	0.024 ± 0.006 a	0.015 ± 0.002 a	0.090 ± 0.044 c	0.069 ± 0.020 bc
18:1ω7c	1.018 ± 0.090 a	2.421 ± 0.011 a	3.103 ± 0.012 a	3.541 ± 1.778 b	4.124 ± 2.151 b
18:1ω7t	1.014 ± 0.070 a	1.881 ± 0.008 a	2.444 ± 0.010 a	2.781 ± 1.387 b	3.172 ± 1.654 b
18:0	0.549 ± 0.107 ab	0.318 ± 0.083 a	0.214 ± 0.037 a	1.282 ± 0.626 c	1.027 ± 0.329 bc
cy19:0	0.157 ± 0.075 ab	0.119 ± 0.041 a	0.079 ± 0.028 a	0.428 ± 0.234 c	0.380 ± 0.159 bc

Means ± standard deviations represent results obtained from sample replicates (n = 6). Statistically significant differences are indicated by alphabetic letters ($p<0.05$). The same letters indicate no significant differences. Key to abbreviations: FAMEs – fatty acid methyl esters; M – mild alkaline methanolysis; MB – mild alkaline methanolysis with silylation; MMB – mild alkaline methanolysis with oximation and silylation.

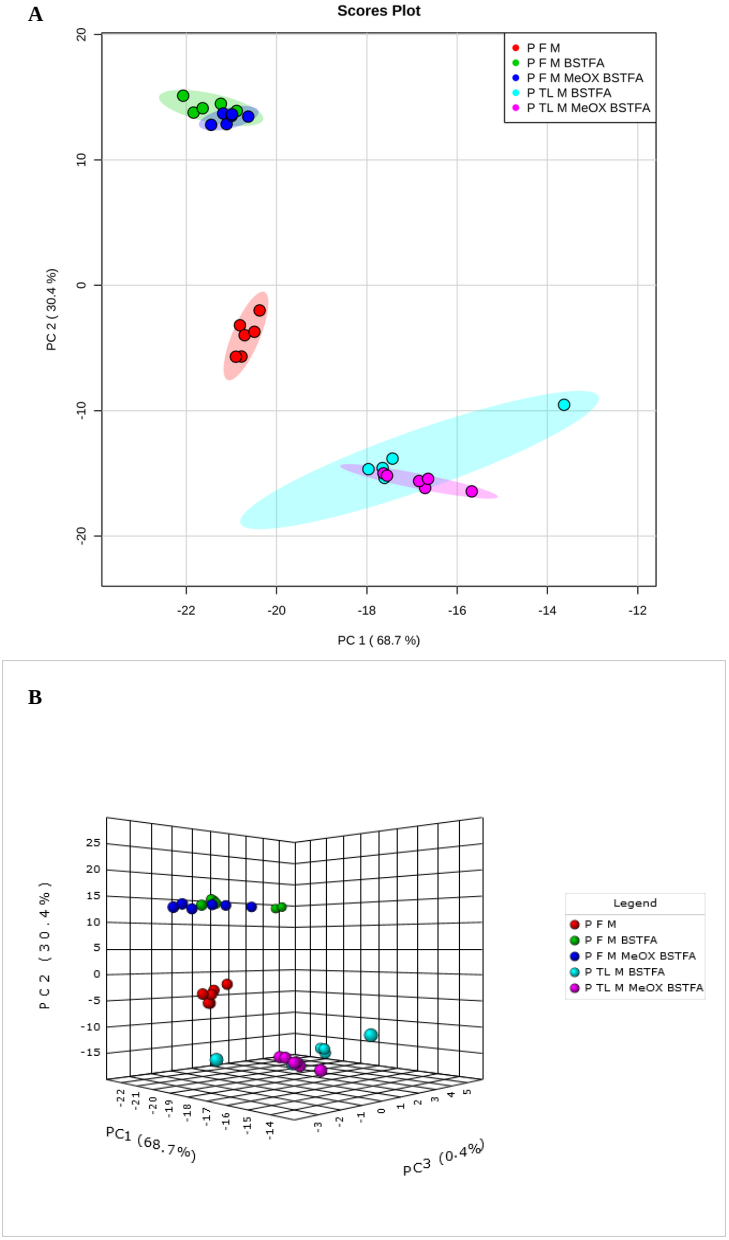

Figure 4.1: Targeted analysis of selected FAMEs from pure cultures using different extraction and derivatisation techniques. A: PCA 2-D scores plot with semi-transparent confidence intervals (95%). B: Corresponding 3-D plot. Key to abbreviations: BSTFA – BSTFA-TMCS; F – fractionated; M – mild alkaline methanolysis; MeOX – methoxyamination solution; P – pure culture; TL – total lipid.

3.2 Most appropriate derivatisation technique for characterisation of pure cultures

There were significant differences between some but not all of the FAME abundances obtained by the various derivatisation techniques (Table 4.2). For the fractionated PLFA samples, the mild alkaline methanolysis (M) technique showed higher FAME concentrations for 13 of the individual FAMEs compared to methanolysis and silylation (MB) or methanolysis with oximation and silylation (MMB). Exceptions included the FAMEs 16:0 (MB provided the highest yield); 18:1ω7c and 18:1ω7t (MMB

gave the highest yield). The MB technique provided higher yields than MMB for the majority of FAMEs in the fractionated samples, except for 18:1ω7c and 18:1ω7t. The MB derivatisation also gave higher concentrations of most FAMEs in the TLE samples, apart from 16:1ω7c, 16:1ω7t, 18:1ω7c and 18:1ω7t that were higher with MMB.

The different derivatisation procedures were compared by using a 2-D PCA scores plot with semi-transparent confidence intervals (95%) (Figure 4.1A). The corresponding interactive 3-D plot is shown in Figure 4.1B. The analyses of the pure cultures show separation of samples based on the various derivatisation techniques applied. The fractionated PLFA samples subjected to methanolysis (P F M) clustered away from the other fractionated samples (P F M BSTFA and P F M MeOX BSTFA). All three derivatisation procedures combined with the fractionated analysis showed high similarity among replicates as indicated by the tight clustering of individual samples. For the TLE samples, methanolysis combined with oximation and silylation (P TL M MeOX BSTFA) provided lower sample-to-sample variability than the combination of methanolysis and silylation (P TL M BSTFA).

3.3 Untargeted analysis of metabolite profiles to characterise pure culture samples

For the untargeted approach (metabolomics analysis), the data obtained from the aqueous and organic phases were combined in a PCA scores plot (Figures 4.2A and 4.2B). Three different derivatisation techniques were included and samples clustered according to the derivatisation technique applied. The cumulative variance for the first two component axes was 79.9%. The samples that were methylated before further derivatisation (P TL M BSTFA and P TL M MeOX BSTFA) grouped away from the other samples. Derivatisation that included MeOX showed less variability (95% confidence) than derivatisation without MeOX (Figure 4.2A).

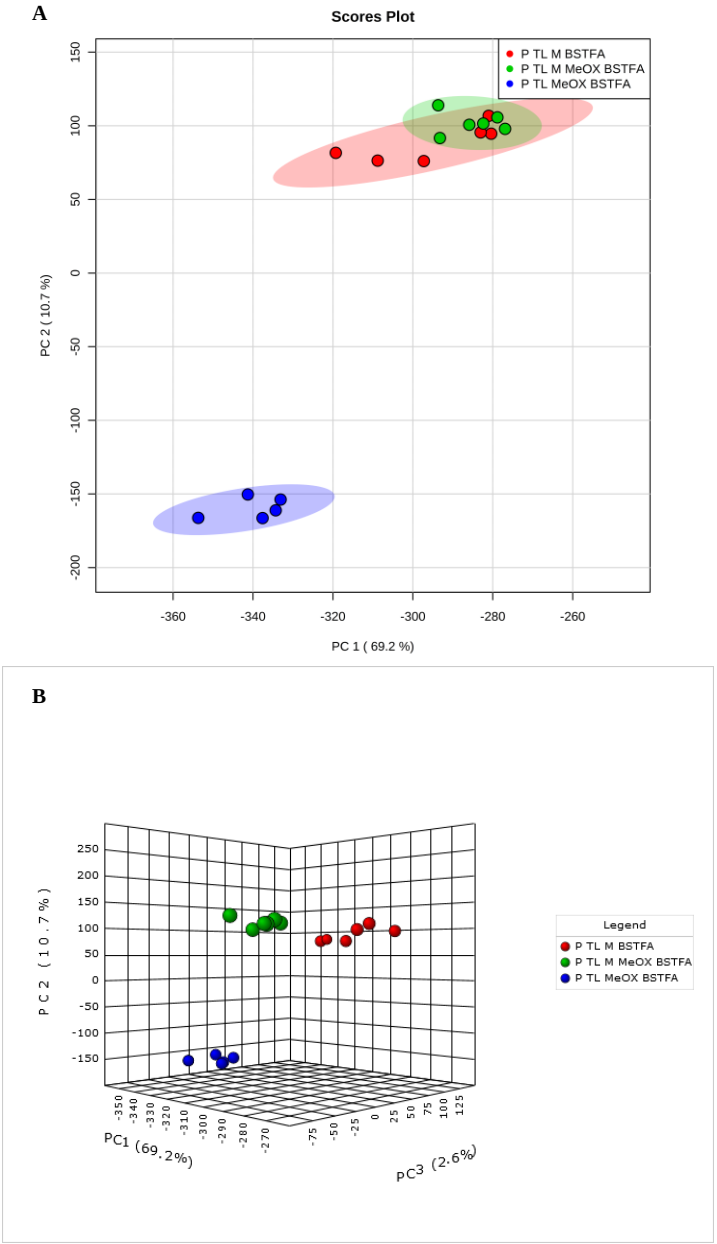

Figure 4.2: Untargeted analysis of metabolite profiles from pure cultures using the aqueous and organic phases subjected to various derivatisation techniques. A: PCA 2-D scores plot with semi-transparent confidence intervals (95%). B: Corresponding 3-D plot. Key to abbreviations: BSTFA – BSTFA-TMCS; M – mild alkaline methanolysis; MeOX – methoxyamination solution; P – pure culture; TL – total lipid.

3.4 Targeted analysis of selected FAMEs to characterise homogenised soil samples

A total of 20 FAMEs, ranging between 14 and 24 carbon atoms, were consistently found in the homogenised soil samples and used as the selection of FAMEs to evaluate the different methods. Similar to the results obtained for the pure cultures, TLE analysis yielded significantly ($p<0.05$) higher abundances of total FAMEs from the homogenised soil samples, compared to fractionated PLFA

analysis (Table 4.3). All the individual FAMEs showed significantly higher abundances with the TLE method, except for 18:2ω6 and 18:0.

Table 4.3: Concentrations of total and selected FAMEs from homogenised soil using various methods

nmol g^{-1}	Soil phospholipid fraction			Soil total lipid extract	
	M	**MB**	**MMB**	**MB**	**MMB**
Total FAMEs	2.130 ± 0.733 a	2.177 ± 0.584 a	1.706 ± 0.372 a	8.735 ± 1.911 b	13.755 ± 2.655 c
14:0	0.013 ± 0.002 a	0.008 ± 0.005 a	0.004 ± 0.004 a	0.085 ± 0.020 b	0.122 ± 0.011 c
i15:0	0.163 ± 0.015 a	0.103 ± 0.055 a	0.098 ± 0.029 a	0.492 ± 0.115 b	0.642 ± 0.050 c
a15:0	0.074 ± 0.007 a	0.049 ± 0.024 a	0.046 ± 0.013 a	0.226 ± 0.053 b	0.300 ± 0.022 c
15:0	0.014 ± 0.001 a	0.011 ± 0.007 a	0.010 ± 0.004 a	0.055 ± 0.014 b	0.068 ± 0.006 b
10Me15:0	0.026 ± 0.003 a	0.021 ± 0.009 a	0.018 ± 0.005 a	0.059 ± 0.013 b	0.076 ± 0.009 c
i16:0	0.117 ± 0.014 a	0.109 ± 0.028 a	0.102 ± 0.020 a	0.267 ± 0.064 b	0.336 ± 0.018 c
16:1ω7c	0.015 ± 0.008 a	0.011 ± 0.000 a	0.010 ± 0.000 a	0.892 ± 0.495 b	2.252 ± 0.719 c
16:1ω7t	0.012 ± 0.006 a	0.009 ± 0.000 a	0.008 ± 0.000 a	0.950 ± 0.507 b	1.732 ± 0.553 c
16:0	0.298 ± 0.037 a	0.343 ± 0.072 a	0.307 ± 0.053 a	1.239 ± 0.322 b	1.552 ± 0.137 c
i17:0	0.067 ± 0.008 a	0.072 ± 0.015 a	0.065 ± 0.011 a	0.157 ± 0.039 b	0.192 ± 0.013 c
a17:0	0.067 ± 0.009 a	0.072 ± 0.014 a	0.066 ± 0.011 a	0.155 ± 0.039 b	0.188 ± 0.012 b
cy17:0	0.063 ± 0.006 a	0.071 ± 0.019 a	0.069 ± 0.016 a	0.164 ± 0.048 b	0.200 ± 0.020 b
17:0	0.015 ± 0.002 a	0.016 ± 0.003 a	0.014 ± 0.002 a	0.045 ± 0.014 b	0.052 ± 0.006 b
10Me17:0	0.061 ± 0.010 a	0.068 ± 0.013 a	0.062 ± 0.011 a	0.157 ± 0.039 b	0.167 ± 0.012 b
18:2ω6	0.482 ± 0.716 a	0.430 ± 0.554 a	0.082 ± 0.081 a	0.442 ± 0.331 a	0.168 ± 0.008 a
18:1ω7c	0.017 ± 0.009 a	0.809 ± 0.010 a	1.039 ± 0.011 a	1.252 ± 0.312 b	2.477 ± 0.791 c
18:1ω7t	0.013 ± 0.007 a	0.628 ± 0.008 a	0.817 ± 0.009 a	0.982 ± 0.244 b	1.905 ± 0.609 c
18:0	0.502 ± 0.111 b	0.668 ± 0.165 a	0.663 ± 0.141 ab	0.457 ± 0.113 ab	0.628 ± 0.051 ab
cy19:0	0.091 ± 0.011 a	0.105 ±0.020 a	0.078 ± 0.040 a	0.402 ± 0.102 b	0.436 ± 0.147 b
24:0	0.022 ± 0.004 a	0.032 ± 0.008 a	0.024 ± 0.009 a	0.259 ± 0.075 b	0.263 ± 0.105 b

Means ± standard deviations represent results obtained from sample replicates (n = 6). Statistically significant differences are indicated by alphabetic letters ($p<0.05$). The same letters indicate no significant differences. Key to abbreviations: FAMEs – fatty acid methyl esters; M – mild alkaline methanolysis; MB – mild alkaline methanolysis with silylation; MMB – mild alkaline methanolysis with oximation and silylation.

As shown by the 2-D and 3-D PCA scores plots in Figures 4.3A and 4.3B respectively, the samples formed separate clusters along the principal component (PC) axis 1 based on the extraction method used. The first axis explained 57% variance and the second axis 19.2%. A total variance of 76.2% was indicated. Two of the three groups for fractionated PLFA analysis, S F M BSTFA and S F M MeOX BSTFA displayed high sample-to-sample variability compared to the other groups as indicated by the semi-transparent confidence intervals (95%) (Figure 4.3A).

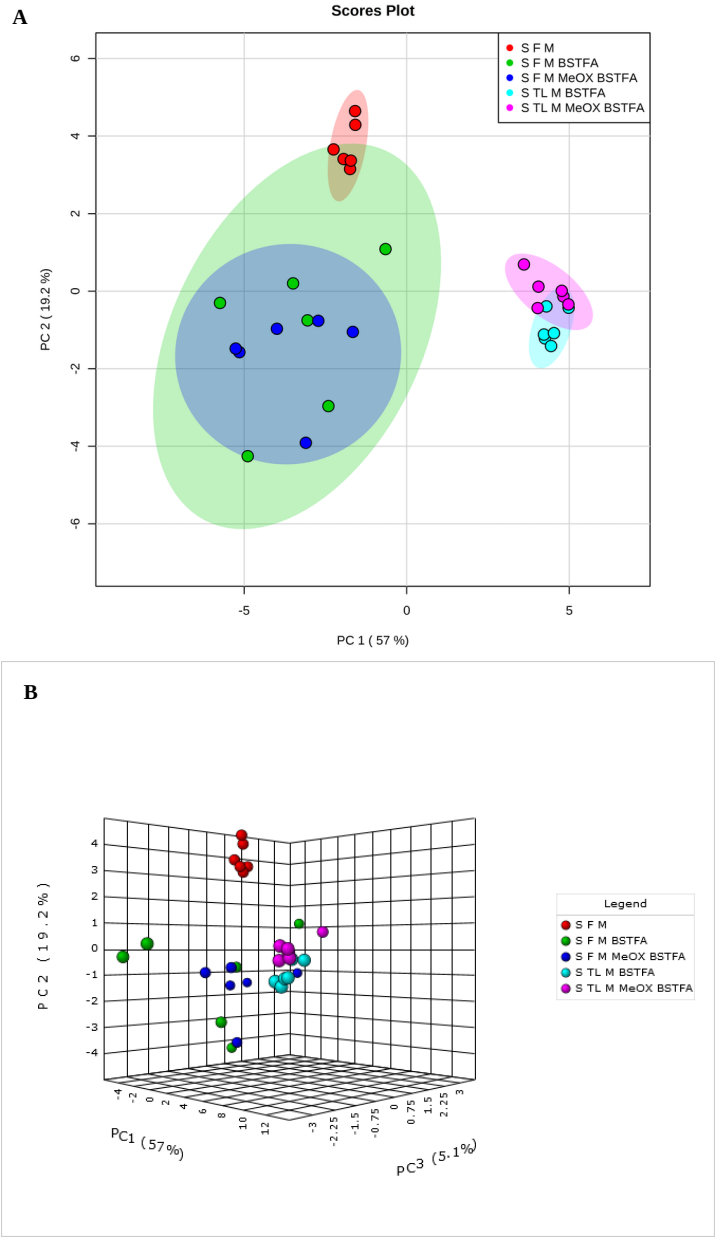

Figure 4.3: Targeted analysis of selected FAMEs from homogenised soil samples using different extraction and derivatisation techniques. A: PCA 2-D scores plot with semi-transparent confidence intervals (95%). B: Corresponding 3-D plot. Key to abbreviations: BSTFA – BSTFA-TMCS; F – fractionated; M – mild alkaline methanolysis; MeOX – methoxyamination solution; S - soil; TL – total lipid.

