Department of Chemistry

The Use of Stable Isotopes for the Characterisation of Natural Organic Matter and Investigation of the Different Organic Precursors of Aquatic Systems

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

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Abstract

Natural Organic Matter (NOM) occurs in all water systems and it can have a major impact on drinking water. Much effort is dedicated to its removal or maintenance at low levels. The complex nature of aquatic NOM necessitates an integrated approach to chemical analysis. The present study assesses the potential of compound specific stable carbon isotope data to complement traditional molecular analysis and to improve the overall characterisation of NOM. The source diagnostic potential of stable isotope characterisation can help identify the origin of NOM components beyond structure alone. Removal strategies will also benefit from a detailed understanding of the origin, characteristics and composition of NOM and its precursors.

A background to NOM and stable isotope analysis is provided in Chapter 1. The Australian drinking water guidelines (NHMRC, 2011) recommend a holistic preventive management approach encompassing all steps in water production from catchment to consumer as the most effective means of assuring drinking water quality and the protection of public health. Following this rationale, this PhD investigates NOM fractions at different stages of the water system from catchment to consumer. The utility of stable carbon isotope analysis for characterising the origins of a variety of NOM fractions and for following their fate from catchment to tap is examined here on a range of samples, including contrasting surface reservoir NOM, biofoulants from water treatment filtration membranes and biofilms from distribution systems.

In Chapter 2, model 'catchment' systems in the form of plant leachates (representing a simplified and concentrated source of allochthonous NOM) were generated under laboratory conditions. The volatile fraction of NOM was targeted by Solid Phase Micro Extraction (SPME) coupled with Compound Specific Isotope Analysis (CSIA). The volatile components of NOM have often been analytically neglected largely due to the laborious isolation techniques they require. A SPME with Gas Chromatography Mass Spectrometry (SPME-GCMS) method previously developed to identify and measure the relative abundances of Volatile and Semi Volatile Organic Compounds (VOCs and SVs, respectively) was modified to allow $\delta^{13}C$ analysis of CSIA of VOCs/SVs. The integrity of these analyses was tested with standards representative of a broad range of biochemical classes common to Dissolved Organic Carbon (DOC) influenced by terrestrial biomass.

The SPME-CSIA analysis of the plant leachates were challenged by significant sensitivity limitations. Multi-fibre SPME desorption proved necessary for CSIA. Analysis of the

working standards and plant leachates showed this approach introduced only negligible isotopic fractionation. Eighteen VOC/SV compounds in the C3 Marri leachate and eight compounds in the C4 Maize, Sorghum and Spinifex leachates were detected with sufficient GC resolution and concentration to allow reliable $\delta^{13}C$ measurement. The $\delta^{13}C$ data obtained provided information about DOC precursors, including primary and secondary metabolites. For example, i) 3-(4-hydroxy-3-methoxy phenyl)-2-propenoic acid (a biomarker of lignocellulose) was 10 ‰ lighter than the bulk biomass, consistent with a lignin source; ii) the close isotopic similarity of 1,6-dimethyl napthalene (-33.9 ‰), 6-methoxy-1-phenyl-3,4-dihydronapthalene (-32.2 ‰) and 1,1,6-trimethyl-1,2-dihydronapthalene (-32.7 ‰) in the Marri leachate identified a common lipid source; and iii) isopropyl-2,5-dimethoxy benzyl acetate was ^{13}C enriched (~28 ‰) relative to the bulk Marri $\delta^{13}C$ value, indicative of a carbohydrate origin. These findings are supported by established $\delta^{13}C$ product/precursor relationships (e.g., Hayes, 2001; Grice & Brocks, 2011).

Other interesting data included consistent ^{13}C depletion with the increasing MW of the lipid products, mirroring trends reported previously for lipid extracts of *Eucalyptus* leaf (Ballentine et al., 1998) and probably indicative of a biosynthetic effect (Collister et al., 1994). Different $\delta^{13}C$ values (caused by differing isotopic discrimination in two different plants) were reflected in a monoterpene of non-ambiguous biological source (cymene). Although the technique shows some promise in providing information about DOC sources and processes, the sensitivity of this analysis will need to be enhanced before SPME-CSIA becomes a practically relevant method of DOC characterisation.

The distribution and $\delta^{13}C$ values of Phospho Lipid Fatty Acids (PLFA) from the biologically active fraction of aquatic NOM samples was the focus of Chapter 3. Here, the microbial communities of the laboratory-leached C3 and C4 plants (Chapter 2) and a suite of biofilms collected from potable and recycled water distribution networks from around Australia were separately studied. This was the first stable isotopic PLFA study of distribution system biofilms.

The PLFA data of the plant leachates reflected a quite diverse microbiota attributed to a plentiful supply of labile organics as well as a relatively refractory pool able to be exploited by niche communities. The PLFAs of Gram-negative bacteria (cy17:0,cy19:0 and Monounsaturated PLFAs) of all leachates were relatively ^{13}C depleted, consistent with utilisation of terpenoids depleted in ^{13}C due to secondary fractionation associated with the enzymatic decarboxylation of pyruvic acid. In contrast, Gram-positive bacteria (*i*14:0, *i*15:0,

*a*15:0, *i*16:0, *i*17:0 and *a*17:0 PLFAs) were relatively ^{13}C enriched suggesting utilisation of more refractory material with relatively ^{13}C enriched values (due to less secondary processing and isotopic fractionation) and possibly also selective preservation of ^{13}C enriched components (e.g., lignin). The preservation of δ^{13}C values indicative of photosynthetic pathways was also evident by greater depletion from C3 plants compared to C4 plants.

The molecular profile and δ^{13}C values of PLFAs from the distribution system biofilm samples were correlated with general water properties, substrate availability and water treatment practices. Water temperature had the biggest influence on the concentrations of PLFAs and microbiota. The higher DOC and nutrient concentration of secondary treated waters supported a high proportion of Gram-positive bacteria and fungi. The δ^{13}C values of diagnostic PLFAs did complement the molecular data in certain instances, however these measurements were also limited to just several PLFAs of highest abundance on account of irMS sensitivity, linearity and GC resolution issues. These analytical restrictions highlight the serious challenges to the practical value and wider acceptance of this analysis approach at its present stage of development. Interesting features of the data obtained included biofilms from recycled wastewater having *i*15:0, a Gram-positive marker, with a distinctly lighter δ^{13}C value than its structural isomer *a*15:0 – indicative of separate microbial subgroups utilising different sources. Similarly, the heavy δ^{13}C value of 18:1ω9*c*, a eukaryotic marker, detected in the biofilms of the recycled waters—suggests utilisation of ^{13}C rich sugars (Leavitt & Long, 1982; Badeck et al., 2005).

The ^{13}C-enrichment of terminally branched PLFAs of two separate recycled water schemes mirrored previously reported tobacco plant data, and has been attributed to valine and *iso*-leucine amino acid precursors of *iso*- and *anteiso*- branched fatty acids, respectively. The utilisation of specific substrates was not evident from the molecular profiles of the PLFAs, illustrating the added value of ^{13}C analysis. However, the aforementioned analytical problems encountered will limit the practical application of δ^{13}C-PLFA analysis to water systems with very high microbial concentrations, such as recycled or waste water systems.

In Chapter 4 the innovative Hydrous Pyrolysis (HyPy) method was developed and tested for its compatibility with CSIA of aquatic biofilms. Pyrolysis of biological material or organic matter from aquatic (and other) environments typically yields complex product distributions which are not compatible with the stringent GC resolution requirements of CSIA. Off-line pyrolysis methods such as HyPy allow additional preparation procedures to

produce simpler fractions of complex OM showing good GC separation between many of the compounds within. Biofoulant material associated with membranes from a water treatment plant was used to fulfil the sample quantity requirements (>30 mg organic-rich sample) of these analyses.