3.5 Most appropriate derivatisation technique for characterisation of homogenised soil

The use of only methylation (M) showed higher abundances for nine of the FAMEs in the fractionated samples, however these did not differ significantly ($p<0.05$) from the abundances obtained with MB or MMB derivatisation (Table 4.3). The other FAMEs and the total FAME concentration, showed higher abundances for the MB technique, except 18:1ω7c and 18:1ω7t that was higher with MMB. Of all the

FAMEs detected and compared, the only statistically significant difference was for the concentration of 18:0 between the M and MB derivatisation techniques applied to the fractionated PLFA samples. In contrast, the TLE analysis showed the highest yields for total and selected FAMEs from the MMB technique. For 11 of the selected FAMEs and the total FAME concentration, statistically significant differences were found, whereas the other FAMEs did not differ significantly. From the PCA scores plot, distinct groups were identified based on the derivatisation procedures applied to the fractionated PLFA and TLE methods, respectively. Tighter clustering and higher similarity among replicates were observed for the fractionated PLFA samples with methanolysis (S F M) than for the other two derivatisation techniques (Figures 4.3A and 4.3B). The latter (S F M BSTFA and S F M MeOX BSTFA) showed high sample-to-sample variability among the replicates as indicated by poor clustering and large semi-transparent confidence intervals (95%). For the TLE analysis, both derivatisation techniques showed low sample-to-sample variability comparable to that obtained for the fractionated samples with only methylation.

3.6 Untargeted analysis of metabolite profiles to characterise homogenised soil samples

A metabolomics analysis was done using the combined data from the aqueous and organic phases of the soil samples. Again, different derivatisation techniques were included. The 2-D PCA scores plot showed clear separation between samples based on the different derivatisation techniques along the PC1 axis (Figure 4.4A). The first axis accounted for 68.7% variance, whereas PC2 and PC3 accounted for 9.7% and 4.1%, respectively. Samples that were methylated before further derivatisation (S TL M BSTFA and S TL M MeOX BSTFA) grouped together and away from those not methylated. On the other hand, the samples that were not methylated before further derivatisation (S TL MeOX BSTFA) showed lower sample-to-sample variability (Figure 4.4A). Also, derivatisation that included MeOX showed less variability (95% confidence) than derivatisation without MeOX as in the case of the pure culture samples.

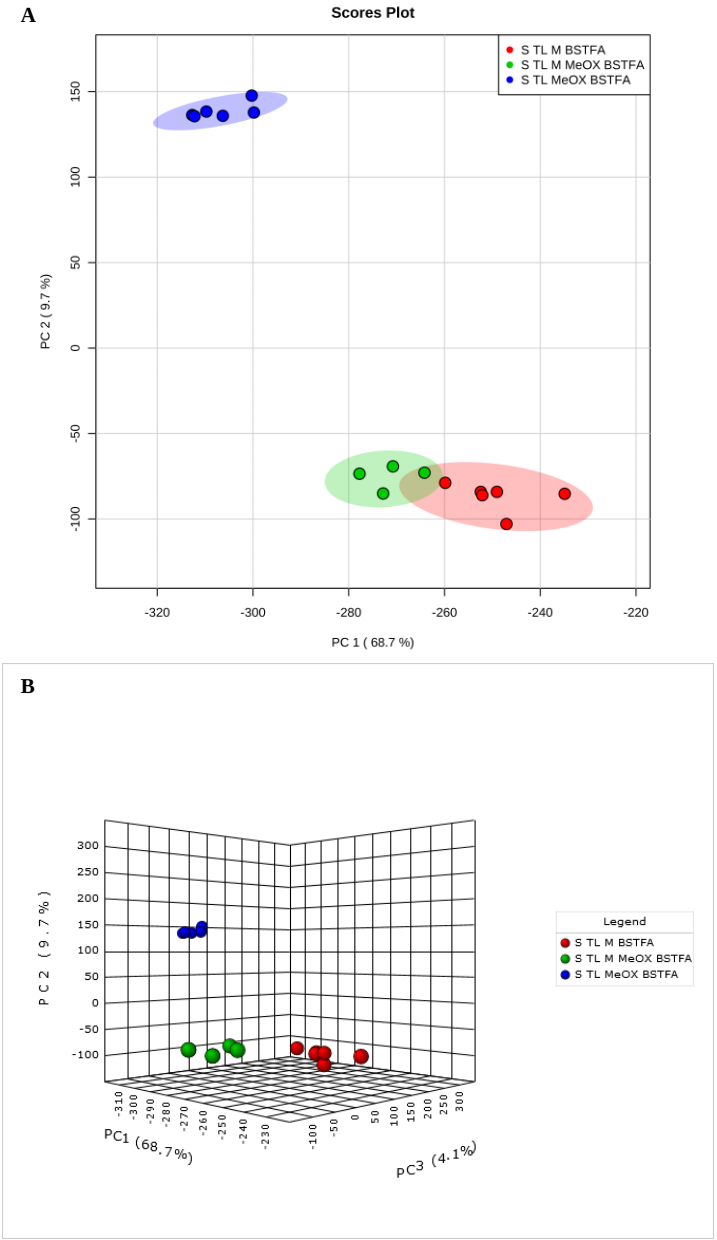

Figure 4.4: Untargeted analysis of metabolite profiles from homogenised soil samples using the aqueous and organic phases subjected to various derivatisation techniques. A: PCA 2-D scores plot with semi-transparent confidence intervals (95%). B: Corresponding 3-D plot. Key to abbreviations: BSTFA – BSTFA-TMCS; M – mild alkaline methanolysis; MeOX – methoxyamination solution; S – soil; TL – total lipid.

4 Discussion

4.1 Targeted analysis of selected FAMEs to characterise pure culture samples

A total of 16 fatty acids, typical of Gram-positive and Gram-negative bacteria (O'Leary and Wilkinson 1988; Wilkinson 1988) were consistently identified in the pure culture samples, irrespective of the method used (Table 4.2). This is in close agreement with previously reported findings on Gram-

positive *B.subtilis* (Rooney *et al.* 2009) and Gram-negative *P.aeruginosa* (Chao *et al.* 2010) cultures. The significantly higher abundances of total FAMEs and individual FAMEs obtained with the TLE method (Table 4.2) can be attributed to the fact that TLE analysis takes neutral, glyco- and polar lipids into account whereas fractionated PLFA analysis depends only on a single class (polar lipids) for FAME characterisation (Zelles 1999; Drenovsky *et al.* 2004). The complexity of the TLE samples may also justify their higher sample-to-sample variability seen in Figures 4.1A and 4.1B. A number of the FAMEs detected in the pure culture samples have been reported to occur in more than one lipid class concurrently, such as 14:0, 16:0, 16:1, 18:0, 18:1 and cy19:0 (Olson and Ingram 1975; Umemoto and Sato 1978).

4.2 Most appropriate derivatisation technique for characterisation of pure cultures

From the results in Table 4.2 it was apparent that the FAME yields were influenced by the derivatisation method used. Using only methanolysis (M) generally gave higher FAME yields for the fractionated PLFA analysis and showed low sample-to-sample variability (Figures 4.1A and 4.1B). Also, the combination of methanolysis and silylation (MB) gave higher FAME abundances for both fractionated samples and TLEs, than methanolysis with oximation and silylation (MMB). However, the MB technique displayed higher sample-to-sample variability. To decide which derivatisation technique was the most appropriate for each method, their advantages and shortcomings had to be considered. Mild alkaline methanolysis can only methylate fatty acids bound to glycerol (Kramer *et al.* 1997), which may lead to high amounts of underivatised compounds in samples ready for GC-MS analysis. These underivatised compounds can cause damage to GC columns and the possibility of compound carry-over and inter-sample contamination exists (Lindeque 2011). To reduce underivatised compounds in samples, the subsequent silylation of compounds containing hydroxyl, carboxylic acid, amine, thiol and phosphate functional groups, with BSTFA containing 1% TMCS was proposed (Gutiérrez *et al.* 2002; Halket and Zaikin 2003). Derivatisation should produce the desired chemical modification of the compounds of interest, and thereby improve the analysis. For example, the use of BSTFA-TMCS to derivatise mono- and disaccharides derived from decomposed organic material and root exudates. According to Jones (1998), root exudates consist of low molecular weight organic compounds such as sugars (e.g. monosaccharides and disaccharides), organic acids and amino acids. During derivatisation with BSTFA-TMCS, multiple TMS ethers of the mono- and disaccharides are formed and separated chromatographically. TMS derivatives of these saccharides may result in multiple peaks due to the tautomeric forms of the reducing sugars (Gullberg *et al.* 2004). This will cause chromatographic separation problems as well as identification and quantification issues. By converting the aldehydes and keto groups to oximes using methoxyamine followed by derivatisation with BSTFA and TMCS, the number of tautomeric forms can be drastically reduced (Fiehn *et al.* 2000). Thus, oximation reduces the number of derivatisation products formed (Zhang *et*

al. 2007; Kanani *et al.* 2008) and the formation of multiple peaks for a single compound can be prevented, resulting in a cleaner chromatogram and thus better quantification (Fiehn *et al.* 2000; Lindeque 2011).

In conclusion, for the FAME characterisation of fractionated pure culture samples, methanolysis is recommended for derivatisation. On the other hand, the combination of methanolysis with oximation and silylation is preferred over methanolysis and silylation for TLEs.

4.3 Untargeted analysis of metabolite profiles to characterise pure culture samples

When an untargeted approach was used to determine the efficiency of the methods to characterise pure culture samples, the TLEs and aqueous phases of the metabolomics analysis was used. Clear separation between the samples, based on the derivatisation technique was observed (Figure 4.2). The combination of oximation and silylation (P TL MeOX BSTFA) generated a different metabolite profile than the other techniques. This can be ascribed to the absence of FAMEs with this technique compared to the other two where methylation was the first step in the derivatisation procedure. The TLE samples that were methylated before further derivatisation (P TL M BSTFA and P TL M MeOX BSTFA) showed more comparable profiles than the P TL MeOX BSTFA samples, which can be linked to the presence of FAMEs in these samples. Also, the clustering of each method's replicate samples (n = 6) provided an indication of the repeatability of that particular method (Venter *et al.* 2015). Compared to the other techniques, methanolysis combined with oximation and silylation showed condensed clustering which indicated higher repeatability (95 % confidence) (Figure 4.2A).

4.4 Targeted analysis of selected FAMEs to characterise homogenised soil samples

The TLE analysis applied to the homogenised soil samples rendered significantly ($p<0.05$) higher concentrations for the total FAMEs and individual FAMEs, than fractionated PLFA analysis (Table 4.3). This was also seen in the pure culture samples. Such higher FAME yields have also been reported by Drenovsky *et al.* (2004) and Acosta-Martínez *et al.* (2010), who both used a MIDI-protocol instead of the TLE analysis used in this study on soil. The MIDI-protocol (like the TLE analysis in this study) does not make use of a fractionation step. Basically, the MIDI-protocol differs from the current TLE analysis as follows: the total lipids are extracted with sodium hydroxide (NaOH) dissolved in aqueous methanol and subsequently methylated either with methanolic KOH or methanolic hydrochloric acid (Schutter and Dick 2000). The high FAME concentrations could be ascribed to the complex mixture of lipids included in the TLEs (as described in section 4.1). The TLE method provided higher repeatability (95% confidence) than the fractionated PLFA analysis with the same derivatisation techniques, namely (i) methylation combined with silylation and (ii) methylation

combined with oximation and silylation (Figures 4.3A and 4.3B). From these results, it is evident that both the fractionated PLFA and TLE analyses possess the ability to detect the selected FAMEs from homogenised soil.

As expected, the pure culture samples had higher concentrations of total and individual FAMEs than the soil samples due to a higher number of cells included in the sample weight. However, it should be noted that the soil matrix can also interfere with the detection and extraction of target compounds (in this case, FAMEs) from the soil (Nielsen and Petersen 2000; Baumann *et al.* 2014; Dominguez *et al.* 2015) and may therefore influence FAME abundance.

4.5 Most appropriate derivatisation technique for characterisation of homogenised soil

The FAME concentrations obtained from the homogenised soil samples were not only determined by the extraction method, but also by the derivatisation technique used. It is evident that the derivatisation technique had a greater influence on the TLE samples than on the fractionated PLFA samples. The TLE samples showed statistically significant ($p<0.05$) differences between the MB and MMB techniques, while the fractionated samples showed no statistically significant differences (Table 4.3). The MMB technique provided higher concentrations of the 18:1 geometric isomers (18:1ω7c and 18:1ω7t), than MB. This was also seen in the pure cultures with the 16:1 (16:1ω7c and 16:1ω7t) and 18:1 (18:1ω7c and 18:1ω7t) isomers. The reason for the higher concentrations of these specific fatty acids with the addition of MeOX prior to silylation is currently unclear.

For the fractionated samples, the mild alkaline methanolysis technique (M) displayed high repeatability among replicates when used without follow-up derivatisation (Figures 4.3A and 4.3B). The concentrations of the total and individual FAMEs did not differ significantly ($p<0.05$) between derivatisation techniques (Table 4.3). The combination of methanolysis and silylation (MB) as well as methanolysis followed by oximation and silylation (MMB) showed higher variability between replicates (Figure 4.3A). These results are contradictory to those of the pure culture samples, where the fractionated samples consistently showed higher repeatability than the TLE samples, irrespective of the derivatisation technique (Figure 4.1A). Based on the results for the TLE samples, the MMB technique is more appropriate for derivatisation since it gave higher FAME concentrations in most cases but similar confidence intervals than the MB technique.

4.6 Untargeted analysis of metabolite profiles to characterise homogenised soil samples

The PCA of the metabolite profiles of the soil samples again demonstrated that the derivatisation technique had a major influence on the clustering observed (Figures 4.4A and 4.4B). The techniques

with methylation before further derivatisation (S TL M BSTFA and S TL M MeOX BSTFA) showed higher similarity, than the samples without methylation (S TL MeOX BSTFA) due to the presence of FAMEs. The techniques that included an oximation step (MeOX) provided a higher repeatability, than those without it, which was also evident in the pure cultures. As explained in 4.2, the addition of MeOX for derivatisation improves the sample analysis since fewer tautomeric TMS derivatives will form and subsequently the resulting chromatogram will be cleaner with less interference from multiple peaks. This will have a positive effect on the qualitative data analysis (i.e. deconvolution, alignment) and give a more reproducible quantitative result.

5 Conclusions

This investigation indicated that the extraction method used for pure cultures or soil influences the FAME yield. The TLE method extracted higher concentrations of FAMEs in the targeted analysis of both pure cultures and homogenised soil, than fractionated PLFA analysis. With the pure culture samples, the TLE method showed lower repeatability than the fractionated method (95% confidence). On the other hand, the TLE method showed repeatability comparable to that of the fractionated method in the soil samples under certain derivatisation conditions. It can be concluded that when analysing pure culture samples, the extraction technique has a greater influence on repeatability than the derivatisation technique. When analysing soil samples, however, the derivatisation technique has a greater influence than the extraction technique. Therefore, sample matrix should be a consideration in determining the most feasible protocol for FAME analysis. Clearly, fractionated and TLE extraction methods may be applied for targeted analysis of FAMEs, however, analyses from a complex matrix such as soil will benefit from the correct derivatisation procedure.

For the untargeted analysis of both pure cultures and homogenised soil samples, the metabolite profiles of the samples were highly related to the derivatisation technique used. Similar results, in terms of the sample clustering and method repeatability, were found for pure cultures and soil samples. The importance of oximation as an additional step before silylation was evident in the higher repeatability of the techniques containing this step.

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Chapter 5
Evaluation of different approaches to distinguish between soil microbial communities after exposure to fumigants

1 Introduction

Fumigation forms an essential part of agricultural practices to ensure the successful control of soil pathogens threatening crop quality and yield. However, due to the high volatility and non-specific targeting of chemical fumigants such as metham sodium, the disturbance and complete destruction of native soil microbial communities by chemical fumigant applications have been reported (Klose *et al.* 2006; Omirou *et al.* 2011). Therefore, the use of green manure crops applied as biofumigants has been widely proposed as an alternative to chemical fumigants (Larkin and Griffin 2007; Wang *et al.* 2009; Szczygłowska *et al.* 2011). Biofumigation involves the suppression of soil-borne pathogens by the incorporation of plant green manure containing glucosinolates, into the soil (Angus *et al.* 1994). Plants belonging to the family Brassicaceae contain glucosinolates that are hydrolysed into isothiocyanates, nitriles and thiocyanates upon tissue degradation (Mithen 2001; Yulianti *et al.* 2007). As these products, especially isothiocyanates, cause the degradation of enzymes by disrupting protein bonds, they are highly toxic to various soil microorganisms and can act as biofumigants (Brown and Morra 1997). Biofumigants have been shown to inhibit the growth of nitrifying bacterial communities (Bending and Lincoln 2000), soil-borne pathogens of potato (Larkin and Griffin 2007) and rhizoctonia root rot of sugar beet (Motisi *et al.* 2013). Also, changes to the native soil microbial communities have been observed during biofumigant incorporations (Omirou *et al.* 2011; Potgieter *et al.* 2013; Fouché 2014). Besides signature lipid biomarker analysis, these changes could also be described by metabolomics analysis. Differentiation by using metabolite profiles has been observed in environmental studies of varying sites or exposures investigating earthworm species (Brown et al. 2014; Lankadurai et al. 2015) and plants (Petersen et al. 2011; Ribeiro et al. 2014) and the potential of soil metabolomics warrants investigation here.

In this chapter, fractionated phospholipid fatty acid (PLFA) and metabolomics analyses were compared for their ability to distinguish between soil microbial communities exposed to different fumigant treatments in a greenhouse experiment. The soil fumigants included the biofumigants canola (*Brassica napus*) and mustard (*Brassica juncea*), as well as the chemical fumigant, metham sodium.

2 Materials and methods

2.1 Chemicals and glassware

The following high purity solvents (Honeywell Burdick & Jackson®) were purchased from Anatech Instruments (Pty) Ltd. (Olivedale, South Africa): acetone, chloroform, hexane, methanol and water. Potassium hydroxide (KOH) and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck Millipore (Modderfontein, South Africa). Glacial acetic acid; silicic acid; 2,2,4-trimethylpentane (isooctane); N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA + TMCS, 99:1), hereafter referred to as BSTFA-TMCS; methoxyamine hydrochloride and pyridine were obtained from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). Internal standards were used for quantification and quality control purposes and also purchased from Sigma-Aldrich (Pty) Ltd. (Johannesburg, South Africa). The internal standard (IS) methyl nonadecanoate (C19:0 FAME) consisted of C19:0 FAME dissolved in isooctane (50 pmol ml^{-1}), whereas the IS mix consisted of L-norleucine, 3-phenylbutyric acid, 2-acetamidophenol and nonadecanoic acid, dissolved in methanol (5 µg ml^{-1}). Glassware as required was purchased from Lasec South Africa (Pty) Ltd. (Midrand, South Africa).

All glassware used for the analytical procedures were washed with tap water and phosphate-free detergent (Liqui-Nox®, Alconox Inc., Separations, Johannesburg, South Africa), rinsed thoroughly with tap, deionised and nano-pure water, air-dried and heated in a muffle furnace at 450°C for 4 h to remove any possible lipid contaminants. The Teflon-lined caps were washed with phosphate-free detergent, rinsed with tap, deionised and nano-pure water, air-dried and sonicated in acetone for 2 min.

2.2 Experimental design

The experiment was conducted in a greenhouse (coordinates 26°41’358’’S, 27°05’437’’E and 1356 m above sea level) at the North-West University, Potchefstroom, South Africa. The greenhouse was maintained at a 15 h:9 h (light:dark) photoperiod and the temperature ranged between 26°C (day) and 20°C (night). A randomised block design was used to place 12 plastic pots (30 cm), filled with homogenously mixed agricultural soil, in the greenhouse. Soil physical and chemical analyses were performed by an independent laboratory, Eco-Analytica (Potchefstroom, South Africa) according to standard procedures and the soil properties were characterised as indicated in Table 5.1. The 12 pots consisted of four treatments of 3 replicates each, which included the treatments: (i) soil only (control), (ii) soil with canola (*B.napus*) green manure, (iii) soil with mustard (*B.juncea*) green manure and, (iv) soil fumigated with metham sodium. After soil fumigant applications, the pots were kept at 50% water holding capacity to ensure sufficient release of isothiocyanates (Morra and Kirkegaard 2002). The experiment was conducted for 28 days (Fouché 2014).

Table 5.1: Selected properties of soil used in the greenhouse experiment

Properties	
pH (H_2O)	6.950 ± 0.042
EC (mS cm^{-1})	0.370 ± 1.155
CEC (cmol kg^{-1})	10.810 ± 0.310
Total C (%)	1.100 ± 0.055
Total N (%)	0.060 ± 0.007
Organic C (%)	1.060 ± 0.060

All values ± standard deviations represent results obtained from replicates (n=3).
Key to abbreviations: C – carbon; CEC – cation exchange capacity; EC – electrical conductivity; H_2O – water; N – nitrogen.

Prior to the start of the experiment, canola (*B.napus*) and mustard (*B.juncea*) (Mayford, Sakata seed Southern Africa (Pty) Ltd., Lanseria, South Africa) were sown in 30 cm plastic pots filled with agricultural soil and maintained until early flowering stage (~80 days of growth) (McCully *et al.* 2008). The glucosinolate levels are the highest in *Brassica* roots during this growth stage (Sarwar and Kirkegaard 1998). On day 0 of the experiment, all the plant material was removed from the pots, chopped into small pieces (the whole plant – roots, shoots and leaves) and incorporated at a rate of 15 g kg^{-1} soil at a depth of 10-20 cm into the relevant treatment pots (Omirou *et al.* 2011). Soil treatments without added green manure were also mixed at a depth of 10-20 cm to ensure similar soil disruption (Potgieter *et al.* 2013). The commercial soil fumigant, metham sodium (HERBIFUME®, 510 g L^{-1}, Nulandis®, Lilianton, South Africa) was diluted to an aqueous solution of 10 g L^{-1} and applied to the treatment pots at a recommended application dosage of 300 μg g^{-1} (Omirou *et al.* 2011), on day 0 of the experiment. The soil was mixed at a depth of 10-20 cm to ensure the even distribution of the chemical fumigant.

The results obtained from the homogeneous soil samples (Chapter 4), showed that the correct derivatisation technique was an important consideration in both targeted and untargeted analyses. Based on these results, mild alkaline methanolysis was applied with the fractionated PLFA method. For the metabolomics analysis, methylation followed by oximation and silylation was applied to the organic phases and oximation followed by silylation to the aqueous phases. The experimental conditions for the analysis of each soil sample are provided in Table 5.2. All procedures were conducted on three replicates of each treatment.

Table 5.2: Experimental conditions for methods compared in the greenhouse experiment

	PLFA analysis	Metabolomics-based approach
Extraction procedure	Modified Bligh & Dyer (phospholipid fraction)	Modified Bligh & Dyer (organic and aqueous phases)
Fractionation procedure	Silicic acid column chromatography	No fractionation
Derivatisation procedure	Mild alkaline methanolysis	Organic phase –mild alkaline methanolysis with oximation and silylation Aqueous phase – oximation and silylation
Quantification	GC-MS	GC-MS

Key to abbreviations: GC-MS – gas chromatography - mass spectrometry; PLFA – phospholipid fatty acid.

2.3 Sample preparation

Soil samples were collected for the control and for each treatment 0, 14 and 28 days after fumigant incorporation. The samples were obtained from the top 0-15 cm soil layer using aseptic methods. On each of the three sampling days, three subsamples were collected from each replicate pot and pooled to obtain one composite sample per pot. Therefore, three composite samples (from three replicate pots) were obtained for each treatment. Soil samples were frozen, lyophilised and stored at -80°C until further use. Before analysis, the samples were mixed thoroughly, homogenised and sieved (2 mm) to remove all visible plant material (Lou *et al*. 2006; Xiaolong *et al*. 2014).

2.4 Extraction procedures

Lyophilised soil samples were extracted with the single-phase chloroform-methanol-phosphate buffer solvent mixture of Bligh and Dyer (1959), as modified by White *et al*. (1979). Briefly, whole lipids were extracted from ~5 g soil with 5 ml chloroform, 10 ml methanol and 4 ml phosphate buffer (50 mM) (1:2:0.8; *v/v/v*). The samples were allowed to extract for 2 h at room temperature before centrifugation for 15 min at 1800 rpm. The supernatants were decanted into clean test tubes. The resulting pellets were re-extracted with 5 ml chloroform and the collected supernatants combined. The addition of 5 ml of water induced phase separation. Tubes were shaken gently and left to separate overnight, at room temperature. The lower organic phase (chloroform) was transferred to a clean test tube, evaporated to dryness at 37°C under a gentle stream of N_2 and stored at -20°C until further use; this represents the TLE. For the metabolomics-based approach, 8 ml of the upper aqueous layer was also collected and evaporated to complete dryness at 37°C. For PLFA analysis, the TLEs obtained from lipid extraction were dissolved in chloroform (3 x 150 µl), loaded onto pre-conditioned glass columns containing 0.5 g activated silicic acid and fractionated into neutral, glyco- and phospholipids, with chloroform (2 x 2.5 ml), acetone (2 x 2.5 ml) and methanol (4 x 2.5 ml), respectively (Guckert *et al*. 1985). For the purpose of this investigation, the neutral and glycolipids were discarded and only the phospholipid fractions were dried down under a gentle stream of N_2 at 37°C and stored at -20°C until further use.

2.5 Derivatisation procedures

The phospholipid fractions for PLFA analysis underwent mild alkaline methanolysis (methanolic KOH) to form fatty acid methyl esters (FAMEs) (White *et al.* 1979). In short, the dried residues obtained from the extraction procedures were suspended in 0.5 ml chloroform and 0.5 ml methanol, to which 1 ml methanolic KOH (0.2 mol L^{-1}) was added, capped and heated for 30 min at 40°C, followed by cooling to room temperature. The FAMEs were extracted by the addition of 2 ml hexane, 200 µl glacial acetic acid (1 mol L^{-1}), 2 ml nano-pure water and centrifugation for 5 min at 2000 rpm. The upper hexane layers were recovered in clean test tubes and the lower phases washed three times with 2 ml hexane. The pooled hexane layers were evaporated under a gentle stream of N_2, transferred to GC-vials by dissolving in 3 x 100 µl hexane and again dried under N_2 at 37°C. Prior to GC-MS analysis of the fractionated samples, the dried FAMEs were redissolved in 50 µl of C19:0 FAME IS.

The TLEs were methylated to form FAMEs, followed by oximation with methoxyamination solution (MeOX) and silylation with BSTFA-TMCS to ensure the formation of trimethylsilyl (TMS)-derivatives of those compounds that were not methylated (Lindeque 2011). The dried TLEs were methylated and evaporated to dryness in GC-vials. To each vial, 150 µl of C19:0 FAME IS and 50 µl of IS mix were added and the total solution dried under N_2 at 37°C. The dried solutions were resuspended in 50 µl MeOX prepared from methoxyamine hydrochloride dissolved in anhydrous pyridine (20 mg ml^{-1}) and incubated for 1 h at 60°C. Next, 50 µl BSTFA-TMCS was added to each sample and incubated for 1 h at 60°C (Venter *et al*. 2015). After incubation, 50 µl isooctane was added to each vial and the samples were ready for GC-MS analysis.

The dried aqueous phases for the metabolomics-based approach (8 ml aliquots) were subjected to oximation and silylation. Briefly, the dried aqueous phases were redissolved in 3 x 100 µl methanol:water (1:1; *v/v*), transferred to GC-vials and dried down under N_2 at 37°C. Each vial received 150 µl of C19:0 FAME IS and 50 µl of IS mix; these standard solutions were evaporated under a gentle stream of N_2 at 37°C. Next, 50 µl MeOX was added to each dry sample and kept at 60°C for 1 h (Venter *et al.* 2015). After the addition of 50 µl BSTFA-TMCS, the samples were heated at 60°C for 1 h, followed by the addition of 50 µl isooctane prior to GC-MS analysis.

2.6 Data acquisition

An Agilent GC-MS instrument (Agilent Technologies, Wilmington, Delaware, USA) consisting of a 7890A gas chromatograph with a split/splitless injector (280°C), equipped with a 7683B autosampler coupled to a 5975B inert XL mass selective detector, was used for lipid analysis. The gas chromatograph was equipped with an SPB-1 column (60 m x 0.25 mm x 0.250 µm film thickness)

(Supelco, Sigma-Aldrich, Johannesburg, South Africa) using helium as the carrier gas at a flow rate of 1.0 ml min^{-1}. The initial oven temperature was 60°C for 2 min, ramping to 150°C at 10°C/min and then to 320°C at 3°C/min, followed by a hold at 320°C for 2 min. The transfer line temperature was set to 280°C, the source temperature at 230°C and the MS Quad at 150°C. The mass analyser performed full scans throughout the run (40-450 m/z) at 175 scans per minute. Electron impact voltage of 70 eV with a dwell time of 100 was used. Samples (1 µl) were injected in splitless mode.

2.7 Data analysis

GC-MS spectra were identified by spectral analysis using the Automated Mass Spectral Deconvolution and Identification System (AMDIS V 2.71) available from National Institute of Standards and Technology (NIST) (Max Planck Institute, Golm, Germany), the NIST 2008 mass spectral library and in-house FAME databases. For the identification of a compound, five of its qualifier ions must be matched with ions in the library. A match factor over 60% is preferred (Reinecke *et al.* 2011). The raw data from the GC-MS was processed by AMDIS and resulted in two types of output files that can be used for statistical analysis, namely the elute data file (.elu) and the find compound data file (.fin). The elute data file contains a list of all the peaks found, including ions and abundances. This file contains unidentified compounds not in the database (Stein 1999; Lindeque 2011). The find data file contains a list of all of the compounds found in the database. The resulting data was imported into Agilent's MassHunter Mass Profiler Professional (MPP) (B.02). First, the found peaks in each data file across all files are aligned. Mass Profiler Professional determines which peaks in each chromatographic run are the same compound and which are different. Compound identification prior to peak alignment makes this task much easier for the identified compounds. Prior identification of compounds using AMDIS is thus very helpful in the statistical analysis of data sets. Once all data were aligned, data were imported into MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) for a number of interpretation algorithms to find the compound relationships in regards to the experiment (Xia *et al.* 2009, 2015). Clustering and score plots obtained by principal component analysis (PCA) were also performed in MetaboAnalyst 3.0. Statistical analyses were performed with Statistica 12.0 (StatSoft, Inc., Tulsa, Oklahoma, USA). One-way analysis of variance (ANOVA) was used, followed by the Tukey's test to determine statistically significant differences ($p<0.05$) between the various methods.

Standard fatty acid nomenclature was used for the representation of all results; this follows the form A: BωC. "A" is the total number of carbon atoms, "B" is the number of double bonds and "C" is the position of the first double bond from the aliphatic (ω) end. The suffixes "c" and "t" indicate *cis* and *trans* conformations. All double bonds are supposedly *cis*. Anteiso- and iso-branching are indicated by the prefixes "a" and "i", "OH" and "Me" refer to a hydroxyl group and a methyl group,

respectively, and the preceding number indicates the position from the carboxyl end of the molecule; "cy" shows a cyclopropyl fatty acid (Guckert *et al*. 1985; Ringelberg *et al*. 1989; Zelles 1999).

3 Results

When it comes to the characterisation of detected analytes in a sample, two metabolomic approaches are generally followed. Targeted analysis focuses specifically on a predefined class of metabolites driven by a hypothesis (Creek and Barrett 2014). In contrast, untargeted analysis provides a comprehensive profile of all the detectable metabolites in a sample, irrespective of class type. Thus, untargeted analysis usually generates a hypothesis (Dunn *et al*. 2013). In this investigation, targeted analysis focused only on FAMEs, while a complete metabolic profile was obtained with untargeted analysis. As described in Chapter 4, the metabolomics-based approach consisted of the separate analysis of the organic phase or TLE (containing apolar metabolites) and the aqueous phase (containing polar metabolites). For the targeted analysis of FAMEs from the metabolomics-based approach, only the TLE results are discussed since the aqueous phase does not contain any FAMEs. In contrast, for the untargeted analysis of metabolites in the soil samples, both the organic (TLE) and aqueous phase results are interpreted as the metabolomics analysis.

3.1 Ability to distinguish between soil microbial communities based on FAME profiles

A total of 26 FAMEs, ranging from 14 to 24 carbon atoms, were detected in the soil samples from the fumigation experiment. The microbial community structure between treatments was compared by grouping individual FAMEs into the structural groups generally applied for this purpose (Table 3.1, Chapter 3). Box-and-whisker plots illustrating the differences among the treatments over the 28 days based on the structural group distributions are shown in Figures 5.1 and 5.2. The FAMEs used for each structural group in the present set of results, are indicated in Table 5.3. Both methods were able to distinguish between the four treatments at different sampling periods (Figures 5.1 and 5.2).