HyPy of a range of membrane biofoulant samples yielded varying proportions of microbially derived products including even-number *n*-alkanes and monomenthyl alkanes (MMAs) in the aliphatic fraction and sugar products (possibly from Extracellular Polymeric Substances, EPS) in the derivatised polar fraction. An even-over-odd carbon preference of *n*-alkanes was indicative of microbial biomass.

The $\delta^{13}C$ values of the *n*-alkanes and several sugar products (i.e., levoglucosan, and an average value for galactofuranose, ribofuranose, arabinose and arabinopyranose) were able to be measured, but none of the components of the aromatic fraction and few from the derivatised polar fraction were able to be sufficiently resolved for uncompromised CSIA.

The stable isotopic data measured from the biofoulants helped characterise bacterial populations and identify a significant contribution to the organic matter from exogenous sources. The bulk $\delta^{13}C$ of the biofoulant was lighter than expected of material rich in relatively isotopically heavy sugars, implying the reworking of ^{13}C depleted carbohydrate Soluble Microbial Products (SMPs). The CSIA data combined with the bulk $\delta^{13}C$ value of the foulants was shown to complement the molecular analysis of membrane foulants and helped illuminate carbon dynamics, biosynthetic processes and potential sources of organics.

Chapter 5 presents a case study investigating the utility of the SPME and PLFA stable isotope methods developed in this research project to practically complement established NOM characterisation techniques. Merits of these analytical methods were assessed through characterisation of the NOM of Bolganup Creek (south west Western Australia) and Harding Dam (north west Western Australia) Reservoirs. These two source waters have contrasting forest and semi-arid grass catchments.

Characterisation of the hydrophobic (HPO) NOM fraction isolated from the two waters showed generally similar qualities. Several minor structural distinctions between the two waters was evident from traditional data including greater aromaticity and O/N alkyl content of Bolganup HPO, measured by both FTIR and ^{13}C-CPMAS NMR. The overall

^{13}C NMR signal intensity of Bolganup was also considerably higher, consistent with the relatively high DOC load (9.8 mg L^{-1}, *Cf*. 3.6 mg L^{-1} of Harding) from the heavily forested catchment. The ^{13}C NMR of Harding did show a relatively high alkyl signal, possibly reflecting algal input.

The $\delta^{13}C$ values of VOC and SV components measured with the SPME method were to a limited degree reflective of primary organic inputs. Due to an overwhelming anthropogenic signal, suspected to originate from the plastic containers used during water sampling and storage then amplified by the reverse osmosis pre-treatment, $\delta^{13}C$ values could only be reliably determined for a few SPME components. The VOC thymol did reflect the $\delta^{13}C$ of the parent HPO and mirrored a similar $\delta^{13}C$ trend between thymol and bulk Marri biomass in the leachate of Chapter 2.

Again, the $\delta^{13}C$ values of only the more concentrated PLFAs were able to be measured. PLFAs of Harding were on average 6.7 ‰ more ^{13}C enriched than Bolganup, probably due to differences in bioavailability of the DOC, providing some information about carbon dynamics and microbial substrate utilisation. The greater ^{13}C depletion of Bolganup PLFAs may also relate to greater anaerobic conditions (associated with stratification) or increased mineralisation of C3 material. Whereas the more ^{13}C enriched PLFAs from the Harding biota may be feeding on relatively enriched sugar substrate from the periodical algal blooms.

The $\delta^{13}C$ PLFA signatures determined for the water microbiota were generally consistent with the bulk $\delta^{13}C$ values of the respective HPO fractions. Thus 'preservation' of the $\delta^{13}C$ value associated with the dominant catchment vegetation was also evident and reflected some allochthonous input to both Harding and Bolganup HPO NOM. The $\delta^{13}C$ PLFA composition holds promise in identifying source inputs, and relationships of organic substrates and their supported biota in surface water catchments.

The application of SPME-GCMS and SPME-CSIA remains at an immature stage of development, providing only a small amount of new information to extend the traditional characterisation of the two NOM samples. Analytical contamination severely interfered with the SPME-GCMS analysis and the trace organic levels present in even the concentrated aliquots of the surface waters posed a series challenge to the sensitivity threshold of the analyses. Nevertheless, new information gleaned from the limited $\delta^{13}C$ data provided by SPME does suggest this approach has some analytical potential.

These analyses do represent a good complement to the resin fractionations now commonly used to isolate organic fractions of source waters, but which miss much of the of low molecular weight volatiles (targeted by SPME-GCMS) as well as the viable microbial biomass (targeted by PLFA analysis). On this basis, further development of the SPME and PLFA methods for isotope analysis is warranted.

In summary, SPME, PLFA and HyPy analytical strategies investigated in this research support the stable isotope analysis of NOM, including difficult to detect moieties, and show potential to contribute to a more holistic characterisation of aquatic NOM. However, further technical improvements (e.g., increased sensitivity or the processing of larger water volumes) will be necessary to extend their practical value. Several ideas on future developments and the potential wider application of the analytical strategies investigated here are provided on conclusion (Chapter 6).

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Publications arising from this thesis

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Chapter 2 CHARACTERISATION OF VOLATILE AND SEMI-VOLATILE COMPONENTS OF DOC

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List of Abbreviations

AC	After cleaning
ADWG	Australian Drinking Water Guidelines
Anthro.	Anthropogenic
AOM	Algogenic Organic Matter
ANOSIM	Analysis of Similarity
ATP	Adenosine Triphosphate
ATR	Attenuated Total Reflection
ATR-FTIR	Attenuated Total Reflection- Fourier Transform Infrared Spectroscopy
BAME	Bacterial Acid Methyl Ester
BC	Before cleaning
BDOC	Bioavailable Dissolved Organic Carbon
BF	Biofilm
BHP	Bacterio hopanepolyols
BOM	Biogenic Organic Matter
BSTFA	*N*-*O*-*bis* (trimethylsilyl)trifluoroacetamide
BTEX	Benzene, Toluene, Ethylbenzene and Xylenes
C	Carbon
C3	Calvin Cycle
C4	Hatch-Slack Cycle
CAM	Crassulacean Acid Metabolism
CAR	Carboxen
Carb.	Carbohydrate
CLPP	Community Level Physiological Profiling
^{13}C-NMR	^{13}C -Nuclear Magnetic Resonance
COM	Colloidal Organic Matter
CoSH	Coenzyme A thiol
CPI	Carbon preference index
CPMAS	Cross-Polarisation Magic Angle Spinning

CSIA	Compound Specific Isotope Analysis
Cyclo	Cyclopropyl
DBP	Disinfection by-product
DCM	Dichloromethane
DGGE	Denaturing Gradient Gel Electrophoresis
DIC	Dissolved Inorganic Carbon
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
D-SPME	Direct Immersion Solid Phase Microextraction
DVB	Divinylbenzene
EA-irMS	Elemental Analyser-Isotope Ratio Mass Spectrometry
EDR	Electrodialysis Reversal
EPA	Environmental Protection Agency
EPS	Extracellular Polysaccharide Substance
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
FID	Free Induction Decay
FIMS	Field Ionisation Mass Spectrometry
FISH	Fluorescence *in situ* hybridisation
FTIR	Fourier Transform Infrared
GC	Gas Chromatography
GC-FID	Gas Chromatography Flame Ionisation Detector
GC-irMS	Gas Chromatography isotope ratio Mass Spectrometry
GCMS	Gas Chromatography Mass Spectrometry
Geosim	*Trans*-1,10-dimethyl-*trans*-9-decalol
H	Hydrogen
HAA	Haloacetic Acid
He	Helium
HMW	Higher Molecular Weight