Table 5.3: Characteristic fatty acids classified into major structural groups

Fatty acid structural groups	Individual fatty acids
Normal saturated - *Nsats*	14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0, 23:0, 24:0
Mid-chain branched saturated - *MBsats*	10Me15:0, 10Me16:0, 12Me16:0, 10Me17:0, 10Me18:0, 12Me18:0
Terminally branched saturated - *TBsats*	i15:0, a15:0, i16:0, i17:0, a17:0
Monounsaturated - *Monos*	16:1ω7c, cy17:0, 18:1ω7c, 18:1ω7t, cy19:0
Polyunsaturated - *Polys*	18:2ω6

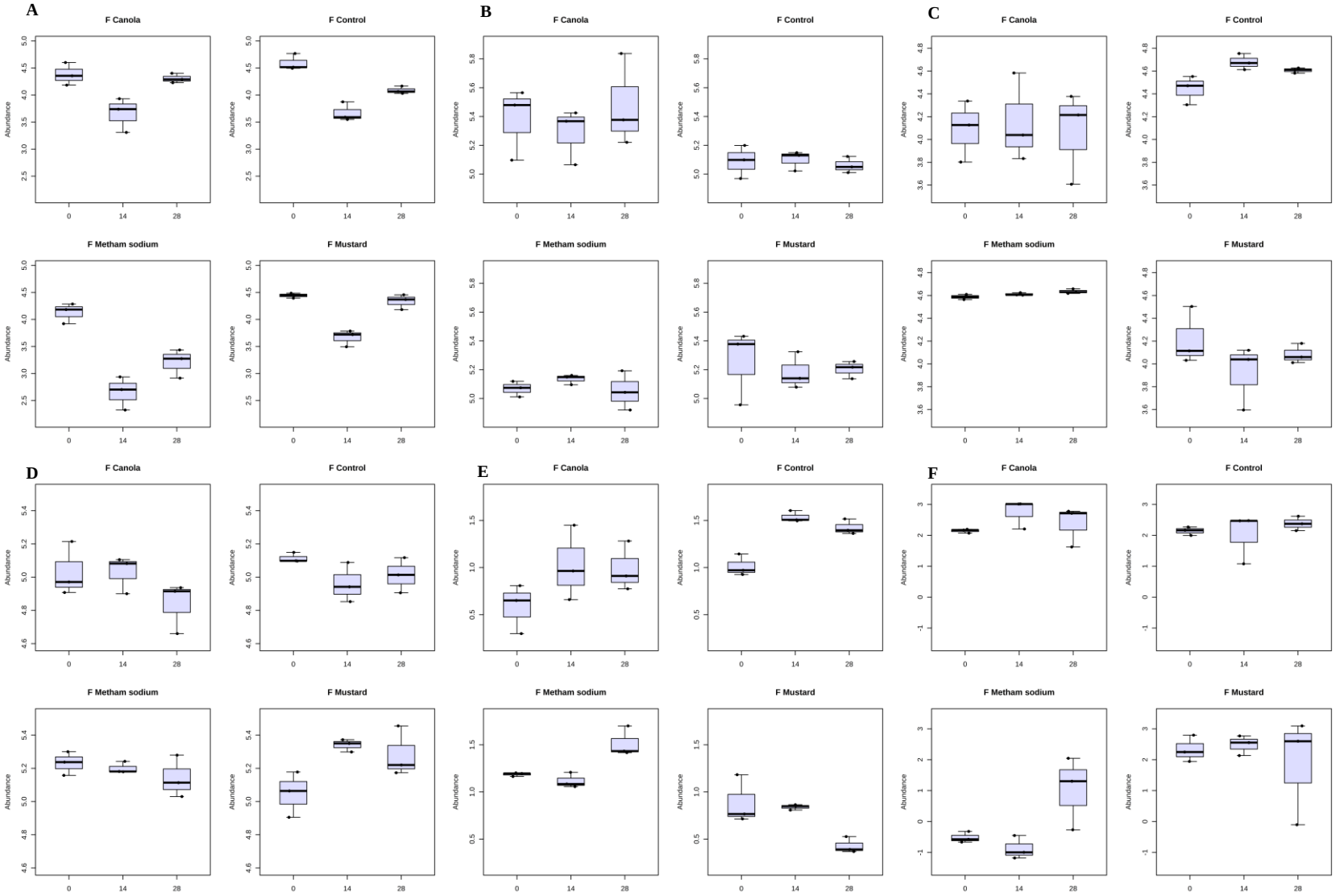

Figure 5.1: Box-and-whisker plots indicating the major structural fatty acid groups for each treatment over time, as obtained by fractionated PLFA analysis. Groups include A) biomass; B) normal saturated; C) mid-chain branched; D) terminally branched; E) monounsaturated and; F) polyunsaturated fatty acids.

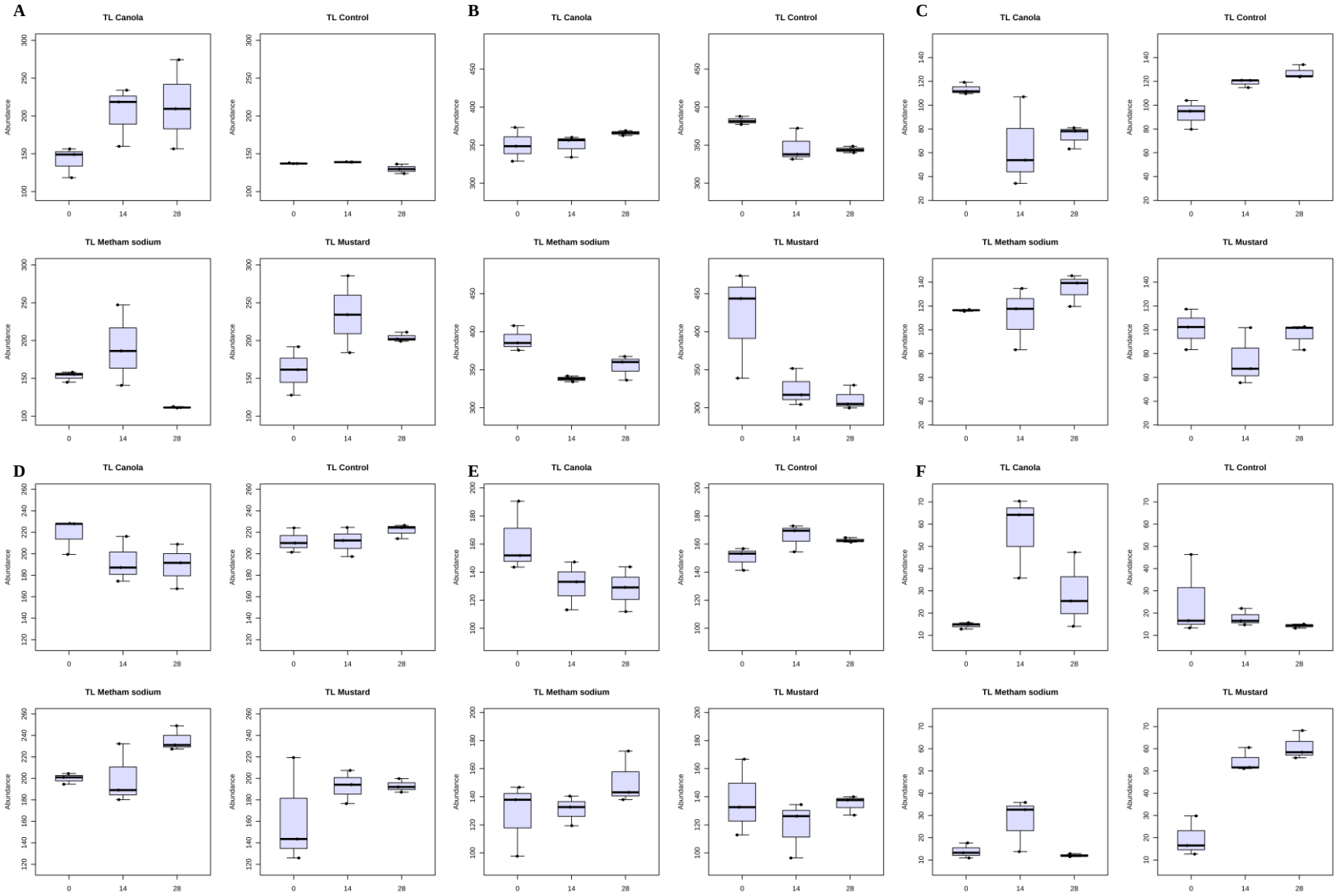

Figure 5.2: Box-and-whisker plots indicating the major structural fatty acid groups for each treatment over time, as obtained by TLE analysis. Groups include A) biomass; B) normal saturated; C) mid-chain branched; D) terminally branched; E) monounsaturated and; F) polyunsaturated fatty acids.

The microbial community structure for the different fumigation treatments based on FAMEs from PLFA and TLE analysis is shown in Table 5.4. The portion of the total fatty acid content represented by each structural group was displayed as a percentage (%) value of the total FAME concentration for each treatment on each sampling day. Significant differences were indicated between PLFA and TLE analysis for the same treatment on the same day by capital letters. For example, with PLFA analysis, Nsats (%) on Day 0 for the control treatment was 34.108 ± 2.690 with the statistically significant grouping indicated by "A". For TLE analysis, Nsats (%) on Day 0 for the control treatment was 42.601 ± 0.584 with the statistically significant grouping indicated by "B". Thus, there was a statistically significant ($p<0.05$) difference between Nsats on Day 0 for the control treatment based on the method used. Statistically significant differences were also indicated for the same method between different treatments by lower case letters shown in brackets. For example, with PLFA analysis, Nsats (%) on Day 0 for the control treatment was 34.108 ± 2.690 with the statistically significant grouping indicated by "(a)". For canola it was 42.066 ± 6.934, also with the statistically significant grouping indicated as "(a)" and similarly for mustard (38.605 ± 6.573) and metham sodium (30.982 ± 1.011) followed by "(a)". This means that there were no statistically significant differences between the treatments on day 0 when analysed with the PLFA method.

The total concentration of FAMEs, which is an indication of viable microbial biomass (White *et al.* 1979), was significantly ($p<0.05$) higher with TLE analysis than with fractionated PLFA analysis for all treatments (indicated by capital letters in Table 5.4). Statistically significant differences ($p<0.05$) in the group abundances between PLFA and TLE analysis were also found. The mid-chain branched saturated fatty acids (MBsats) and terminally branched saturated fatty acids (TBsats) contributed significantly higher fractions of the total fatty acid content when PLFA analysis was used in comparison to TLE analysis. Polyunsaturated fatty acids (Polys) were also higher with PLFA than with TLE, although not significantly in all cases. Conversely, TLE analysis extracted higher normal saturated fatty acids (Nsats) and monounsaturated fatty acids (Monos) than PLFA analysis. These differences were statistically significant in all cases for Monos, but not for Nsats.

Results (Table 5.4, lower case letters in brackets), showed no significant ($p<0.05$) differences between the four treatments on day 0. This was expected, since the experiment started on day 0 and the same soil was used in all treatment pots. The results showed that PLFA analysis was able to detect a much higher number of statistically significant differences between treatments on the same day than TLE analysis. With TLE analysis, the only significant differences between treatments were on day 14 for Polys where canola and mustard differed significantly from the control and the metham sodium treatment. PLFA analysis showed significant differences between all treatments on day 14 for Polys and on day 14 and 28 for total FAMEs, MBsats, TBsats and Monos.

Table 5.4: Microbial community structure for different fumigation treatments based on FAMEs from PLFA and TLE analysis

nmol g^{-1}	Control			Canola			Mustard			Metham sodium		
	Day 0	14	28	0	14	28	0	14	28	0	14	28
PLFA												
Total FAMEs	2.422±0.264 A(a)	1.279±0.160 A(a)	1.705±0.087 A(a)	2.097±0.307 A(a)	1.284±0.271 A(a)	1.981±0.123 A(a)	2.175±0.071 A(a)	1.274±0.132 A(a)	2.025±0.193 A(a)	1.904±0.214 A(a)	0.639±0.129 A(b)	0.935±0.167 A(b)
Nsats (%)	34.108±2.690 A(a)	34.340±1.618 A(a)	33.331±1.358 A(a)	42.066±6.934 A(a)	39.238±5.065 A(a)	45.337±10.478 A(a)	38.605±6.573 A(a)	36.383±3.284 A(a)	36.876±1.543 A(a)	30.982±1.011 A(a)	35.125±1.052 A(a)	33.251±3.154 A(a)
MBsats (%)	24.780±2.337 B(a)	27.128±1.295 B(a)	26.477±0.530 B(b)	19.150±3.420 A(a)	19.037±5.592 A(ab)	18.470±5.165 A(a)	21.127±3.976 B(a)	15.599±2.833 A(b)	17.528±1.030 B(a)	24.413±0.444 B(a)	25.396±0.607 B(a)	26.050±0.578 B(b)
TBsats (%)	34.663±0.738 B(a)	31.222±2.596 B(a)	32.266±2.353 B(ab)	32.838±3.781 B(a)	32.719±2.490 B(ab)	28.685±2.962 A(a)	33.212±3.142 B(a)	40.527±1.044 B(c)	39.071±4.199 B(b)	34.739±2.242 B(a)	36.755±0.689 B(bc)	35.355±3.164 B(ab)
Monos (%)	2.024±0.167 A(a)	2.902±0.118 A(b)	2.683±0.158 A(bc)	1.516±0.267 A(a)	2.086±0.588 A(a)	2.007±0.373 A(ab)	1.873±0.346 A(a)	1.792±0.035 A(a)	1.345±0.081 A(a)	2.103±0.030 A(a)	2.169±0.132 A(ab)	2.870±0.325 A(c)
Polys (%)	4.425±0.412 A(a)	4.408±1.991 A(ab)	5.243±0.856 B(a)	4.429±0.203 B(a)	6.920±2.004 A(a)	5.501±2.106 A(a)	5.183±1.590 A(a)	5.699±1.230 A(a)	5.179±3.882 A(a)	7.763±2.065 B(a)	0.555±0.156 A(b)	2.473±1.645 A(a)
TLE												
Total FAMEs	16.200±0.057 B(a)	16.035±0.091 B(a)	16.685±2.930 B(a)	16.367±2.604 B(a)	25.950±6.242 B(a)	18.263±4.708 B(a)	19.040±4.523 B(a)	22.551±5.004 B(a)	17.572±1.444 B(a)	17.850±0.969 B(a)	16.298±0.969 B(a)	16.993±1.583 B(a)
Nsats (%)	42.601±0.584 B(a)	40.031±2.386 B(a)	45.158±7.571 A(a)	40.542±3.249 A(a)	43.437±3.780 A(a)	47.032±10.056 A(a)	49.647±10.107 A(a)	41.668±2.960 A(a)	44.950±2.529 B(a)	45.495±1.989 B(a)	42.621±1.978 B(a)	38.864±0.454 B(a)
MBsats(%)	14.184±1.418 A(a)	14.444±0.548 A(a)	13.783±0.896 A(a)	13.823±0.306 A(a)	9.523±3.575 A(a)	13.300±1.926 A(a)	12.829±1.654 A(a)	10.803±2.055 A(a)	14.594±0.266 A(a)	14.652±0.167 A(a)	12.879±0.938 A(a)	14.239±0.735 A(a)
TBsats (%)	23.614±1.258 A(a)	24.379±1.690 A(a)	20.911±4.553 A(a)	25.260±1.647 A(a)	23.772±1.481 A(a)	20.941±4.911 A(a)	19.093±5.074 A(a)	23.492±2.369 A(a)	23.339±0.791 A(a)	23.356±0.763 A(a)	25.640±1.351 A(a)	25.344±0.905 A(a)
Monos (%)	16.766±0.910 B(a)	19.097±1.166 B(a)	18.293±3.036 B(a)	18.704±2.552 B(a)	16.179±1.347 B(a)	16.575±2.565 B(a)	16.138±2.576 B(a)	16.625±0.901 B(a)	15.151±3.316 B(a)	14.870±2.999 B(a)	17.227±1.038 B(a)	18.948±1.397 B(a)
Polys (%)	2.835±2.035 A(a)	2.048±0.433 A(a)	1.854±0.147 A(a)	1.671±0.178 A(a)	7.089±2.499 A(b)	2.152±1.063 A(a)	2.294±0.961 A(a)	7.412±2.819 A(b)	1.966±0.638 A(a)	1.627±0.404 A(a)	1.632±0.220 B(a)	2.606±1.394 A(a)

Means ± standard deviations represent results obtained from sample replicates (n = 3). Statistically significant differences ($p<0.05$) are indicated by alphabetic letters: the same letters indicate no significant difference; capital letters indicate significant differences between PLFA and TLE analysis for the same treatment on the same day; lowercase letters in brackets indicate significant differences between the different treatments on the same day obtained with PLFA or TLE analysis. Standard fatty acid nomenclature was used (Guckert *et al.* 1985; Ringelberg *et al.* 1989; Zelles 1999). Key to abbreviations: FAMEs – fatty acid methyl esters; MBsats – mid-chain branched; Monos – monounsaturated; Nsats – normal saturated; Polys – polyunsaturated; TBsats – terminally branched.

An interactive Principal Component Analysis (iPCA) was conducted on the FAME profiles of the various treatments over 28 days, as extracted by fractionated PLFA analysis. In Figure 5.3, principal component (PC) axis 1 explained 57.4% of the total variance, whereas PC2 and PC3 explained 15.8% and 7.7% respectively. Clustering according to soil treatments was observed, with some clustering patterns being influenced by time trends. For all four treatments, the samples taken on day 0 grouped closer together, while more variability was observed for the fumigant treatments on day 14 and 28. Mustard and canola treatments displayed comparable patterns over the course of the experiment, and clearly differed from the metham sodium and control treatments. Due to their less condensed clustering, both the mustard and canola showed high sample-to-sample variability. In contrast, the control treatment had a more condensed clustering, which indicated lower sample-to-sample variability.

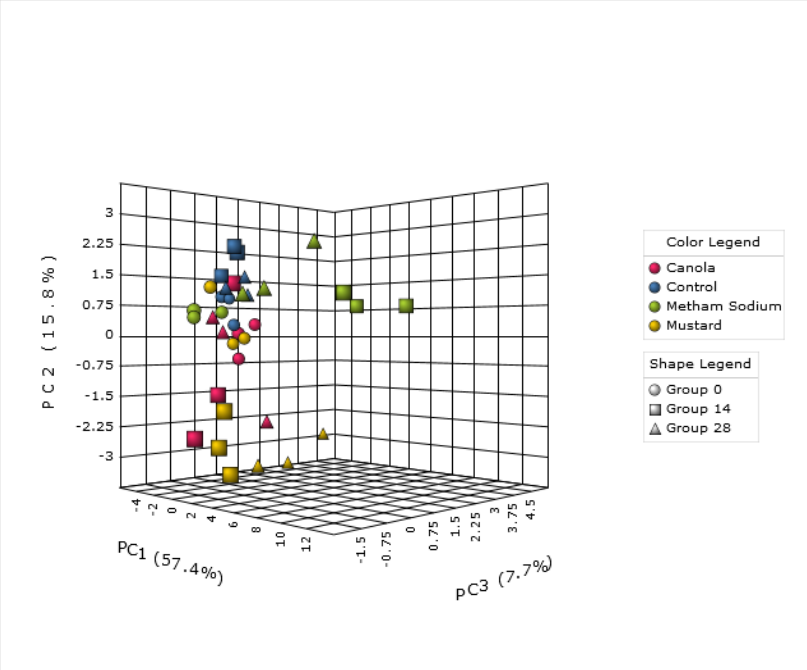

Figure 5.3: 3-D iPCA plot indicating the distribution of the samples for the different treatments over 28 days, as obtained by fractionated PLFA analysis. The three PC axes accounted for a total variance of 80.9%.

Even though the FAME profiles of the samples extracted by TLE analysis also grouped according to soil treatment, the iPCA plot (Figure 5.4) illustrated more overlaps among the different treatment distributions. In Figure 5.4, PC1 explained 41.8% of the total variance, whereas PC2 and PC3 explained 21.3% and 10.7% respectively. When compared to the fractionated PLFA analysis, the TLE method provided less distinguishable separation among the different treatments and the total variance accounted for was lower (73.8% compared to 80.9% for the PLFA). Again, the distributions of the mustard and canola treatments were more similar to each other than to the control and the metham

sodium treatment. Moreover, the clustering of individual samples of these two treatments showed higher sample-to-sample variability. The clustering of the control treatment indicated lower sample-to-sample variability.

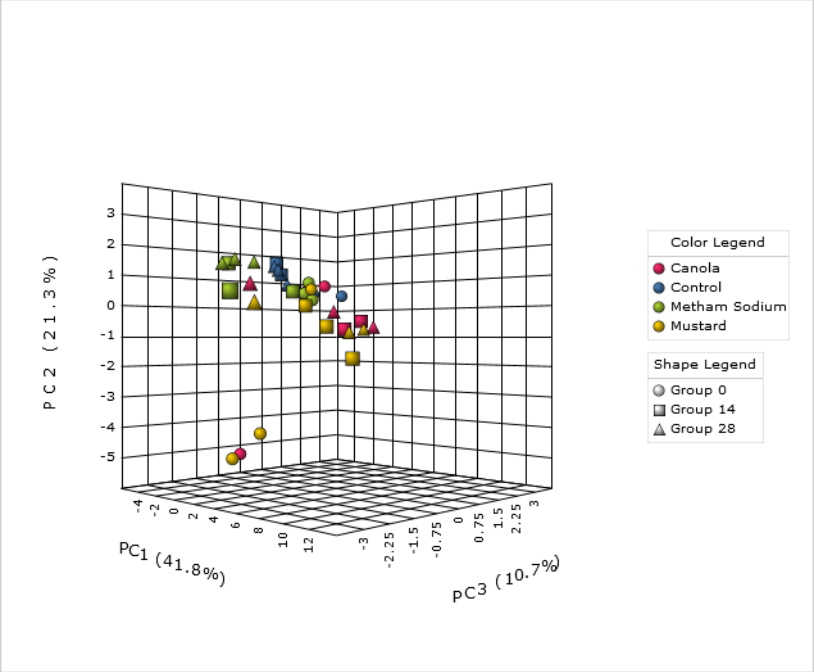

Figure 5.4: 3-D iPCA plot indicating the distribution of the samples for the different treatments over 28 days, as obtained by TLE analysis. The three PC axes accounted for a total variance of 73.8%.

3.2 Ability to distinguish between soil communities based on metabolite profiles

The average distributions of the four treatments, based on their metabolite profiles obtained from the organic and aqueous phases are shown in Figure 5.5. PC1 explained 13.3% of the total variance, whereas PC2 and PC3 explained 7.5% and 4.6% respectively. These values are much lower than those obtained in the iPCAs for the targeted analyses (Figures 5.3 and 5.4). The component axes combined, only accounted for 25.4% of the total variance. In the same way as the targeted analysis, the untargeted analysis showed similar distribution patterns for the canola and mustard treatments. These biofumigant treatments differed from the metham sodium treatment and all three fumigation treatments differed from the control.

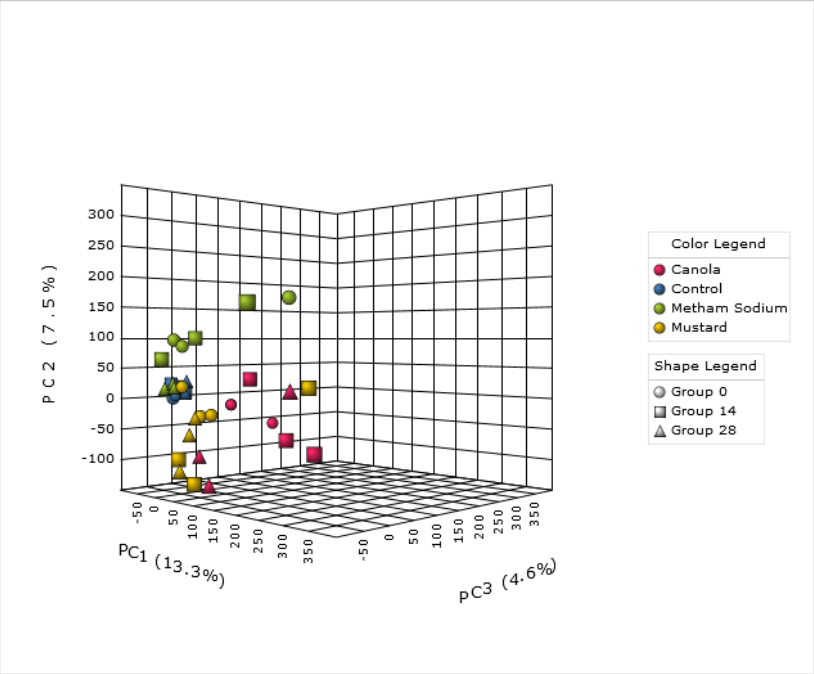

Figure 5.5: 3-D iPCA plot indicating the distribution of the samples for the different treatments over 28 days, as obtained by untargeted analysis. The three PC axes accounted for a total variance of 25.4%.

4 Discussion

4.1 Ability to distinguish between soil microbial communities based on FAME profiles

The TLE analysis extracted over six times more total fatty acids than fractionated PLFA analysis for each treatment over time (Table 5.4). This supports the observations of Drenovsky *et al.* (2004) who found on average seven-fold higher fatty acid concentrations in total soil FAME samples than PLFA samples. Given that TLE analysis extracts fatty acids from the neutral, glyco- and polar lipid fractions, whereas PLFA analysis uses only the phospholipid fraction (Kaur *et al.* 2005), the yield differences can be explained. Several of the detected fatty acids have been reported to occur in more than one lipid fraction such as 16:0, i16:0, 16:1ω7c and 18:2ω6 (Drijber *et al.* 2000; Bradley *et al.* 2007).

Certain fatty acids act as signature biomarkers for a specific group of microorganisms (Frostegård and Bååth 1996). By dividing these fatty acids into major structural groups, the distribution of microbial groups within the soil microbial community can be characterised. Fractionated PLFA analysis provided significantly ($p<0.05$) higher mole% values for the MBsats (representative of Actinomycetes), TBsats (indicative of Gram-positive bacteria) and Polys (indicative of fungi), than TLE analysis (Table 5.4). The Nsats (found in all bacteria) and Monos (indicative of Gram-negative

bacteria) where higher with TLE in comparison to PLFA analysis. Fernandes *et al.* (2013) and Drenovsky *et al.* (2004) also noted the higher concentrations of FAMEs indicative of Actinomycetes and other bacteria in PLFA extracted samples. Neutral lipids are usually present in high amounts in eukaryotic organisms (Bååth 2003) and will be extracted by TLE but not by PLFA analysis. Therefore, the yields of certain groups expressed as a percentage of the total FAMEs, will differ for the different extraction techniques. Both methods were able to distinguish between the four treatments at specific sampling times, based on the structural group distributions.

By comparing the iPCAs displayed separately for both methods (Figures 5.3 – PLFA and 5.4 – TLE), it was evident that the extraction method influenced the ability to discriminate between treatments. With TLE analysis differentiation between treatments was lower than for PLFA analysis. This observation is supported by the results in Table 5.4 that indicate very few statistically significant differences between treatments based on TLE analysis while PLFA analysis of the same treatments showed significant differences between all treatments on day 14 and 28 for the majority of microbial structural groups. This corresponded with the results found by Drenovsky *et al.* (2004). However, both Schutter and Dick (2000) and Drijber *et al.* (2000) noted comparable treatment discrimination between PLFA and TLE analysis. Both methods showed similar distribution patterns for the canola and mustard treatments and indicated higher sample-to-sample variability among the replicates of these two treatments compared to the control and the metham sodium treatment.

4.2 Ability to distinguish between soil communities based on metabolite profiles

Data obtained from the metabolomics analysis of the organic and aqueous phases were combined for the untargeted analysis. The TLE consisted of FAMEs and all apolar metabolites able to form TMS-derivatives, whereas the aqueous phase included polar metabolites (Chen *et al.* 2013). Even though the total variance explained by the component axes were much lower than for the targeted analysis, the clustering obtained from the untargeted analysis was similar to the targeted analysis and it was possible to distinguish between different treatments. From these results, the conclusion can be drawn that the effect of the chemical fumigant, metham sodium, was markedly different to that of the biofumigants (canola and mustard). Furthermore, it is clear that all the fumigants had an effect on the metabolite profiles obtained since these differed from the control. Omirou *et al.* (2011) and Fouché (2014) also observed considerable differences between the effects of chemical fumigants compared to biofumigants on the native soil microbial communities. It is also possible that the glucosinolates formed by the two *Brassica* species had an effect on the untargeted metabolite profiles because they would form TMS-derivatives. Here, it is important to note that the differences between treatments cannot be attributed solely to the soil microbial community since the metabolites of microorganisms cannot be distinguished from those of plants or other organisms that live in the soil. Therefore, the

contributors should rather be referred to as "soil communities" (Jones *et al.* 2014), than soil "microbial" communities. Indeed, Jones *et al.* (2014) proposed the term "community metabolomics" for the application of metabolomics analysis to soil. The study in question showed distinct groupings for soil communities living in different contaminated sites based on metabolite profiles. To the contrary, a study by Kakumanu *et al.* (2013) that investigated microbial community responses to desiccation in soil refers to the extraction of *microbial metabolites*. Such interpretations should be considered with caution, since it is unclear how these authors distinguish between metabolites originating from microorganisms and those from other biological sources.

5 Conclusions

This comparative investigation allowed the evaluation of the efficiency of PLFA analysis and a metabolomics-based approach to discriminate between soil microbial communities exposed to different fumigants in a greenhouse experiment. Two interpretational approaches were followed, namely targeted and untargeted analysis of the data. The targeted approach focused on a specific group of metabolites, in this case FAMEs. In contrast, the untargeted approach considered all metabolites in the samples. With the targeted approach the TLE analysis extracted higher concentrations of the total and individual FAMEs compared to PLFA analysis; however, the inclusion of non-microbial FAMEs is possible. Compared to PLFA analysis, TLE analysis provided a lower discrimination potential between the four soil treatments over time.

The untargeted approach showed potential to distinguish between samples based on different treatments in a similar way as the targeted approach with the important distinction that the untargeted metabolomics analysis should not be interpreted as a microbial profile alone. Also, changes in metabolite profiles do not necessarily signify changes in community structure as with signature lipid biomarkers but rather points to functional changes, which may or may not accompany structural changes. In this regard, the potential of metabolomics-based approaches to enhance the characterisation of soil communities, albeit not only microbial communities, is clear. However, since the distribution patterns in this study are quite similar for the targeted and untargeted approaches, it begs the question of just how great the contribution of microbial communities is in terms of the combined soil biological metabolite profile. If this is considered, the manner in which Kakumanu *et al.* (2013) refers to the extraction of microbial metabolites, may be more accurate than it seemed initially.

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Chapter 6
General discussion and conclusions

1 General discussion

This work started with literature-based comparisons of experimental procedures used for microbial signature lipid biomarker analysis (Chapter 2 and Chapter 3) and led to focused experimental work to compare different approaches – traditional and modern – to investigate soil microbial communities. During this investigation, the quantitative and qualitative data generation potential of a metabolomics-based approach to characterise the FAMEs of soil microbial communities were evaluated against that of traditional microbial lipid analyses, including fractionated PLFA and TLE analyses. The metabolomics-based approach entailed the analysis of both polar and apolar metabolites; these metabolites were analysed as two separate phases to ensure optimal recovery. The organic phase or total lipid extract (TLE) contained the apolar metabolites, including FAMEs, while the aqueous phase (water and methanol) contained the polar metabolites. Targeted and untargeted data analyses were used. For the targeted analysis the focus was set on FAMEs only, whereas untargeted analysis provided a representation of all the measurable metabolites in the samples.

The different methods (fractionated PLFA, TLE and metabolomics analysis) were evaluated for their capacity to analyse pure cultures and homogenised soil samples. During this experiment, different derivatisation techniques were also evaluated to identify the most appropriate technique for each method. The results obtained from this investigation, set out in Chapter 4, were considered and in Chapter 5 the ability of the fractionated PLFA and metabolomics analysis to discriminate between soil communities exposed to different fumigant treatments was assessed in a greenhouse experiment.

After consideration of the results obtained from this investigation, a standard operating procedure (SOP) for the analysis of microbial lipids as FAMEs was implemented in the Soil Microbiology Laboratory at the North-West University, Potchefstroom Campus. To achieve this final aim, several specific objectives were put forward at the start of the investigation and their outcomes are described below. The specific objective is given in bold format, followed by a brief discussion of how this objective was achieved.

- **A literature-based comparison of methods available for PLFA analysis on environmental samples focusing on method origin, modifications made to original methods and the advantages and limitations of each (Chapter 2)**

Despite the vast body of literature available on PLFA analysis, a comprehensive review of the range of methods and their modifications used to perform PLFA analysis on environmental samples, proved valuable. The most important contributions of this review included clarifications on the effectiveness of different modifications. The review was structured to evaluate different methods and their modifications in terms of the main steps of the analysis, namely extraction, fractionation, derivatisation and quantification. The reason for the variety of methods and for the many variations to existing methods is the common objective to achieve higher sample throughput with more accuracy, in a shorter period of time and with reduced solvent volumes. The conclusion was drawn that future advances and improvements on conventional signature lipid biomarker analysis will continue to be important. Even though it is not new, this approach is still relevant since the complexity of microbial communities in environmental samples means that no single type of analysis is sufficient for comprehensive characterisation. Signature lipid biomarker analysis therefore remains a firm component in the suite of approaches required for environmental studies of microbial communities.

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Willers, C., Jansen van Rensburg, P.J. and Claassens, S. (2015) Microbial signature lipid biomarker analysis – an approach that is still preferred, even amid various method modifications. *Journal of Applied Microbiology* **118**, 1251-1263.

- **A literature-based comparison of the current interpretations of generated PLFA data as applied in environmental studies (Chapter 3)**

The purpose of this review was to address the many controversies surrounding the interpretation of PLFA data. This was done by examining recent applications of PLFA analysis in environmental studies with specific reference to how interpretations were made and which original sources were referred to. It was found that different interpretations of the similar data sets can lead to conflicting conclusions in comparable studies. As the interpretations do not always correlate between investigations, discrepancies exist regarding the use of PLFA analysis to indicate viable microbial biomass, community structure based on signature lipid biomarkers and the physiological status of microbial communities. Several suggestions were provided to reduce the occurrence of such inconsistencies and refine the overall PLFA interpretations. Given that contrasting findings have been found on the turnover rates of PLFAs in changing environmental conditions, the term "viable" microbial biomass should be used with care. Also, to ensure comparable results among complex

sample matrices, microbial biomass should only be expressed as the total amount of extractable PLFAs (nmol or pmol) per weight of the sample. When the need arises for the estimation of the dominance of particular microbial groups within a microbial community, a range of fatty acid biomarkers should rather be used than a single biomarker by itself. In certain investigations the origin of the sample, be it aqueous or sedimentary, can also clarify the designation of a fatty acid if indicative of more than one microbial group. Though the shifts in the composition of fatty acids are still widely applied as physiological stress indicators, the possibility of these shifts being due to changes in the species composition should be kept in mind. Overall, the trends in similar studies regarding the structure and physiological state of microbial communities should be identified, before blindly interpreting PLFA results.

This chapter was published as:

Willers, C., Jansen van Rensburg, P.J. and Claassens, S. (2015) Phospholipid fatty acid profiling of microbial communities – a review of interpretations and recent applications. *Journal of Applied Microbiology* **119**, 1207-1218.

- **An evaluation of the targeted analysis of selected FAMEs to characterise both homogenised soil and pure bacterial cultures as obtained from fractionated PLFA, TLE and metabolomics analysis, respectively (Chapter 4)**

For the application of a targeted approach that considers only the FAMEs in a sample, only the TLE or organic phase from the metabolomics analysis could be used since no FAMEs are present in the aqueous phase. Therefore, the TLE extraction method and the extraction of the organic phase from the metabolomics analysis are essentially the same. A comparison was thus made between the FAMEs detected by PLFA and TLE. The TLE method extracted higher concentrations of FAMEs from both soil and pure cultures than the PLFA extraction method. However, the repeatability (sample-to-sample variability) differed with the PLFA method showing higher repeatability than TLE for the pure culture samples. For the soil samples, TLE was comparable to PLFA in terms of repeatability, but only under certain derivatisation conditions. Thus, when comparing extraction efficiency between methods, the derivatisation technique should also be included in the evaluation.

In order to not completely disregard the metabolomics approach, the untargeted analysis of the soil and pure culture samples were also investigated. These results could also only be considered meaningfully with the inclusion of the derivatisation technique and will be discussed under the next objective.