Hop	Hopanoid
HP	Hewlett Packard
HPC	Heterotrophic Plate Counts
HPI	Hydrophilic
HPO	Hydrophobic
HPSEC	High Performance Size Exclusion Chromatography
HS	Head Space
HS-SPME	Head Space Solid Phase Microextraction
HyPy	Hydropyrolysis
ICP-AES	Inductive Coupled Plasma-Atomic Emission Spectrometry
irMS	isotope ratio Mass Spectrometry
KIE	Kinetic Isotope Effect
K_m	Michaelis constant
LC	Liquid Chromatography
LCMS	Liquid Chromatography Mass Spectrometry
LLE	Liquid-Liquid Extraction
LMW	Low Molecular Weight
MCB	Mid-chain Branched
MDS	Multi-dimensional Scaling
MF	Microfiltration
MIB	2-methyl*iso*borneol
ML	Mega Litres
MMA	Mono-methyl alkane
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
MSD	Mass Selective Detector
MSSV	Micro-scale Sealed Vessel
MSSVpy	Micro-scale Sealed Vessel pyrolysis
MTBE	Methyl Tertiary Butyl Ether

MU	Mono-unsaturated
MVOC	Microbial Volatile Organic Compound
MW	Molecular Weight
N	Nitrogen
NAD	Nicotinamide adenine dinucleotide
NADP-ME	Nicotinamide adenine dinucleotide phosphate -Malic enzyme
Nd	Not detected
NF	Nanofiltration
NHMRC	National Health and Medical Research Council
NMR	Nuclear Magnetic Resonance
NOM	Natural Organic Matter
NPOC	Non-Purgable Organic Carbon
O	Oxygen
OH	Hydroxyl
OM	Organic Matter
P	Phosphorous
PA	Polyacrylate
P&T	Purge and Trap
PC	Principal Component
PCA	Photosynthetic Carbon Assimilation
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PEPCase	Phosphoenolpyruvate Carboxylase
PLFA	Phospholipid Fatty Acid
POM	Particulate Organic Matter
Pot	Potable
PU	Polyunsaturated
Py	Pyrolysis
Py-GCMS	Pyrolysis Gas Chromatography Mass Spectrometry

Py-GC-irMS	Pyrolysis Gas Chromatography isotope ratio Mass Spectrometry
Rec	Recycled
RO	Reverse Osmosis
RT	Retention Time
RuBP	Ribulose-1,5-bisphosphate
RubisCO	Ribulose-1,5-bisphosphate-Carboxylase-Oxygenase
S	Sulfur
Sat	Straight Chain
Sd	Standard deviation
SEC	Size Exclusion Chromatography
SMP	Soluble Microbial Products
SOM	Soil Organic Matter
SOM-C	Soil Organic Matter Carbon
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
SPME-GCMS	Solid Phase Microextraction Gas Chromatography Mass Spectrometry
SSB	Spinning Side-band
Ste	Steroid
SUVA	Specific Ultraviolet Absorbance
SV	Semi-Volatile
SVOC	Semi -Volatile Organic Compound
TB	Terminally Branched
TCU	Total Colour Units
TDN	1,1,6-trimethyl-1,2-dihydronaphthalene
THM	Trihalomethane
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TMAH	Tetramethylammonium Hydroxide
TMH	Tetramethylhydroxide
TMS	Tri-methylsilyl

TMSH	Trimethylsulfonium hydroxide
TOC	Total Organic Carbon
TPI	Transphilic
U	Unsaturated
UCM	Unresolved Complex Mixture
UF	Ultrafiltration
UHP	Ultra High Purity
UV	Ultraviolet
VHA	Very Hydrophobic Acids
VPDB	Vienna Peedee Belemnite
Vis	Visible
VOC	Volatile Organic Compound
WPWSS	West Pilbara Water Supply System

Chapter 1

Introduction

1.1 Background

1.1.1. Natural Organic Matter

1.1.1.1 Natural Organic Matter in the Aquatic Environment

Natural Organic Matter (NOM) is a complex mixture of organic compounds, highly variable in composition and functionality. These compounds originate from decaying organic matter (OM) or from anthropogenic inputs, precursors which impact nearly all natural waters (Amy & Cho, 1999; Croué et al., 1998; Croué et al., 1999b; Frimmel, 1998). In a watershed, NOM includes all OM such as the biomass of microorganisms, Dissolved Organic Carbon (DOC) and colloidal material (Thurman, 1985).

There are many ways of categorising NOM, one common method is by origin. Allochthonous NOM enters the water system from the terrestrial environment, whereas autochthonous NOM is derived *in-situ* from aquatic biota such as algae (Lee et al., 2006). These sources are discussed in Sections 1.1.2.3 and 1.1.2.4, respectively. The range of organic components in NOM varies between water sources and with seasons. This leads to variations in the nature and reactivity of NOM over time and space.

NOM plays an important role in the aquatic environment, influencing the physico-chemical properties of water, participating in carbon and nutrient cycling, interacting with metals and affecting the mobility, bioavailability and toxicity of anthropogenic pollutants (Frimmel, 1998).

1.1.1.2 The Importance of NOM in Drinking Water Sources

Safe drinking water is essential to sustain life. Every effort therefore needs to be taken to ensure that the drinking water industry provides consumers with high purity water. The adoption of chlorination of drinking water supplies in the 20th century has been responsible for a substantial decrease in infectious diseases (NHMRC, 2006). Disinfection

kills the majority of known bacterial pathogens and greatly reduces the numbers of viral pathogens, including most protozoans (NHMRC, 2006).

Disinfectants (in Australia this is most commonly chlorine) can react with NOM to produce a complex mixture of disinfection-by-products (DBPs). Since the discovery of 'haloforms' in drinking water (Rook, 1976), there has been concern over the combined health effects of these chemicals. Several published epidemiologic studies have identified relationships between chlorinated drinking water and adverse health outcomes (Simmons et al., 2002). The DBPs most commonly regulated by the Department of Health are trihalomethanes (THMs) and haloacetic acids (HAAs; NHMRC, 2006). Other by-products are also produced, and these may include compounds which present a greater risk to drinking water quality than THMs and HAAs. The study of established and emerging DBPs is an area of active research (Richardson et al., 2007; Plewa et al., 2008; Dotson et al., 2009; Krasner, 2009; Kristiana et al., 2009; Hu et al., 2010). In the drinking water industry the quantity of NOM is generally inferred from DOC measurements. It has been estimated that 90 % of NOM is present as DOC (Amy, 1993). DOC commonly is operationally defined as the organic carbon fraction that can pass through a 45 µm filter (Clesceri et al., 1998).

DOC affects water quality of drinking water by increasing the disinfectant and coagulant demand, providing precursor material for DBPs (Singer, 1999) and enhancing regrowth in distribution systems (Volk & LeChevallier, 2002). DOC can also detrimentally affect potable water quality as it enhances transportation and distribution of organic micropollutants and lowers the efficiency of treatment processes (Amy & Cho, 1999).

The treatability of water is ultimately dependant on the composition, structure and behaviour of NOM and in particular, DOC (Bursill et al., 1985; Drikas, 2003). Consequently there has been an expanding interest from water utilities in elucidating specific structural data and properties (e.g., reactivity) of NOM. There has also been an interest in understanding the relationship between structural moieties of NOM and specific organic precursors. Given the numerous potential sources (i.e. specific terrestrial, microbiological or anthropogenic source) of aquatic organics, the exact identity of the main precursors of DOC in a particular water source is often uncertain (Guggenberger & Zech, 1994). This is compounded by the fact that different organic species can impact water systems at different times and to different degrees (Croué et al., 1998) and that DOC has varying degrees of bioavailability (Tranvik & Hofle 1987; Moran & Hodson, 1990; McArthur &

Richardson, 2002). Accurate identification of the major organic inputs to source water is crucial for catchment and reservoir strategies aimed at minimising organic loads (Newcombe et al., 1997).