- **An evaluation to determine the most appropriate derivatisation technique for each of the three approaches by comparing the various derivatives produced during: mild alkaline methanolysis, a combination of mild alkaline methanolysis and silylation, mild alkaline methanolysis combined with oximation and silylation, and oximation followed by silylation (Chapter 4)**

To determine the best derivatisation technique for fractionated PLFA, TLE and metabolomics analysis, respectively, a targeted (FAMEs only) and an untargeted (all metabolites) approach was conducted. The FAME concentrations as well as sample-to-sample variability was considered. This investigation showed that when characterising pure culture samples, the extraction technique has a greater influence on repeatability than the derivatisation technique. However, when analysing soil samples, the derivatisation technique has the greater influence and therefore sample matrix should be considered in determining the most feasible protocol for FAME analysis.

For the characterisation of FAMEs from fractionated pure cultures (PLFA method), mild alkaline methanolysis without further derivatisation is recommended, while methanolysis with oximation and silylation is preferred for FAMEs from TLEs. Characterisation of FAMEs from soil samples with the PLFA method will benefit from methanolysis only, while TLE analysis showed the best overall results with methanolysis followed by oximation and silylation. The untargeted approach showed better results with the inclusion of an oximation step and this was the case for pure cultures and soil samples as well as for both the organic and aqueous phases.

- **An evaluation of the efficiency of fractionated PLFA and metabolomics analysis, respectively, to distinguish between soil microbial communities (based on FAMEs) exposed to different fumigants in a greenhouse experiment (Chapter 5)**

The comparison of the PLFA and metabolomics analyses was combined with two interpretational approaches of the data, namely targeted and untargeted analysis. With the targeted approach, the TLE from the metabolomics analysis gave higher concentrations of total and individual FAMEs compared to PLFA analysis but discrimination between the control and fumigation treatments were much lower. Therefore, PLFA analysis is recommended to detect changes in microbial communities over time and between treatments when considering only FAMEs. The untargeted approach showed potential to distinguish between different treatments but it is important to remember that this approach considers all metabolites and not only FAMEs. Therefore, the approach does not characterise microbial communities *per se*, but may be valuable to enhance investigations of soil communities that include other organisms.

- **The formulation of a standard operating procedure for optimised lipid profiling of microbial communities and the implementation of this method in the Soil Microbiology Laboratory at the North-West University, Potchefstroom Campus (Appendix A)**

Based on the results obtained in the previous objectives, a collection of protocols was implemented in the Soil Microbiology Laboratory at the North-West University, Potchefstroom Campus, South Africa. These serve as the standard operating procedures for lipid profiling of microbial communities and contain instructions for different combinations of protocols, the most appropriate of which will be chosen based on the sample matrix and type of analysis required in a specific study. In addition, protocols for untargeted analysis are provided in an attempt to facilitate future investigations and improvements in the application of metabolomics to soil biological communities.

2 Concluding observations

When analysing lipids or a wider range of metabolites from any sample matrix, extraction (which includes appropriate derivatisation techniques) is a critical step in obtaining useful and reliable data (Tambellini *et al*. 2013). This is not only applicable to newer approaches such as metabolomics analysis but also to older, well used methods. In this context, methods that have been in use for a long time merits re-evaluation in certain instances. With the advances in equipment and statistical software programmes, it is possible to analyse samples and data in a different manner and often more accurately and comprehensively than when the methods were originally developed.

This investigation showed that interpretations of microbial community structure may be influenced by the method used to analyse the samples and that this observation also holds for newer approaches such as metabolomics analysis. With the latter approach, cognisance should be taken of the results indicating function rather than structure – whether of microorganisms (as in pure culture samples) or the biological community (in soil). Contextualisation of the metabolomics data is therefore important for accurate interpretation. Interpretation can also be improved by using targeted and untargeted analysis from the same samples. If targeted and untargeted analysis can be done using the same sample, it would mean simpler and less time-consuming procedures to obtain data. Also, microbial ecologists will have access to more comprehensive soil data to decipher the intricate interactions of microbial communities in the environment.

Metabolomics analysis holds potential for studying complex soil systems in the same manner that whole lipid analyses are more applicable to detect differences in soil types and land management (Fernandes *et al*. 2013) than to indicate shifts in microbial communities. There is no contention on the

fact that analyses that aim to characterise microbial communities in soil samples are notoriously difficult – not only in terms of community structure, but even more so in terms of community function. Metabolite profiling methods, particularly from soil can provide valuable information about biological compounds that affect microbial communities even if all of them do not originate from microbial communities. As with all other approaches used to study microbial communities, metabolite profiling and more specific techniques such as metabolite tracing (Sévin *et al*. 2015) should not be used in isolation, but should be combined with complementing approaches to give a holistic view of microbial community dynamics that include function and structure.

Refinement of metabolomics-based approaches that focus on the interpretation of changes in metabolite profiles will promote applicability. Additionally, targeted and untargeted approaches have advantages and shortcomings and would be of more value if applied together in soil studies. By employing an untargeted analysis on soil samples of disturbed and undisturbed sites, changes in metabolite profiles caused by the particular disturbance can be discovered. Therefore, untargeted analysis generates a hypothesis of the possible cause of metabolite profile differences (Dunn *et al.* 2013; Creek and Barrett 2014). To further characterise and quantify the relevant metabolites, targeted analysis should then be used. If applied to microbial ecology, it would then also be necessary to look at more target groups than, for example, only FAMEs.

Finally, modern analyses can benefit greatly from, amongst others, networking algorithms and isotope labelling techniques. These create the possibility to correlate metabolic turnover and metabolite prevalence in complex samples such as soil, with environmental changes, shifts in community structure and the dominance of certain species in the biological community (Larsen *et al.* 2011; Mosier *et al.* 2013; Garg *et al.* 2015).

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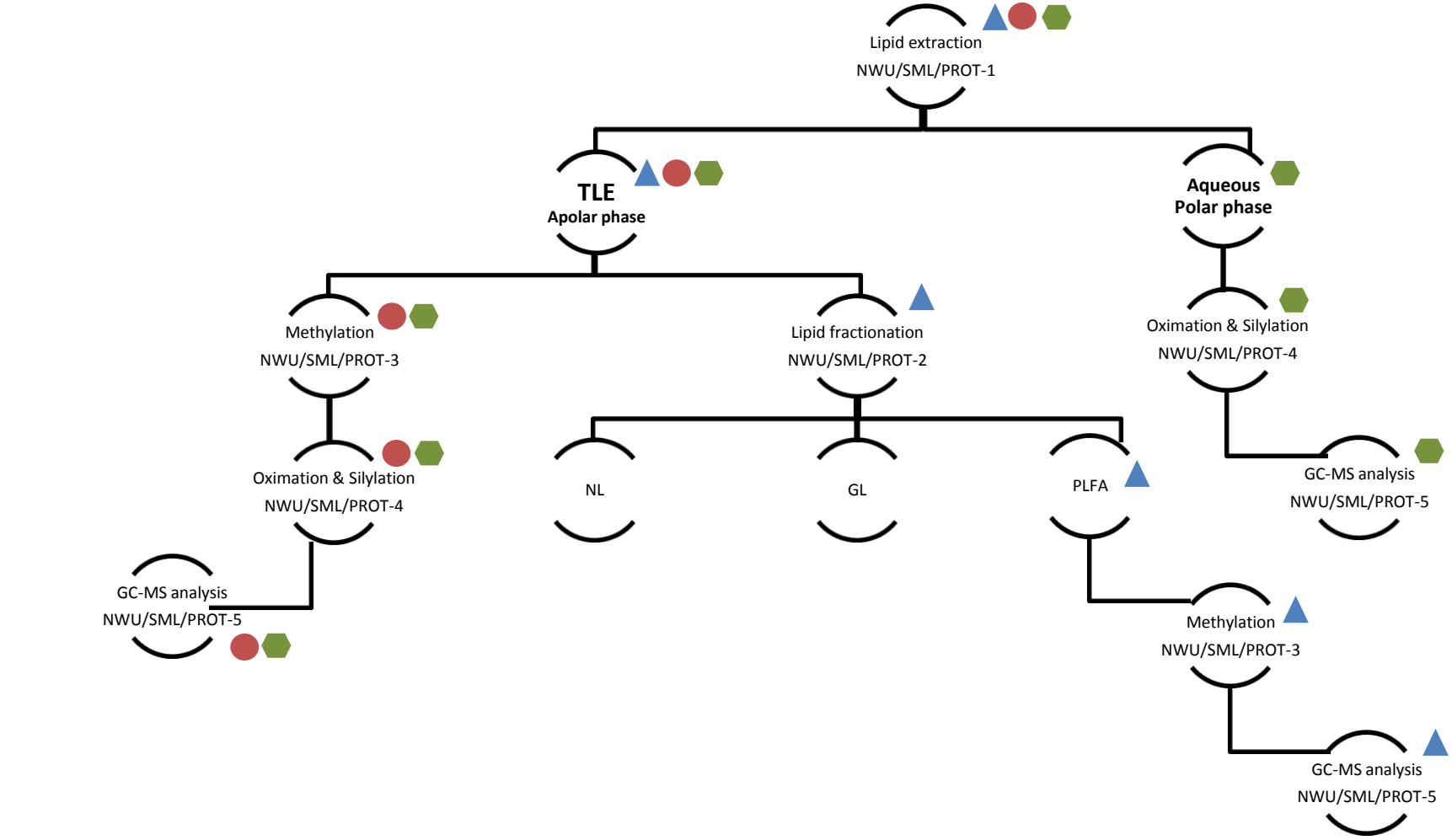
Appendix A

Standard operating procedure for targeted fatty acid methyl esters and proposed untargeted metabolomics

Protocols and work instructions

Complete protocol workflow for targeted fatty acid methyl esters and proposed untargeted metabolomics



Workflow order

Fatty acid methyl esters from fractionated phospholipids (see protocols for specifics)

- Lipid extraction (See protocol NWU/SML/PROT-1 and work instruction NWU/SML/WI-1)
- Use the resulting total lipid extract for lipid fractionation (See protocol NWU/SML/PROT-2 and work instruction NWU/SML/WI-2)
- Use the resulting phospholipid fraction and methylate to produce fatty acid methyl esters (See protocol NWU/SML/PROT-3 and work instruction NWU/SML/WI-3)
- Perform GC-MS analysis (See protocol NWU/SML/PROT-5 and work instruction NWU/SML/WI-5)

Fatty acid methyl esters from total lipid extract (see protocols for specifics)

- Lipid extraction (See protocol NWU/SML/PROT-1 and work instruction NWU/SML/WI-1)
- Use the resulting total lipid extract and methylate to produce fatty acid methyl esters (See protocol NWU/SML/PROT-3 and work instruction NWU/SML/WI-3)
- Use the resulting methylated sample and oximate and silylate (See protocol NWU/SML/PROT-4 and work instruction NWU/SML/WI-4)
- Perform GC-MS analysis (See protocol NWU/SML/PROT-5 and work instruction NWU/SML/WI-5)

Metabolomics approach (see protocols for specifics)

- Lipid extraction (See protocol NWU/SML/PROT-1 and work instruction NWU/SML/WI-1)
- Use the resulting total lipid extract and methylate to produce fatty acid methyl esters (See protocol NWU/SML/PROT-3 and work instruction NWU/SML/WI-3)
- Use the resulting methylated sample and oximate and silylate (See protocol NWU/SML/PROT-4 and work instruction NWU/SML/WI-3)
- Perform GC-MS analysis (See protocol NWU/SML/PROT-5 and work instruction NWU/SML/WI-5)

Lipid extraction protocol (NWU/SML/PROT-1)

1 Scope

Total lipids are extracted from lyophilised soil samples by using a modified Bligh and Dyer (1959) procedure with a single-phase chloroform, methanol, and phosphate buffer (1:2:0.8; *v/v/v*) solvent mixture (White *et al.* 1979).

2 Equipment

Product	Model / catalogue number	Company
Analytical balance	SBC 32	Scaltec Instruments GmbH, Germany
Centrifuge	Harrier 15/80	MSE (UK) Ltd., London, UK
Glassware (different volumes)	Beakers, measuring cylinders, Pasteur-pipettes (150 mm), screw cap test tubes	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Nitrile gloves	#RLAS1GL014-0000M	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Magnetic stirrer	MSH 10	Labcon, Maraisburg, South Africa
Muffle furnace	Nabertherm	Naber Industrieofenbau, Lilienthal/Bremen, Germany
Oven (105°C)	Scientific Series 9000	Scientific Engineering (Pty) Ltd., Roodepoort, South Africa
pH meter	CyberScan pH 510	Eutech Instruments, Thermo Fisher Scientific Inc., USA
Solvent evaporator	GD102-30	Agela Technologies Inc., Delaware, USA
Sonicator	PS-40	Jeken Ultrasonic Cleaner Ltd., Dong guan, China
Vortex mixer	VM-300	Gemmy Industrial Corporation, Taiwan

3 Chemicals

Product	Details / catalogue number	Supplier
Acetone	Honeywell Burdick & Jackson high purity, #010-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Chloroform	Honeywell Burdick & Jackson high purity, #049-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Di-potassium hydrogenphosphate	# 1051090500	Merck Millipore, Modderfontein, South Africa
Hexane	Honeywell Burdick & Jackson high purity, #212-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Hydrochloric acid	#320331-2.5L	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Methanol	Honeywell Burdick & Jackson high purity, #230-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Water	Honeywell Burdick & Jackson high purity, #365-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa

4 Procedure

- Weigh ~5 g of lyophilised soil sample on aluminium foil (or ~30 mg dry weight cells) and add to a 50 ml red-capped test tube (1st tube). Note the exact weight for future record.
- Add 5 ml chloroform, 10 ml methanol and 4 ml phosphate buffer to the sample, to provide a solvent ratio of 1:2:0.8 (*v/v/v*).
- Cap the tubes; sonicate the samples for no more than 2 min, vortex for 30 s and let stand at room temperature for 2 h, to allow single-phase extraction to take place.
- After the 2 h extraction, centrifuge the tubes (15 min at 1800 rpm) for the proper separation of the pellet and solvents, and decant the single-phase solvent system into a clean test tube (2nd tube).

- Wash the pellet to remove residual lipids by adding 5 ml chloroform to the 1st tube, cap and vortex for 30 s, followed by centrifugation for 15 min at 1800 rpm.
- By using a clean Pasteur-pipette, transfer the chloroform layer to the 2nd tube containing the single-phase solvents and add 5 ml water to the 2nd tube to provide a final solvent volume ratio of 2:2:1.8 (*v/v/v*).
- Cap and let stand overnight for the final phase separation to occur.
- Transfer the complete lower organic phase into a clean screw cap test tube (labelled with a green sticker for identification) using a clean Pasteur-pipette. Hold the pipette at a 90° angle and blow lightly while descending to the bottom phase – bubbles will appear. To avoid contamination, use a new pipette for each sample. This phase represents the total lipid extract (TLE).
- Dry the collected TLE under a gentle stream of N_2-gas at 37°C. Following solvent evaporation, close the tubes tight and store at -20°C until further use.

5 Notes

- Do not use any plasticware during lipid analysis – only glass, washed and baked according to the prescribed procedures and solvent-rinsed Teflon may come into contact with samples. Potential contaminants for lipid analysis include all lipids from fingers, hair, hand lotions, oils and hydrocarbons – wear gloves throughout the procedures and do not touch your skin or hair.
- While sampling, keep soil samples on ice to prevent the biological communities from undergoing dramatic changes. Immediately after sampling, freeze soil samples at -80°C overnight and lyophilise. Keep the samples at -80°C until analysis is possible. Before extraction, mix soil samples thoroughly on aluminium foil and homogenise by sieving through a 2 mm sieve to remove all the visible plant materials that may contribute to the lipid yield.
- Wash glassware meticulously in tap water and phosphate-free detergent (Liqui-Nox ALC-1232, Alconox Inc., Separations, South Africa) to remove unwanted contaminants (White and Ringelberg 1998). Rinse the glassware ten times each with tap and deionised water and five times with nano-pure water. Let the glassware air-dry before wrapping it in aluminium foil and baking at 450°C in a muffle furnace for at least 4 h. Disposable Pasteur-pipettes need not be washed, but should also be baked at the above mentioned conditions. Wash and rinse Teflon-lined caps in the same manner as the glassware, but let air-dry. Sonicate for 2 min in acetone and remove with tweezers. Place in a glass beaker covered with foil and bake in oven (105°C) until dry.
- Rinse solvent bottles respectively with hexane, methanol and lastly with the solvent that it will be used for.
- When drying solvent under nitrogen-gas (N_2), take care not to exceed the evaporation block temperature of 37°C as heat breaks down unsaturated fatty acids. Furthermore, lipids should not be exposed to air. Oxygen in the air reacts with the double bonds in the unsaturated lipids (Kates 1986; Christie 1989).
- Prepare phosphate buffer (50 mM) as follows: dissolve 8.7 g K_2HPO_4 in 995 ml water in a 1000 ml volumetric flask. Add a clean stir bar (washed in acetone) to the solution and place the flask on a magnetic stirrer (stir without heating). Measure the pH of the solution and rinse the pH meter probe with distilled water, before and after each reading. Adjust the solution to a pH of 7.4 by adding HCl (3M – 24.8 ml 37% HCl added to 75.2 ml water) drop-wise to the solution with a clean Pasteur-

pipette. Place the prepared phosphate buffer into a 1000 ml bottle and add 50 ml chloroform. Allow to extract overnight, before using the buffer. Store the buffer in the refrigerator.

6 Waste disposal

Dispose of organic solvents in organic waste containers and of used Pasteur-pipettes (or broken glass) in sharp-object waste containers.