In some regions of Australia, high ambient temperatures coupled with long distribution systems necessitate the use of relatively high concentrations of disinfectant to maintain continuous and effective disinfection. In such situations, a high concentration DOC typically equates to an increased demand for chemical disinfectants (depending on the biological availability of the DOC) and increased potential for DBP formation. To avoid this situation and minimise consumption of expensive chemicals, the chlorine dosing level of treatment processes is continually regulated (Newcombe et al., 1997; Amy & Cho, 1999; Croué et al., 1999a; Croué et al., 1999b; Newcombe et al., 2002a; Newcombe et al., 2002b). Thus, understanding the concentrations, composition and sources of NOM is imperative to efficient water treatment protocols.

1.1.2 The Composition and Sources of DOC in Drinking Water Systems

1.1.2.1 General

DOC in aquatic systems is a complex matrix comprising reactive and refractory components. DOC is routinely divided into two fractions (humic and non humic). The humic fraction, commonly comprises around 50 % of the DOC (Thurman, 1985), and can be divided into humic and fulvic acids on the basis of their acid solubility. Fulvic acids make up the bulk of the humic fraction. They are characteristically more soluble, and have lower average molecular weights (600-1000 Da) compared to humic acids (Thurman, 1985; Malcolm, 1990). Humic acids are insoluble in water below pH 1 (Thurman & Malcolm, 1981), have higher average molecular weights (1000–10 000 Da) and make up a smaller (10–20 %) portion of the humic fraction (Malcolm, 1990). Non-humic substances make up the remaining DOC, which includes 30 % as hydrophilic acids (Thurman, 1985) – a mixture of simple organic acids and complex polyelectrolytic acids (Leenheer, 1981). The remaining ~20 % of the DOC consists of basic structures including proteins, peptides, lipids, carbohydrates, carboxylic acids, amino acids, hydrocarbons and other Low Molecular Weight (LMW) organic molecules like water soluble carbohydrates, cellulose and lignin-rich substrates (Findlay & Sinsabaugh, 2003).

DOC composition can vary between different water bodies. For example, in autochthonous dominated lakes, substances such as neutral sugars, amino sugars and amino acids can account for up to a third of the DOC (Tranvik & Jorgensen, 1995; Weiss & Simon, 1999). In some highly coloured surface water reservoirs humic substances account for 90 % of the DOC (Croué et al., 1999a). Rivers and streams generally have much higher DOC concentrations than in estuarine and coastal seas (Thurman, 1985).

1.1.2.2 Microbial Biomass

The metabolism of DOC affects nutrient balance and the ratio of DOC to inorganic nutrients can promote either autotrophic or heterotrophic activity (Biddanda et al., 2001). Microorganisms can significantly impact the nature of aquatic DOC (Boon et al., 1996). They biodegrade many organic substrates to produce metabolites which may cause other physical and chemical changes to their immediate environment. Biogeochemical cycling processes within freshwater aquatic environments are discussed in detail in Chapter 3. Microbiology is important to all stages of potable water systems, exerting a strong influence on source organics, drinking water treatment (discussed in Chapter 4) and distribution (discussed in detail in Chapter 3).

1.1.2.3 Allochthonous Sources of DOC

DOC released from fresh plant material is subject to many simultaneous transformations and biogeochemical reactions (Gregorich et al., 2000). The structural chemistry of leaf litter determines its susceptibility to decomposition and the cycling of nutrients in aquatic systems (Suberkropp & Chauvet, 1995; Hedde et al., 2007). Leaf litter begins to lose soluble organic and inorganic materials shortly after immersion in water, referred to as the initial phase of litter decomposition (Berg & McClaugherty, 2003). This is followed by a gradual decline in the rate of loss of organics for an extended period (Briggs & Maher, 1983). This latter (sometimes referred to as 'second') and more complex phase of decomposition is dependent on many environmental variables such as nutrient content, microbial community composition, historical factors and temperature as well as plant type, growth stage and vegetation assemblage (Webster & Benfield 1986; Berg & McClaugherty, 2003; Kalbitz et al., 2004; Kalbitz et al., 2006).

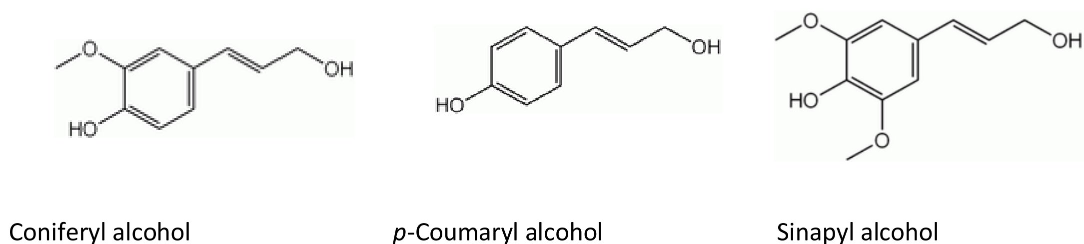
Previous research on the release of DOC by River Red Gums has shown that approximately 20 % of the litter mass may be lost in the first 24–72 hours of a flood (Briggs & Maher, 1983; Boulton & Boon, 1991; Zander et al., 2005). In contrast to this rapid loss of the

quantitatively significant soluble organic fraction, degradation of structural polymers in leaf material is relatively slow. For example, hemicellulose and cellulose degrade gradually (3–14 days) over the course of leaf decomposition (Meyer & Johnston, 1983; Webster & Benfield 1986; Gessner, 1991). Lignin degradation is very slow compared to other leaf components (Suberkropp et al., 1976; Webster & Benfield 1986). Guggenberger and Zech (1994) reported that most of the polar and mobile metabolites of plant litter are soluble and contribute to DOC. Other factors can affect breakdown rates including; temperature, dissolved nutrient concentrations, dissolved oxygen, pH and habitat (i.e., fresh water *vs* terrestrial *vs* wetland).

Consequently, DOC is a complex heterogeneous substance in constant flux, and difficult to characterise. Molecular (biochemical) indicators (e.g., lignin phenols) have been used to successfully relate DOC to specific terrestrial source as well as the diagenetic state of vascular plant tissue (Hedges & Mann, 1979; Ertel & Hedges, 1985). For example, ratios of vanillic acid to vanillin and syringic acid to syringaldehyde have been used as indices of lignin decay (Opsahl & Benner, 1995).

The major HMW components significant in the DOC of allochthonous dominated water bodies include lignin, tannins, polyphenols, aromatic acids and terpenoids (Spitzy & Leenheer, 1991). These compounds are relatively resistant to diagenetic alteration and may preserve a reliable link to their biological source (Amon & Benner, 1996).

Lignins are aromatic heteropolymeric macromolecules of high structural variability related to differences in the relative proportions of three hydroxycinnamyl alcohol monomeric units (*p*-coumaryl, coniferyl and sinapyl alcohols, Fig. 1.1).



Coniferyl alcohol *p*-Coumaryl alcohol Sinapyl alcohol

Figure 1.1 The monomers of lignin

Lignin has a key role in regulating litter decomposition and is important in the production of DOC (Guggenberger & Zech, 1994; Berg & McClaugherty, 2003). Although the mechanism

by which this occurs is not yet fully understood, submerged leaf litter bag studies of maple, ash, beech, spruce and pine have shown that this process controls the ‘second phase’ of litter decomposition (Kalbitz et al., 2006).

Tannins derived from plants can be either of hydrolysable or condensed form. Gallic acid (Fig. 1.2) is esterified with monosaccarides to make up the hydrolysable tannins. Condensed tannins however are made up of flavonol units that are polymerised and conjugated to other plant components such as carbohydrates and terpenoids (Robinson, 1991). Tannins are particularly abundant in leaves and needles with contents reported as high as 20 % (Hernes et al., 2001). In allochthonous systems, aromatic acids and phenols are typically attributed to the degradation of lignin and tannin (Pomes et al., 2000).

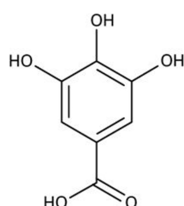


Figure 1.2 Gallic acid

Terpenoids are also significant constituents of DOC (Leenheer et al., 2003). This class comprises compounds made up of isoprene units, the numbers of which form the basis of their categorisation. Terpenes are a major input to the DOC of allochthonous systems particularly those dominated by *Eucalyptus*, and therefore are a particularly prominent source in Australian waters (Zander et al., 2005; Canhoto & Laranjeira, 2007; Goncalves et al., 2007). 1,8-Cineole (Figure 1.3) is a principal terpene found in *Eucalyptus* sp. (Boland et al., 1991).

Carbohydrates and Nitrogen containing products (N-products) are also important components of the DOC in allochthonous systems. These represent most of the biologically available fraction of DOC (Munster 1991, 1993; Tranvik & Jorgensen 1995; Weiss & Simon 1999). Thurman (1985) reported that ~30 % of the mass of terrestrial flora ends up in the aquatic environment, and half of this input is carbohydrate material.

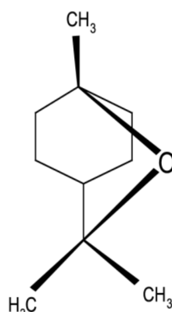


Figure 1.3 1,8-Cineole (eucalpytol)

N-products are chemically diverse, ranging from simple free amino-acids to complex polypeptides, protein, and humic-bound amino acids (Keil & Kirchman 1993; Hubberten et al. 1994). Significant biochemical classes of allochthonous precursors are discussed in detail in Chapter 2.

1.1.2.4 Autochthonous Sources of DOC

Autochthonous DOC forms within the water body typically as a heterogeneous mixture of Algogenic Organic Matter (AOM) or Biogenic Organic Matter (BOM). Complex pelagic ecosystems develop in aquatic ecosystems and AOM specifically derives from the activity of phytoplankton. BOM refers to material arising from bacterial degradation, mineralisation and from the death and decay by bacteria.

Algal blooms comprising for example, cyanobacteria and green algae, are major sources of AOM, often occurring in bloom proportions in lakes and other stagnant waters. Metabolic excretion rich in polysaccharides (80 to 90 %; Myklestad, 1995) and autolysis of cells are major processes associated with AOM formation. Consequently, AOM is rich in proteins, polysaccharides, nucleic acids and lipids (Fogg, 1983) and typically have a low aromatic content (McKnight et al*.*, 1994).

Actinomycetes and some cyanobacteria also produce *nor*-terpenoids, responsible for naturally-occurring off-tastes and odours in drinking water (Mallevialle & Suffet, 1987). Some of the most well-known are ‘geosmin’ (trans-1,10-dimethyl-trans-9-decalol) and ‘MIB’ (2-methyl iso borneol).

1.1.2.5 DOC in Water Treatment

Advanced characterisation of DOC has also improved our understanding of removal mechanisms during water treatment and to a lesser extent membrane fouling processes. Detailed compositional data have been used to optimise treatment processes and reduce

membrane fouling, respectively. However, there is still some ambiguity over much of NOM (and hence DOC) behaviour, including for example, the specific components or moieties of NOM responsible for fouling, biofilm growth and DBP formation.

The role of DOC in the fouling of membranes has been the subject of several reports (Combe et al., 1999; Yuan & Zydney, 1999; Jones & O'Melia, 2000; Lee et al., 2006). Fouling contributes significantly to the deterioration of membrane performances, consequentially increasing operation and maintenance costs and eventually shortening membrane life (Lee et al., 2005). The practical utilisation of membrane technology has been greatly limited by fouling (Violleau et al., 2005).

The fouling issue has attracted many investigations which have identified that hydrodynamics, feed water properties and membrane type can all have an influence on fouling (Bessiere et al., 2009). Both the humic (Aoustin et al., 2001; Jucker & Clark, 1994; Yuan & Zydney, 1999) and non-humic (Lin et al., 2000) fractions of DOC can contribute to fouling. Yet the origin and mechanisms of fouling remain difficult to identify and quantify (Bessiere et al., 2009). Enhanced characterisation of the sources and structures of foulants is required to fully evaluate the impact of surface water pre-treatment and catchment management strategies on the performance of membrane filters. A sophisticated understanding of the chemical and biological nature of a foulant will also assist targeted treatment to mitigate its impact. Fouling is discussed in more detail in Chapter 4.

1.1.2.6 DOC in Water Distribution

Drinking water quality can deteriorate rapidly along pipeline distribution (LeChevallier et al., 1987). A biofilm layer inside of the pipes (Characklis, 1973; van der Wende et al., 1989) can contribute to DOC, entrained inorganics, extracellular products and harmful biological activity such as pathogenicity (Zacheus et al., 2001) to the post treated water (Beech & Sunner, 2004). The concentration of free bacterial cells detected in treated water in distribution pipes increases as erosion, sloughing and abrasion of the biofilm occurs (van der Wende et al., 1989), commonly as a consequence of changes in flow velocity (Donlan, 2002). Biofilms are microbially diverse and heterogeneous, mostly comprising complex distributions of bacteria. Their impact on distribution systems is discussed further in Chapter 3.

1.2 The Analysis of DOC in Drinking Water

The complex structure of DOC presents a significant analytical challenge, which necessitates the use of a combination of sophisticated characterisation techniques. Many analytical methods include initial isolation techniques to produce less complex fractions more amenable to analyses.

Column chromatography is a common Isolation method with different stationary phases helping to concentrate different physico-chemical fractions. Other common isolation methods include freeze drying (to remove H_2O), reverse osmosis, and ultrafiltration.

Analytical pyrolysis techniques such as flash pyrolysis, and the adjunct technique of thermochemolysis combined with GCMS are popular spectrometry methods for studying the speciation of DOC. Other commonly applied methods of spectrometry analysis include: Size Exclusion Chromatography (SEC), most commonly with UV detection, but also possible now with DOC detection, Liquid Chromatography Mass Spectrometry (LCMS), Nuclear Magnetic Resonance (NMR) and Fourier Transform Infra-Red (FTIR) spectroscopy.

1.2.1 DOC Isolation Procedures

Lyophilisation, membrane techniques and sorption processes are all conventional DOC isolation procedures (Leenheer, 1981; Croué et al., 2000). Both lyophilisation and membrane separations avoid the use of extreme (high and low) pH values which can alter the structural properties of DOC (Croué et al., 2000). Membranes also offer the advantage of rapidly processing large volumes of water. However, none of these techniques removes inorganic salts from the isolated DOC which can prove problematical for many analytical methods.