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Lipid fractionation protocol (NWU/SML/PROT-2)

1 Scope

The total lipid extracts (TLEs) are fractionated into neutral, glyco- and phospholipids by silicic acid column chromatography (Guckert *et al.* 1985). Neutral lipid fractions contain sterols that can be used for fungal biomass estimation (Olsson *et al.* 1995; White and Ringelberg 1998), whereas the glycolipid fraction contains polyhydroxyalkanoates which can serve as a measure of unbalanced microbial growth (Findlay and White 1983; Nichols and White 1989). For phospholipid fatty acid analysis, only the phospholipid fraction is used for methyl ester formation and the other two fractions are discarded as waste.

2 Equipment

Product	Model / catalogue number	Company
Analytical balance	SBC 32	Scaltec Instruments GmbH, Germany
Desiccator	Vacuum desiccator	DURAN Group GmbH, Germany
Glassware (different volumes)	Beakers, measuring cylinders, Pasteur-pipettes (150 mm), screw cap test tubes	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Nitrile gloves	#RLAS1GL014-0000M	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Muffle furnace	Nabertherm	Naber Industrieofenbau, Lilienthal/Bremen, Germany
Oven (105°C)	Scientific Series 9000	Scientific Engineering (Pty) Ltd., Roodepoort, South Africa
Solvent evaporator	GD102-30	Agela Technologies Inc., Delaware, USA
Syringes	Hamilton®, 700 series, removable needle, 250 µl, #24538-U	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Vortex mixer	VM-300	Gemmy Industrial Corporation, Taiwan

3 Chemicals

Product	Details / catalogue number	Supplier
Acetone	Honeywell Burdick & Jackson high purity, #010-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Chloroform	Honeywell Burdick & Jackson high purity, #049-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Glass wool	#18421	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Hexane	Honeywell Burdick & Jackson high purity, #212-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Methanol	Honeywell Burdick & Jackson high purity, #230-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Silicic acid	100-200 mesh powder, #SIL350-1KG	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa

4 Procedure

- Prepare silicic acid slurry by adding 5 ml chloroform to activated silicic acid and pack a glass column using a Pasteur-pipette. Suck chloroform up while stirring and scrape it on the sides of the column and onto the glass wool plug. Do not allow the packing bed to dry out once the procedure has begun.
- Flush the column twice with 2.5 ml acetone and 2.5 ml chloroform, respectively. Allow the first aliquot to elute through, before adding the second. Collect the solvents and discard as waste.
- Redissolve the dried TLE (tube labelled with green sticker) with 150 µl chloroform, using a Hamilton syringe. Vortex briefly and load onto the packed column with a Pasteur-pipette. Repeat three times for a quantitative transfer (total - 450 µl for each sample).

- Add a series of three solvents of increasing polarity onto the column to separate the three lipid classes. Collect each lipid class in separate test tubes positioned below the columns.
- Elute the column twice with 2.5 ml chloroform and collect the neutral lipid fraction in screw cap test tubes (labelled with yellow stickers).
- Elute the column twice with 2.5 ml acetone and collect the glycolipid fraction in screw cap test tubes (labelled with orange stickers).
- Elute the column with 4 x 2.5 ml aliquots of methanol and collect the phospholipid fraction in screw cap test tubes (labelled with blue stickers).
- If further analysis of the neutral and glycolipid fractions is required, evaporate the solvents under a gentle stream of N_2-gas, cap and store at -20°C for further analysis. For the scope of this method, discard these fractions as waste.
- Dry the phospholipid fraction under N_2-gas flow at 37°C, cap the tubes and store at -20°C until further use.

5 Notes

- Place a piece of glass wool in a custom-made glass column and bake in the muffle furnace at 450°C for at least 4 h. The glass wool serves as a filtering plug.
- Weigh 0.5 g silicic acid in screw cap test tubes, cap, bake at 105°C for 2 h and store in a desiccator until cooled. This activates the silicic acid.
- When packing the columns, make sure that no air pockets form within the packed bed. If air bubbles appear, agitate the bed with a clean Pasteur-pipette until the bubbles rise to the surface.
- During elution of the columns, allow the first aliquot to elute through before adding the second aliquot. The conditioning and equilibration of the columns, by flushing with acetone and chloroform beforehand, ensures proper equilibration in terms of pH and solvent strength, for optimal retention. The sorbent bed should never run dry causing the pores to de-wet. As soon as air fills the pores, the applied analyte will pass directly through, without being retained. This will result in poor target analyte recovery (Arsenault 2012).
- Do not use any plasticware during lipid analysis – only glass, washed and baked according to the prescribed procedures and solvent-rinsed Teflon may come into contact with samples. Potential contaminants for lipid analysis include all lipids from fingers, hair, hand lotions, oils and hydrocarbons – wear gloves throughout the procedures and do not touch your skin or hair.
- Wash glassware meticulously in tap water and phosphate-free detergent (Liqui-Nox ALC-1232, Alconox Inc., Separations, South Africa) to remove unwanted contaminants (White and Ringelberg 1998). Rinse the glassware ten times each with tap and deionised water and five times with nano-pure water. Let the glassware air-dry before wrapping it in aluminium foil and baking at 450°C in a muffle furnace for at least 4 h. Disposable Pasteur-pipettes need not be washed, but should also be baked at the above mentioned conditions. Wash and rinse Teflon-lined caps in the same manner as the glassware, but let air-dry. Sonicate for 2 min in acetone and remove with tweezers. Place in a glass beaker covered with foil and bake in oven (105°C) until dry.
- Rinse solvent bottles respectively with hexane, methanol and lastly with the solvent that it will be used for.

- When drying under nitrogen-gas (N_2), take care not to exceed the evaporation block temperature of 37°C as heat breaks down unsaturated fatty acids. Furthermore, lipids should not be exposed to air. Oxygen in the air reacts with the double bonds in the unsaturated lipids (Kates 1986; Christie 1989).

6 Waste disposal

Dispose of organic solvents in organic waste containers and of used Pasteur-pipettes (or broken glass) in sharp-object waste containers.

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Methylation protocol (NWU/SML/PROT-3)

1 Scope

The dry lipid residues (either total lipid extracts (TLEs) or phospholipid fractions) are subjected to mild alkaline methanolysis, using methanolic potassium hydroxide (KOH), to form fatty acid methyl esters (FAMEs) (White *et al.* 1979).

2 Equipment

Product	Model / catalogue number	Company
Analytical balance	SBC 32	Scaltec Instruments GmbH, Germany
Centrifuge	Harrier 15/80	MSE (UK) Ltd., London, UK
Desiccator	Vacuum desiccator	DURAN Group GmbH, Germany
GC-vials	Screw top, 2 ml, clear, #5182-0714	Agilent Technologies, California, USA
GC-vial caps	Blue screw cap, red septa, #5182-0717	Agilent Technologies, California, USA
Glass inserts	250 µl pulled point, #5183-2085	Agilent Technologies, California, USA
Glassware (different volumes)	Beakers, measuring cylinders, Pasteur-pipettes (150 mm), screw cap test tubes	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Nitrile gloves	#RLAS1GL014-0000M	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Oven (40°C)	Gallenkamp vacuum oven	Fistreem International Ltd., Leicestershire, UK
Pipette	Acura® manual 835, 0.2-2 ml	Socorex Isba SA, Ecublens, Switzerland
Solvent evaporator	GD102-30	Agela Technologies Inc., Delaware, USA
Syringes	Hamilton®, 700 series, removable needle, 250 µl, #24538-U	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Vortex mixer	VM-300	Gemmy Industrial Corporation, Taiwan

3 Chemicals

Product	Details / catalogue number	Supplier
Chloroform	Honeywell Burdick & Jackson high purity, #049-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Glacial acetic acid	# ARK2183-1L	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Hexane	Honeywell Burdick & Jackson high purity, #212-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Methanol	Honeywell Burdick & Jackson high purity, #230-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Methyl nonadecanoate	#N5377	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Potassium hydroxide	#1050330500	Merck Millipore, Modderfontein, South Africa
Isooctane	#360066	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Water	Honeywell Burdick & Jackson high purity, #365-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa

4 Procedure

- Redissolve the dry lipid residues in 0.5 ml chloroform, 0.5 ml methanol and 1 ml methanolic KOH. Make sure the screw cap test tubes are closed tight.
- Vortex for 30 s, incubate for 30 min at 40°C for methylation to take place and allow cooling to room temperature.
- Add 2 ml hexane, mix, add 200 µl of glacial acetic acid with a Hamilton syringe to neutralise the sample, and add 2 ml water to induce phase separation.
- Vortex samples for 30 s and centrifuge for 5 min at 2000 rpm to separate the organic phase (containing the FAMEs) from the aqueous phase.

- Transfer the upper organic phase into a clean screw cap test tube (labelled with a white sticker) and re-extract the bottom phase with 2 ml hexane, vortex for 30 s and centrifuge for 3 min at 2000 rpm.
- Again, transfer the upper phase into the same clean tube as used above (labelled white) and repeat the hexane extraction twice more (total 6 ml).
- Dry under a gentle stream of N_2-gas at 37°C.
- Dissolve the dried FAMEs in 100 µl hexane using a Hamilton syringe, vortex briefly and transfer to a GC-vial with a clean Pasteur-pipette. Repeat this transfer procedure twice more. Dry under N_2-gas at 37°C and store at -20°C.
- When only the phospholipid fraction is used, resuspend the FAMEs in 50 µl methyl nonadecanoate internal standard for GC-MS analysis.

5 Notes

- As KOH is hygroscopic, the pellets should be kept in a desiccator to prevent water contamination. Prepare a fresh 0.2 mol L^{-1} solution daily. Dissolve 0.28 g KOH-pellets in 25 ml methanol. It is difficult to weigh the KOH precisely, as the pellets may differ in size. Calculate the exact volume of methanol required as follows: (amount weighed / 0.28 g) x 25 ml = volume methanol required (ml).
- The use of glacial acetic acid (1N) to neutralise the sample is necessary, because methanolysis is incomplete at a higher pH and the FAMEs affinity for water is higher at an elevated pH (White and Ringelberg 1998).
- To prepare methyl nonadecanoate (C19:0 FAME) internal standard solution (50 pmol ml^{-1}), dissolve 1.56 mg methyl nonadecanoate in 100 ml isooctane. Store in the refrigerator in a volumetric flask.
- Do not use any plasticware during lipid analysis – only glass, washed and baked according to the prescribed procedures and solvent-rinsed Teflon may come into contact with samples. Potential contaminants for lipid analysis include all lipids from fingers, hair, hand lotions, oils and hydrocarbons – wear gloves throughout the procedures and do not touch your skin or hair.
- Wash glassware meticulously in tap water and phosphate-free detergent (Liqui-Nox ALC-1232, Alconox Inc., Separations, South Africa) to remove unwanted contaminants (White and Ringelberg 1998). Rinse the glassware ten times each with tap and deionised water and five times with nano-pure water. Let the glassware air-dry before wrapping it in aluminium foil and baking at 450°C in a muffle furnace for at least 4 h. Disposable Pasteur-pipettes need not be washed, but should also be baked at the above mentioned conditions. Wash and rinse Teflon-lined caps in the same manner as the glassware, but let air-dry. Sonicate for 2 min in acetone and remove with tweezers. Place in a glass beaker covered with foil and bake in oven (105°C) until dry.
- Rinse solvent bottles respectively with hexane, methanol and lastly with the solvent that it will be used for.
- When drying under nitrogen-gas (N_2), take care not to exceed the evaporation block temperature of 37°C as heat breaks down unsaturated fatty acids. Furthermore, lipids should not be exposed to air. Oxygen in the air reacts with the double bonds in the unsaturated lipids (Kates 1986; Christie 1989).

6 Waste disposal

Dispose of organic solvents in organic waste containers and of used Pasteur-pipettes (or broken glass) in sharp-object waste containers.

References

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Oximation and silylation protocol (NWU/SML/PROT-4)

1 Scope

By adding methoxyamine hydrochloride dissolved in pyridine to a sample, aldoses and ketoses are converted to acyclic methoximes (Lindeque 2011). During silylation the active hydrogen atoms in –OH, =NH, -NH_2, -SH and –COOH groups in a compound, are replaced with a trimethylsilyl group (Halket and Zaikin 2003). This yields a more volatile, less polar and more thermally stable derivative for gas chromatography-mass spectrometry (GC-MS) analysis.

2 Equipment

Product	Model / catalogue number	Company
Analytical balance	SBC 32	Scaltec Instruments GmbH, Germany
Screw top GC-vials	#5182-0714	Agilent Technologies, California, USA
GC-vial caps	#5182-0717	Agilent Technologies, California, USA
Pulled point glass inserts	#5183-2085	Agilent Technologies, California, USA
Glassware (different volumes)	Beakers, measuring cylinders, Pasteur-pipettes (150 mm)	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Nitrile gloves	#RLAS1GL014-0000M	Lasec South Africa (Pty) Ltd., Midrand, South Africa
Oven (60°C)	Gallenkamp vacuum oven	Fistreem International Ltd., Leicestershire, UK
Solvent evaporator	GD102-30	Agela Technologies Inc., Delaware, USA
Syringes	Hamilton®, 700 series, removable needle, 250 µl, #24538-U	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Vortex mixer	VM-300	Gemmy Industrial Corporation, Taiwan

3 Chemicals

Product	Details / catalogue number	Supplier
2-acetamidophenol	#A7000, Aldrich	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
L-norleucine	#74560, Fluka	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Methanol	Honeywell Burdick & Jackson high purity, #230-4	Anatech Instrument (Pty) Ltd., Olivedale, South Africa
Methoxyamine hydrochloride	#226904, Aldrich	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Methyl nonadecanoate	#N5377	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
N, O-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane	#33148, Supelco	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Nonadecanoic acid	#N5252, Sigma	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
3-phenylbutyric acid	#116807, Aldrich	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Pyridine	#270970, Sigma-Aldrich	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa
Isooctane	#360066	Sigma-Aldrich (Pty) Ltd., Johannesburg, South Africa

4 Procedure

- Using a Hamilton syringe, add 150 µl of methyl nonadecanoate internal standard and 50 µl of internal standard mix to the dried fatty acid methyl esters (FAMEs prepared from total lipid extract) or to the dried aqueous phase (for metabolomics analysis).
- Dry under a gentle stream of N_2-gas at 37°C.
- Add 50 µl of oximation reagent to the vial, cap, vortex briefly and incubate for 1 h at 60°C.
- After cooling, add 50 µl BSTFA-TMCS to the samples, cap, vortex briefly and incubate for 1 h at 60°C.
- Add 50 µl of isooctane to each vial for GC-MS analysis.

5 Notes

- Prepare methyl nonadecanoate (C19:0 FAME) internal standard solution (50 pmol ml^{-1}) by dissolving 1.56 mg methyl nonadecanoate in 100 ml isooctane. Keep refrigerated in a volumetric flask.
- For the internal standard mix (5 µg ml^{-1}) dissolve the following in 100 ml methanol: 0.5 mg L-norleucine, 0.5 mg 3-phenylbutyric acid, 0.5 mg 2-acetamidophenol and 0.5 mg nonadecanoic acid (Venter *et al.* 2015).
- Prepare oximation reagent (20 mg ml^{-1}) by dissolving 200 mg methoxyamine in 10 ml pyridine.
- Do not use any plasticware during this analysis – only glass, washed and baked according to the prescribed procedures and solvent-rinsed Teflon may come into contact with samples. Potential contaminants include all lipids from fingers, hair, hand lotions, oils and hydrocarbons – wear gloves throughout the procedures and do not touch your skin or hair.
- Wash glassware meticulously in tap water and phosphate-free detergent (Liqui-Nox ALC-1232, Alconox Inc., Separations, South Africa) to remove unwanted contaminants (White and Ringelberg 1998). Rinse the glassware ten times each with tap and deionised water and five times with nano-pure water. Let the glassware air-dry before wrapping it in aluminium foil and baking at 450°C in a muffle furnace for at least 4 h. Disposable Pasteur-pipettes need not be washed, but should also be baked at the above mentioned conditions. Wash and rinse Teflon-lined caps in the same manner as the glassware, but let air-dry. Sonicate for 2 min in acetone and remove with tweezers. Place in a glass beaker covered with foil and bake in oven (105°C) until dry.
- When drying solvent under nitrogen-gas (N_2), take care not to exceed the evaporation block temperature of 37°C as heat breaks down unsaturated fatty acids. Furthermore, lipids should not be exposed to air. Oxygen in the air reacts with the double bonds in the unsaturated lipids (Kates 1986; Christie 1989).

6 Waste disposal

Dispose of organic solvents in organic waste containers and of used Pasteur-pipettes (or broken glass) in sharp-object waste containers.

References

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Data acquisition protocol (NWU/SML/PROT-5)

1 Scope

A gas chromatograph coupled to a mass spectrometer is used to identify and quantify the analytes within each sample.

2 Equipment

Product	Model / catalogue number	Company
Autosampler	7683B	Agilent Technologies, California, USA
Capillary column	SPB-1, 60 m x 0.25 mm x 0.25 µm film thickness	Supelco, Sigma-Aldrich, Johannesburg, South Africa
Carrier gas	Helium	Afrox Ltd., Potchefstroom, South Africa
Gas chromatograph	7890A, split/splitless injector	Agilent Technologies, California, USA
Mass spectrometer	5975B inert XL	Agilent Technologies, California, USA

3 Conditions

Gas chromatograph

- Injector temperature - 280°C
- Injector mode - splitless
- Injection volume - 1 µl
- Carrier gas flow rate - 1.0 ml min^{-1}
- Oven temperature - 60°C for 2 min; 10°C/min to 150°C; 3°C/min to 320°C; hold for 2 min at 320°C

Mass spectrometer

- Transfer line temperature - 280°C
- Source temperature - 230°C
- MS Quad temperature - 150°C
- Ionisation - electron impact at 70 eV with dwell time of 100
- Full scan monitoring - 40-450 m/z at 175 scans/min

4 Procedures

- Load the vials onto the auto sampler in the next available empty positions of the tray.
- All samples are acquired using the FAME method (FAME.M).
- Under the Sequence menu choose “Load Sequence” and select the previous day’s FAME sequence.
- Under the Sequence menu choose “Edit Sample Log Table”. Delete previous day’s samples and starting with a QC sample, update vial positions to reflect current run. Add new samples by clicking on the “Repeat” button which will update vial positions and data file names by one. Continue adding

samples and changing data file names and Sample names until all samples are entered into the sample log table. Click "OK".