Sorption-based isolation techniques simultaneously concentrate and separate DOC and into fractions based on their hydrophobic-hydrophilic and acid-base properties (Leenheer, 1981; Thurman & Malcolm, 1981). Non-ionic, macroporous XAD-type resins, particularly the acrylic ester XAD-8 resin in combination with the cross-linked aromatic polymer XAD-4 resin, have been widely used for the isolation of aquatic DOC. The original procedure of Leenheer and Huffman (1976) for separating the hydrophobic (HPO) and hydrophilic (HPI) fractions forms the basis of currently used methods. The HPO fraction contains humic material, which adsorbs onto the XAD-8 resin at acidic pH and is desorbed at basic pH. At

acidic pH the HPI fraction of non-humic material, does not adsorb to the XAD-8 resin, but does adsorb to the XAD-4 resin. Further separation of the TPI fraction by desorption with base will produce a transphilic (TPI) fraction which elutes with the solvent (Malcolm & MacCarthy, 1992; Croué et al., 1993). The HPO and HPI fractions collected using this method are free from salts, however, only about 75 % recovery of the total DOC is typical (Leenheer, 1981, Croué et al., 2000). This procedure is used in the present study to isolate DOC fractions from two Western Australian source waters (Chapter 5).

The TPI fraction is enriched in salts, but can be fractionated further into acidic, basic and neutral fractions using quite complex ion-exchange and precipitation procedures (Croué et al., 2000). But given the intricate efforts required and the very small yields obtained (Croué et al., 2000) these fractions are not routinely isolated.

1.2.2 Conventional DOC Characterisation Procedures

1.2.2.1 Elemental Analysis

Elemental analysis is often the starting point for NOM characterisation (Croué et al., 2000). Specific ratios of the major elements (i.e., C, H, N, O, S) can indicate the DOC type. For example, a higher C:H ratio is indicative of greater aromatic content (Abbt-Braun & Frimmel, 1999). Similarly, the C:O and C:N ratios can reflect the extent of oxygen and nitrogen containing functional groups, although these groups are usually better measured by other methods as described below.

1.2.2.2 NMR Spectroscopy

NMR spectroscopy is a non-destructive technique that provides a quantitative measure of organic structural configurations. Different carbon structures give rise to different chemical shifts. Both solution and solid state ^{13}C NMR spectroscopy produce chemical shifts in the same spectral region that allows semi-quantitative and qualitative assignment of structural features (Nanny et al., 1997). ^{13}C solid state NMR spectroscopy has been used effectively for NOM characterisation for some time (Schafer & Stejskal, 1976; Hatcher et al., 1980; Newman et al., 1980). Incomplete cross polarisation of certain carbon atoms by solid state NMR which, in particular could underestimate aromatic (Wilson, 1997), was overcome by high resolution solid state ^{13}C Cross-Polarisation Magic Angle Spinning (CPMAS).

CPMAS has become the standard mode for analysing complex naturally occurring organics (Croué et al., 2000; Smernik & Oades, 2000a&b; Leenheer et al., 2003) and is used in the present study to assist characterisation of source waters (Chapter 5).

1.2.2.3 FTIR Spectroscopy

FTIR spectroscopy uses the absorption of infrared radiation by the vibrational modes of bonded atoms to study molecular structure (Bloom & Leenheer, 1989). Since the vibrational dipole change of paramagnetic oxygen molecules is particularly pronounced, FTIR is particularly useful for examining the nature of oxygen in organic molecules, a structural feature with which several analytical methods are not suitable. For instance, NMR spectroscopy provides only indirect spectral information via the effects of oxygen on ^{13}C nuclei (Bloom & Leenheer, 1989).

FTIR spectra are, however, only semi-quantitative due to the wide range of absorption responses representing different structural moieties. The absorptivity of each group in the molecule would need to be known for precise quantitation (Bloom and Leenheer, 1989). The complexity of functional groups present in NOM represents an additional challenge to structural characterisation (Bloom and Leenheer, 1989). Nevertheless, FTIR data complements the information provided by NMR and analytical pyrolysis, and the data from these three methods are often integrated to provide a more holistic assessment of NOM (and hence DOC) structure.

1.2.2.4 UV and Visible Spectroscopy

UV/vis spectroscopy is often used to provide a fast indication of aromaticity, however the typically broad featureless spectra of DOC provide little further structural detail (Wang et al., 1990). Absorbance of UV are generally attributed to the aromatic chromophores present in aquatic DOC (Christman et al., 1989; Chin et al., 1994), whilst absorbance at 400 nm (referred to as 'colour') is generally attributed to the presence of conjugated ketone and quinone-like structures (Stevenson, 1982).

Specific UV Absorbance (SUVA), a common method of DOC analysis because of its simplicity, rapidness and field application, is a measure of the UV absorbance at 254 nm per mg of DOC in 1 L of water. SUVA values have been directly correlated to the aromatic character of DOC (Traina et al., 1990, Novak et al., 1992).

1.2.2.5 Size Exclusion Chromatography

The molecular weight distribution of DOC, largely dependent on the structures of the main source precursors (e.g., lignin, polysaccharides, proteins) and diagenetic action (Yau et al., 1979), can have a significant influence on its properties. For example, molecular size can be a major factor in the effectiveness of drinking water treatment processes. LMW DOC components are generally more bioavailable than HMW species (Volk et al., 2000, Hem and Efraimsen, 2001) and are also the most difficult to remove using conventional coagulation treatment (Chow et al., 1999, Drikas et al., 2003).

Several techniques have been used to characterise MW distribution, foremost being High Performance Size Exclusion Chromatography (HPSEC) which was able to measure the average MW distributions on minimally prepared samples of low volume in a relatively short time period. The HPSEC technology has also recently been extended from the UV selective response of MW separated fractions, to direct measurement of their DOC concentrations (e.g., Allpike et al., 2005).

1.2.3. Characterisation of Organic Matter by GCMS

Gas Chromatography Mass Spectrometry (GCMS) in combination with a range of sample introduction techniques is routinely used to identify and quantify the organic constituents present in DOC. Well established sample introduction techniques include analytical pyrolysis of consolidated fractions and Solid Phase Micro-Extraction (SPME, discussed in Chapter 2) of volatile and semi-volatile constituents.

Several of the techniques now used for molecular studies of DOC first evolved in other Earth science disciplines, including soil science and organic geochemistry where, for example, the isolation and characterisation of kerogen represented a major analytical challenge. Kerogen is the recalcitrant part of sedimentary OM (SdOM) that is insoluble in non-oxidising acids, bases and organic solvents (Dow, 1977). DOC also contains a quantitatively significant macro molecular moiety which presents a similar analytical challenge. Several different thermal and chemical degradation methods have been developed to reduce the size of macromolecular organic material and meet the molecular size limitations of GCMS (e.g., Douglas & Grantham, 1974; Irwin, 1979, Hedges & Ertel, 1982; Leenheer et al., 1989; Bruchet et al., 1990; Challinor, 1995).

1.2.3.1 Thermal Degradation

Analytical pyrolysis which uses thermal energy to break-down macromolecules emerged in the late seventies (e.g., Irwin, 1979) and has since been widely used to help characterise a large range of environmental samples including plant biomass, Soil Organic Matter (SOM) and aquatic NOM.

1.2.3.1.1 Analytical Pyrolysis

Pyrolysis involves the thermal treatment of organic macromolecules in an inert environment, with many of the resultant fragments (m/z < 1000) amenable to GCMS detection (Py-GCMS). Relative abundances of pyrolysis products can be determined from the peak areas of GCMS chromatograms, which in turn can provide a measure of the major biopolymers present in NOM (Bruchet et al., 1990). Py-GCMS is now routinely used to facilitate the molecular characterisation of aquatic NOM (Bruchet, 1985, Gadel & Bruchet, 1987; Abbt-Braun et al., 1989; Bruchet et al., 1990, Schulten and Gleixner, 1999, González-Vila et al., 2001, Templier et al., 2005) and terrestrial NOM. Saiz-Jimenez and deLeeuw (1986b) reported the detection of 322 compounds in the pyrolysates of soil humic fractions, with the major biochemical precursors established to be polysaccharides.

Many of the pyrolysis GCMS products of environmental OM have been correlated with biochemical groups such as amino acids, proteins, carbohydrates, amino sugars, lignin and tannins (Saiz-Jimenez & de Leeuw, 1986b; Bruchet, 1985, Gadel & Bruchet, 1987; Bruchet et al., 1990; Page et al., 2002). Lignin produces a characteristic distribution of hydroxy- and methoxy- aromatic pyrolysisates (Hedges & Mann, 1979; Saiz-Jimenez & de Leeuw, 1984; 1986a; Opsahl & Benner, 1997). Carbohydrates typically yield furans and furaldehydes on pyrolysis (Pouwels et al., 1987; 1989; Pastorova et al., 1994) and proteins produce pyridines, pyrroles and other low MW nitrogen containing compounds (Bruchet, 1985; Saiz-Jimenez & de Leeuw, 1986b; Chiavari & Galletti, 1992) and amino sugars produce acetamide derivatives (Bruchet, 1985; Stankiewicz et al., 1996; Christy et al., 1999).

However, Py-GCMS is often challenged by the high complexity and heterogeneity of NOM. The GC resolution of many NOM pyrolysates, particularly those with functional groups can be challenging (Larter & Senftle, 1985; Dignac et al., 2006). Polar structures, intermolecularly bound by hydrogen bonds, are often of low thermal volatility (Leenheer & Noyes, 1984; Saiz-Jimenez, 1994; del Rio et al., 1996) and prone to condensation on the GC transfer line (Parsi et al., 2007; Saiz-Jimenez, 1994). The high reactivity of polar pyrolysates

also makes them vulnerable to secondary reactions during the pyrolysis step (Saiz-Jimenez, 1994; Hatcher et al., 2001). The inefficient pyrolysis detection of polar structures represents the largest inconsistency with molecular data from FTIR and ^{13}C-NMR. For example, these spectroscopic studies have identified aliphatic and aromatic carboxylic groups as major components of humic substances and several aquatic NOM samples (e.g., Leenheer et al., 1995; Leenheer et al., 2003), whereas only minor yields of pyrolysates typical of these precursors were detected from analytical pyrolysis of these and similar samples (Saiz-Jimenez, 1993; Saiz-Jimenez, 1994; del Rio et al., 1996) whilst carbohydrates are susceptible to decarboxylation on pyrolysis.

The direct mass spectrometric detection of pyrolysates (i.e., without any chromatographic separation) has been tried at different times with limited success. In the absence of GC separation the analysis time can be fast, but the superimposed mass spectra of the multitude of DOC products can be very challenging to resolve. Some specialised MS approaches, for example, Field Ionisation Mass Spectrometry (FIMS), have provided marginal analytical improvements by producing largely molecular ions, and this data (when integrated with data obtained from complementary analytical techniques) has provided some useful insights about SOM. Leinweber & Schulten (1999) for example, characterised the SOM of nine soil horizons using a combination of chemical degradation ^{13}C NMR and Py-FIMS and found distinct differences in the dominant carbohydrate derived compounds and sterols depending on the extent of podsolisation of the soil. Additionally, they were able to characterise conversions in DOM components during composting (e.g., an increase in fatty acid and carbohydrate derived products during the thermophillic composting phase, ascribed to enhanced bacterial activity). These findings allowed inferences to be made regarding the fate of manure DOM in soils.

1.2.3.1.2 Offline Pyrolysis

Pyrolysis can also be used as an offline system which can be scaled up for larger product yields. This allows further preparations such as column chromatography (e.g., Douglas & Grantham, 1974) or subsequent derivatisation with chemical agents (e.g., Dungait et al., 2008b). Derivatisation of polar structural groups may make a large range of additional products amenable to GC resolution. Derivatisation agents typically have an affinity for specific functional groups which can help reduce hydrogen bonding that may inhibit chromatographic separation of compounds (Leenheer et al., 1989), and/or protect 'labile' (i.e., easily altered) groups from alteration (Griffith & Schnitzer, 1989). Offline pyrolysis is

therefore an attractive tool for producing fractions of NOM or other complex biological or environmental organic matter for Compound Specific Isotope Analysis (CSIA). Internal standards can be added to fractions post pyrolysis to support quantification and large pyrolysis quantities are advantageous for multiple analyses (Faix et al, 1987).

1.2.3.1.3 Thermochemolysis

Many Py-GCMS products of NOM samples are too polar or insufficiently volatile for GC analyses. Thermochemolysis GCMS with Tetramethylammonium hydroxide (TMAH) is a simultaneous degradative and derivatisation technique. The activity of TMAH is higher at elevated thermal conditions such as provided by pyrolysis. This method was introduced by Challinor (1995) for forensic analyses and is now a well-established analytical method applied to a wide range of organic materials including aquatic NOM (Hatcher & Clifford, 1994; Martin et al., 1995; McKinney &Hatcher, 1996; Hatcher & Minard, 1996; del Rio et al., 1998; Mannino & Harvey, 1999; van Heemst et al., 2000).

Whilst thermochemolysis is a good compliment to other molecular analysis, it may lead to detection of experimental artefacts from reactions such as saponification, methylation or methyl group rearrangement (Poerschmann et al., 2005). Thus, its application to complex organic matter and the interpretation of data obtained needs to be carefully considered (Saiz- Jimenez, 1994).

1.2.3.2 Chemical Degradation

In addition to derivatisation, some chemical reagents can target the selective degradation of NOM. A number of different agents have been used, with the main reaction processes:

- Oxidation (using alkaline permanganate, alkaline cupric oxide, alkaline nitrobenzene) frequently used in the investigation of lignin structures in complex natural polymers (Hedges & Ertel, 1982; Ertel et al., 1984). Chlorine oxidation has been used to study DBP formation (Larson & Weber, 2004).
- Reduction (catalytic hydrogenation, hydriodic acid, *n*-butylsilane, sodium amalgam, and zinc dust; e.g., Stevenson, 1982; Nimmagadda & McRae, 2007). Phenol cleavage can be selectively targeted with a *p*-toluene sulfonic acid catalyst (Jackson et al., 1972).
- Hydrolysis – with bases (alkali hydroxides, ammonia) which promote the cleavage of activated ethers (e.g., of lignin) and ester bonds, or acids (6M hydrochloric acid,

4M methanesulfonic acid) which promote peptide bond cleavage. A review of hydrolytic degradation of NOM was presented by Parsons (1989).

- Sulfurisation – degradation conducted using a mixture of sodium hydroxide and sodium sulfide (also known as the Kraft process, used in wood delignification, and dominates commercial pulping processes). During the process hydroxide and hydrosulfide anions react to cleave inter-unit linkages of the lignin polymer, resulting in smaller fragment units. It has been used to isolate and assist in the characterisation of lignin, and has proved useful in investigating aromatic linkages of lignin (Hayes & O'Callaghan, 1989).

Chemical degradation can provide useful molecular information about humic substances (Hayes et al., 1989) and aquatic NOM (Ertel et al., 1984), however, most approaches suffer from being laborious, hazardous and inefficient or give low yields due to accumulative losses at the many sequential steps typically involved (Farrimond et al., 2003).

1.3 Characterisation of NOM using Stable Carbon Isotope Analysis

1.3.1 Unique Insights provided by Stable Isotope Analysis

The techniques described so far have focused on studying the molecular structure of NOM. Molecular products or structural features provided by the analytical methods described in Section 1.2 can provide insight into the biosynthetic origins and diagenetic processes of NOM. Stable carbon isotopic ($\delta^{13}C$) values measureable by isotope ratio Mass Spectrometry (irMS) can complement molecular data with additional source and diagenetic information.