- From the Sequence menu choose "Save sequence as" and rename the sequence with current date. E.g.130915A (if it is the first sequence of the day) or 130915B (if the second sequence of the day. Create a folder to save all data of the day in c:\msdchem\1\data\FAME\
- From Sequence menu, click on "Run", then "run sequence". The software will process the sequence line by line until completed.
- Data management
  - To avoid loss of data, data **must** be transferred daily to an external hard drive
  - It is the responsibility of the user to ensure transfer of files.

5 Data processing

GC-MS spectra are identified by spectral analysis using the Automated Mass Spectral Deconvolution and Identification System (AMDIS V 2.71). The NIST 2008 mass spectral library and in-house FAME databases are used for identification. The resulting data are imported into Agilent's MassHunter Mass Profiler Professional (MPP) (B.02). The found peaks in each data file across all files are aligned. MPP determines which peaks in each chromatographic run are the same compound and which are different. Once all data are aligned, data are imported into MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) for a number of interpretation algorithms to find the compound relationships in regards to the experiment (Xia *et al*. 2009, 2015). Clustering and score plots obtained by Principal Component Analysis (PCA) and Analysis of variance (ANOVA) can also be performed in MetaboAnalyst 3.0.

References

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Work instruction NWU/SML/WI-1

Lipid extraction

1 Apparatus and chemicals

2 mm soil sieve
Aluminium foil
Analytical balance
50 ml red-capped test tubes
Chloroform
Methanol
Phosphate buffer (50 mM)
Sonicator
Vortex mixer
Centrifuge
Pasteur-pipettes
Water
Screw cap test tubes
Solvent evaporator with N_2-gas

2 Procedure

- Freeze soil samples in the -80°C freezer overnight.
- Lyophilise soil samples overnight.
- Grind soil and sieve through a 2 mm sieve. Remember to use aseptic techniques between samples.
- Weigh ~5 g of soil on aluminium foil or ~30 mg dry weight cells directly in the red-capped test tube.
- Add sample to 50 ml red-capped test tube (1^{st} tube).
- Add 5 ml chloroform, 10 methanol and 4 ml phosphate buffer to each sample.
- Cap the tubes and sonicate for no more than 2 min.
- Vortex for 30 s.
- Let stand for 2 h.
- Centrifuge @ 1800 rpm for 15 min.
- Decant upper layer into a clean red-capped test tube (2^{nd} tube).
- Add 5 ml chloroform to the original tube (1^{st} tube).
- Cap and vortex for 30 s.
- Centrifuge @ 1800 rpm for 15 min.
- Transfer upper layer into 2^{nd} tube by using a clean Pasteur-pipette each time.
- Add 5 ml water to 2^{nd} tube, cap and shake.
- Let stand overnight for phases to separate.
- With a clean Pasteur-pipette, transfer the bottom phase into a clean screw cap test tube (green sticker). Hold pipette at a 90° angle and blow until the bottom phase is reached – bubbles will appear. Use a new pipette for each sample.
- Dry under a gentle stream of N_2-gas @ 37°C, cap tubes and store @ -20°C until further use.

Work instruction NWU/SML/WI-2

Lipid fractionation

1 Apparatus and chemicals

Glass wool
Custom-made glass columns
Muffle furnace
Analytical balance
Silicic acid
Screw cap test tubes
Oven @ 105°C
Desiccator
Chloroform
Pasteur-pipettes
Acetone
Hamilton syringe
Vortex mixer
Methanol
Solvent evaporator with N_2-gas

2 Procedure

- Place some glass wool in a glass column, wrap in aluminium foil and bake in muffle furnace @ 450°C for 4 h.
- Weigh 0.5 g silicic acid in test tube, cap and bake for 2 h @ 105°C. Store in desiccator until cooled.
- Add 5 ml chloroform into the tube with silicic acid.
- Pack the column with the above mentioned mixture using a Pasteur-pipette. Suck chloroform up while stirring and scrape it on the sides of the column and onto the glass wool plug. There should be no sign of air pockets within the bed. Packing should not be allowed to dry or the surface of the bed disturbed once the procedure has begun.
- Flush columns 2 x 2.5 ml acetone (1^{st} aliquot has to go through, before adding the next).
- Flush columns 2 x 2.5 ml chloroform (1^{st} aliquot has to go through, before adding the next).
- Discard these solvents as waste.
- Redissolve the dry lipid extract (green sticker) with 150 µl chloroform using a Hamilton syringe, vortex and load onto the column. Repeat twice more for a quantitative transfer (total 450 µl).
- Position clean pre-labelled screw cap test tubes underneath the columns for the collection of the separate lipid classes.
- Add 2 x 2.5 ml chloroform to column. Collect neutral lipids in yellow labelled tubes. Dry for further use or discard as waste.
- Add 2 x 2.5 ml acetone to column. Collect glycolipids in orange labelled tubes. Dry for further use or discard as waste.
- Add 4 x 2.5 ml methanol to column. Collect phospholipids in blue labelled tubes.
- Dry lipid fractions under a gentle stream of N_2-gas @ 37°C, cap tubes and store @ -20°C until further use.

Work instruction NWU/SML/WI-3

Methylation

1 Apparatus and chemicals

Pipette
Pasteur-pipettes
Chloroform
Methanol
Methanolic KOH – freshly prepared (daily)
Screw cap test tubes
Vortex mixer
Oven @ 40°C
Hexane
Hamilton syringe
Glacial acetic acid (1N)
Water
Centrifuge
Solvent evaporator with N_2-gas
GC-vials and caps
Internal standard: methyl nonadecanoate
Glass insert

2 Procedure

- Add 0.5 ml chloroform and 0.5 ml methanol to the tube with the dry lipid residue (green / blue sticker).
- Add 1 ml methanolic KOH and close the tube tight.
- Vortex for 30 s.
- Incubate for 30 min @ 40°C. Allow to cool.
- Add 2 ml hexane and mix.
- Use a Hamilton syringe to add 200 µl of 1N glacial acetic acid.
- Add 2 ml water and cap the tube.
- Vortex for 30 s.
- Centrifuge for 5 min @ 2000 rpm.
- Use a clean Pasteur-pipette to transfer the upper phase into a clean screw cap test tube (white sticker).
- Add 2 ml hexane to the original tube, vortex for 30 s and centrifuge for 3 min @ 2000 rpm.
- Transfer the upper phase into the labelled tube using a Pasteur-pipette.
- Repeat the hexane extraction step twice more – total 6 ml hexane. Use a clean Pasteur-pipette each time.
- Dry FAMEs under a gentle stream of N_2-gas @ 37°C.
- Using a Hamilton syringe, dissolve the dried FAMEs in 3 x 100 µl hexane, vortex briefly and transfer to a GC-vial with a Pasteur-pipette.
- Dry under N_2-gas and store @ -20°C.
- When using the phospholipid fraction, add 50 µl internal standard to GC-vial with a Hamilton syringe, vortex briefly and transfer with a Pasteur-pipette to a glass insert for GC-MS analysis.

Work instruction NWU/SML/WI-4

Oximation and silylation

1 Apparatus and chemicals

Hamilton syringe
Internal standard: methyl nonadecanoate
Internal standard mix
Solvent evaporator with N_2-gas
Oximation reagent
Vortex mixer
Oven @ 60°C
BSTFA-TMCS (99:1)
Isooctane
Pasteur-pipettes
Glass inserts

2 Procedure

- Using a Hamilton syringe, add 150 µl of internal standard and 50 µl of internal standard mix to the dried residues in the vials.
- Dry the total solution under a gentle stream of N_2-gas @ 37°C.
- With a Hamilton syringe, add 50 µl of oximation reagent.
- Cap the vials and vortex briefly for 30 s.
- Incubate for 1 h @ 60°C.
- Add 50 µl BSTFA-TMCS to the cooled vials.
- Cap the vials and vortex.
- Incubate for 1 h @ 60°C.
- Add 50 µl of isooctane to the vials.
- Vortex for 30 s.
- Transfer the solution to a glass insert with a Pasteur-pipette for GC-MS analysis.

Work instruction NWU/SML/WI-5

Data acquisition

1 Apparatus

An Agilent GC-MS instrument consisting of a 7890A gas chromatograph with a split/splitless injector equipped with a 7683B auto sampler coupled to a 5975B inert XL mass selective detector.

2 Procedure

- Load the method "file" "open method" select FAME.m
- Load the samples in the first available open positions on the auto sampler tray.
- Under the Sequence menu choose "Load Sequence" and select the previous day's FAME sequence.
- Under the Sequence menu choose "Edit Sample Log Table". Delete previous day's samples and starting with a QC sample, update vial positions to reflect current run. Add new samples by clicking on the "Repeat" button which will update vial positions and data file names by one. Continue adding samples and changing data file names and Sample names until all samples are entered into the sample log table. Click "OK".
- From the Sequence menu choose "Save sequence as" and rename the sequence with current date. E.g.130915A (if it is the first sequence of the day) or 130915B (if the second sequence of the day. Create a folder to save all data of the day in c:\msdchem\1\data\FAME\
- From Sequence menu click on "Run", then "run sequence". The software will process the sequence line by line until completed.

Appendix B

Supplementary information for Chapter 4

1 Repeatability of homogenisation

(Section 2.3.2, p.67, Chapter 4)

A low percentage relative standard deviation (% RSD) indicates high repeatability among replicates (Waliszewski and Szymczynski 1991). The values of the soil samples were acceptable (<25%). The RSD value differs depending on the analytical range, where the % RSD is high among samples near the detection limit (which may be the case with certain FAMEs).

Table B1: Repeatability of soil samples

nmol g^{-1}	**Mean ± stdev**	**% RSD**
Total FAMEs	1.958 ± 0.124	6.345
14:0	0.016 ± 0.003	21.566
i15:0	0.118 ± 0.006	4.838
a15:0	0.058 ± 0.004	6.192
15:0	0.011 ± 0.001	11.513
10Me15:0	0.021 ± 0.002	8.938
i16:0	0.098 ± 0.006	6.186
16:0	0.260 ± 0.018	7.015
10Me16:0	0.078 ± 0.008	9.814
12Me16:0	0.011 ± 0.000	2.999
i17:0	0.058 ± 0.004	6.048
a17:0	0.058 ± 0.004	7.431
cy17:0	0.053 ± 0.005	9.124
17:0	0.012 ± 0.001	6.830
10Me17:0	0.053 ± 0.003	6.034
18:2ω6	0.366 ± 0.053	14.367
18:1ω7c	0.008 ± 0.002	28.618
18:1ω7t	0.007 ± 0.002	21.975
18:0	0.563 ± 0.035	6.162
cy19:0	0.067 ± 0.008	12.026
20:0	0.012 ± 0.002	14.883
22:0	0.014 ± 0.003	21.975
23:0	0.003 ± 0.000	9.430
24:0	0.012 ± 0.003	21.638

Means ± standard deviations represent results obtained from sample replicates (n = 6). Standard fatty acid nomenclature was used (Guckert *et al.* 1985; Ringelberg *et al.* 1989; Zelles 1999). Key to abbreviations: FAMEs – fatty acid methyl esters; RSD – relative standard deviation; stdev – standard deviation.

2 Aqueous phase aliquot volume

(Section 2.5, p.69, Chapter 4)

Table B2: Compound count and abundance obtained from different sample volumes of the aqueous phase

Sample	**AQ1 (1ml)**	**AQ2 (2ml)**	**AQ4 (4ml)**	**AQ8 (8ml)**
Compound count	22	38	42	42
Total abundance	124 991 331	241 808 971	463 592 451	684 483 558
Estimated total concentration (pmol)	291.13	573.33	1 166.87	1 793.37
Increase in abundance (ratio)	1	2	4	5

Key to abbreviations: AQ – aqueous phase

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Appendix C

Title pages of published articles

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REVIEW ARTICLE

Microbial signature lipid biomarker analysis – an approach that is still preferred, even amid various method modifications

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Keywords
derivatization, environmental samples, fatty acid methyl ester, fractionation, lipid extraction, lipid quantification, microbial lipid analysis.

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Summary

The lipid composition of microbial communities can indicate their response to changes in the surrounding environment induced by anthropogenic practices, chemical contamination or climatic conditions. A considerable number of analytical techniques exist for the examination of microbial lipids. This article reviews a selection of methods available for environmental samples as applied for lipid extraction, fractionation, derivatization and quantification. The discussion focuses on the origin of the standard methods, the different modified versions developed for investigation of microbial lipids, as well as the advantages and limitations of each. Current modifications to standard methods show a number of improvements for each of the different steps associated with analysis. The advantages and disadvantages of lipid analysis compared to other popular techniques are clarified. Accordingly, the preferential utilization of signature lipid biomarker analysis in current research is considered. It is clear from recent literature that this technique stays relevant – mainly for the variety of microbial properties that can be determined in a single analysis.

Introduction

In the past 40–50 years, the importance of micro-organisms as a biomonitoring tool for environmental changes has been recognized. This can be ascribed to their wide-spread distribution, rapid reproduction rate and high susceptibility to environmental disturbances (Fang and Findlay 1996; Ruess and Chamberlain 2010). Considering the role of micro-organisms in the biogeochemical cycling of nutrients and the degradation of organic contaminants, it is evident that the characterization of the microbial community can provide insight into the functionality of an environment (Mummey *et al.* 2002). Seeing that <1% of all naturally occurring micro-organisms can be cultivated on synthetic media (Vestal and White 1989; Hill *et al.* 2000; Gans *et al.* 2005), the use of culture-independent techniques such as signature lipid biomarker analyses, are widely applied (Kaur *et al.* 2005; Joergensen and Emmerling 2006).

Lipids in microbial cells function as the main constituents of membranes, act as carbon storage bodies, facilitate signal transduction and can be used to identify bacteria on species level (Ishida *et al.* 2006). The quantification and identification of these lipids contribute to an understanding of the functioning of microbial metabolic genes and pathways (Basconcillo and McCarry 2008; Bühring *et al.* 2012), can be applied in the screening of pathogenic bacteria (Müller *et al.* 1998; Kellogg *et al.* 2001) and the measurement of microbial community structure and diversity (White *et al.* 1996; Ringelberg *et al.* 1997; Jungblut *et al.* 2009; Naeher *et al.* 2012). For the accurate profiling of microbial lipids from various sample matrices, several important processes are required (Huang *et al.* 2002; Poerschmann and Carlson 2006), including: (i) the complete removal or extraction of the lipids from the samples without any damage; (ii) the fractionation of the extracted whole lipids into various lipid classes; (iii) the conversion of the lipids into their respective methyl derivatives through methylation or derivatization; and (iv) the quantification and characterization of the methyl esters. No single experiment can comprehensively

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REVIEW ARTICLE

Phospholipid fatty acid profiling of microbial communities–a review of interpretations and recent applications

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Keywords
fungal to bacterial ratio, microbial activity, microbial biomass, microbial community structure, phospholipid fatty acid, physiological stress ratio.

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Summary

Profiling of microbial communities in environmental samples often utilizes phospholipid fatty acid (PLFA) analysis. This method has been used for more than 35 years and is still popular as a means to characterize microbial communities in a diverse range of environmental matrices. This review examines the various recent applications of PLFA analysis in environmental studies with specific reference to the interpretation of the PLFA results. It is evident that interpretations of PLFA results do not always correlate between different investigations. These discrepancies in interpretation and their subsequent applications to environmental studies are discussed. However, in spite of limitations to the manner in which PLFA data are applied, the approach remains one with great potential for improving our understanding of the relationship between microbial populations and the environment. This review highlights the caveats and provides suggestions towards the practicable application of PLFA data interpretation.

Introduction

Microbial ecologists have long been interested in the relationship between microbial communities and their environments. The development of culture-independent techniques, such as phospholipid fatty acid (PLFA) analysis lead to major changes in the manner in which microbial communities were studied. David C. White and colleagues, at the Florida State University, Florida, USA and later at the University of Tennessee, Knoxville, USA, initiated the use of phospholipid biomarkers for microbial community characterization. The first report of the use of PLFA biomarker analysis dates back to 1979 when it was used to estimate microbial biomass from marine and estuarine sediments (White *et al.* 1979b). This approach was a modification of an original procedure to extract fish tissue lipids (Bligh and Dyer 1959) and pioneered the use of microbial lipids as biomarkers for microbial community structure and metabolic activity in subsequent environmental studies (Bobbie and White 1980; Guckert *et al.* 1985; Findlay and White 1987; Findlay *et al.* 1990; Tunlid and White 1992; Zelles *et al.* 1994; Frostegård and Bååth 1996). These early studies were associated with the need to find an appropriate indicator of biomass (Frostegård *et al.* 2011), as defined by Jenkinson and Ladd (1981): (i) the measured component should be present in all portions of the environmental biomass in known concentrations; (ii) the component should only be present in viable organisms; (iii) it should be quantitatively extractable from the environment and (iv) an accurate technique should be available for estimating the component concentration in the environmental samples. Phospholipid fatty acid analysis proved to be highly successful as such an indicator and has been popular in environmental studies for determining microbial community structure ever since. This is evident by the large number of recent publications (since 2011) applying this approach. However, there are numerous limitations associated with the method that relate to specific extraction, fractionation and analysis methods and have been