Some vegetation, micro-organisms and physico-chemical processes are characterised by diagnostic $\delta^{13}C$ values. $\delta^{13}C$ data may also help resolve the connectivity of organic products with different elements of aquatic systems – e.g., bioavailable substrates consumed in water treatment processes; the seasonal flux of different organic seasons (Hayes, 1993; Grice, 2001; Grice & Brocks, 2011); or the residence time of particular organic compounds (e.g. Lu et al., 2003).

1.3.2 Background to Stable Isotope Analysis

Hydrogen, carbon, nitrogen, oxygen and sulfur present in OM each occur as a mixture of stable isotopes. Carbon has two stable isotopes, ^{12}C and ^{13}C, with approximate average

natural abundances of 98.89 % and 1.11 %, respectively. The lightest isotope (e.g., ^{12}C) is the most naturally abundant, however, small variations in the abundance of the heavier isotope (e.g., ^{13}C) arise as from differences in the inter and intramolecular binding forces between the isotopes.

The values of major stable isotopic elements can be determined on whole material (bulk isotopic analysis), and several (C, H and very recently S) can also be determined on single compounds by CSIA (Grice & Brocks, 2011). The present study investigates the contribution of bulk and compound specific $\delta^{13}C$ analysis to NOM characterisation.

The ratio between ^{12}C and ^{13}C is determined by irMS measurement of ions *m/z* 44 (i.e., $^{12}C^{16}O_2$) and *m/z* 45 (i.e., $^{13}C^{16}O_2$) and *m/z* 46 (i.e., $^{12}C^{16}O^{18}O$) following complete oxidation of the organic sample to CO_2. The stable carbon isotopic value ($\delta^{13}C$) is expressed in the units 'per mill' (‰) according to the formula:

$$\delta^{13}C = [(R_{sample}/(R_{standard})-1] \times 1000$$

R_{sample} is the ratio of ^{13}C to ^{12}C in the sample being analysed. $R_{standard}$ is the ratio of ^{13}C to ^{12}C of an international reference standard. The original reference standard was a PeeDee belemnite sample obtained from *Belemnitella americana*, a Cretaceous marine fossil from the PeeDee Formation in South Carolina (Urey 1951; Craig 1957). However, following the complete consumption of this original standard, it has been replaced by the 'Vienna Pee Dee Belemnite' (V-PDB) standard (Coplen, 1995) which was previously correlated against PeeDee belemnite. The V-PDB reference standard has been assigned a $\delta^{13}C$ value of zero. The ^{13}C:^{12}C ratio of these standards is higher than most natural organic compounds, which are therefore expressed with a negative delta (δ) value.

1.3.2.1 Isotopic Fractionation in Plants

The photosynthetic mechanisms used by plants include the Calvin cycle (C3), the Hatch-Slack cycle (C4) and Crassulacean Acid Metabolism (CAM). Bonds with ^{12}C are relatively weaker than those with ^{13}C and thus more reactive, hence isotopic discrimination in favour of the lighter isotope occurs during physical and enzymatic processes (e.g., during diffusion of atmospheric CO_2 across stomata of C3 plants an apparent fractionation of ~4 ‰ occurs; Lajtha & Marshall, 1994). Differences in isotopic fractionation during photosynthesis give rise to distinct $\delta^{13}C$ values of different photosynthetic plant types (Bender, 1971). C3 plants, for example, incorporate atmospheric CO_2 into a C3 compound via the enzyme

Ribulose-1,5-bisphosphate-Carboxylase-Oxygenase (RubisCO), after which it is directly introduced to the Calvin cycle.

RubisCO discriminates against the heavier isotope, and consequently, C3 plants have low $\delta^{13}C$ values (i.e., bulk biomass values of -32 ‰ to -20 ‰; O'Leary, 1981; O'Leary, 1988; Ehleringer et al., 1991; Ehleringer & Monson). In contrast, C4 plants incorporate CO_2 into a C4 compound via the primary enzyme phosphoenolpyruvate (PEP) carboxylase, which does not discriminate against the heavier isotope as much as the C3 compound, resulting in relatively enriched bulk $\delta^{13}C$ values (-9 ‰ to -17 ‰ for bulk biomass; O'Leary, 1981; O'Leary, 1988; Hattersley, 1982). Some CAM plants switch between C3 and C4 metabolism and consequently have a wide range of bulk $\delta^{13}C$ values (-28 ‰ to -10 ‰ for bulk biomass; Osmond et al., 1976; Osmond & Holtum, 1981; O'Leary, 1981; O'Leary, 1988).

Bulk $\delta^{13}C$ values represent the average isotopic value of all biochemical constituents of all plant tissues. A few early studies investigated the isotopic composition of different biochemical classes. Wilson and Grinstead (1977) for example, investigated the stable isotopic compositions of the lignin and cellulose components (separated from wood samples using conventional wood chemistry techniques at the time; Green, 1963) to test if an observed difference between $\delta^{13}C$ values of cellulose and lignin reflected temperature variance. They studied 15 samples covering three annual rings of a Monterey Pine and measured ^{13}C values of both cellulose and lignin isolates from winter-wood, which were ^{13}C depleted relative to wood lain in summer. They determined a temperature coefficient of at least 0.2 ‰ per °C, ascribed to changes in air temperature affecting the biosynthesis of cellulose and lignin.

The photosynthesis of plants is of particular relevance to the $\delta^{13}C$ value of NOM since it represents a major allochthonous source of NOM. In general the isotopic composition of the soil reflects the photosynthetic pathway of the dominant species in the plant community (Boutton, 1991). Other organisms in soil (e.g., bacteria) may also influence a soils isotopic composition, particularly in anaerobic environments (e.g., Quay et al., 1988).

There is a range of environmental variables which can subtly affect the $\delta^{13}C$ values of plants. This includes altitude, humidity, water stress, salinity and nutrient status. When interpreted with knowledge of environmental influences, isotopic analysis can therefore assist environmental and ecological research, as well as palaeo-environmental

reconstructions (Grice et al., 2009; Grice & Brocks, 2011). For example, stable isotope analysis can help establish C3 and C4 land use cover through time and space and this information can then be used to investigate soil organic matter turnover rates (Bonde et al., 1992; Arrouays et al., 1995; Krull et al., 2004; Creamer et al., 2011).

Carbohydrates, cellulose and hemi-cellulose are relatively enriched in ^{13}C compared to bulk plant tissues (Schweizer et al., 1999; Boutton, 1991). Consequently the relative high proportions of carbohydrates in tissues such as tubers and sap are usually enriched in ^{13}C, and conversely tissues with a greater predominance of lipids and waxes (e.g., leaves) and are generally depleted in ^{13}C (Gleixner et al., 1993; Scartazza et al., 2004; Brandes et al., 2006 and Gessler et al., 2007). Similar differences actually occur intra-molecularly, with carboxyl and other oxidised functional groups relatively more enriched in ^{13}C than reduced (methyl) groups (Abelson and Hoering, 1961; Weilacher et al, 1996) and lignin metabolites relatively depleted in ^{13}C (Benner et al., 1987). Abelson and Hoering (1961) specifically investigated $\delta^{13}C$ values of amino acids isolated from photosynthetic organisms, reporting a wide range of carbon isotope discrimination of individual amino acids as well as distinct differences in the $\delta^{13}C$ values of carboxyl groups of individual amino acids. For example -17.1 ‰ was the average total glutamic acid $\delta^{13}C$ value (determined in 6 organisms) but the corresponding $\delta^{13}C$ value for carboxyl carbons of glutamic acid was +0.2 ‰. This contrasts markedly with the average total $\delta^{13}C$ value determined for leucine (-21.8 ‰), whereas the $\delta^{13}C$ value of the carboxyl carbons of leucine was -18.2 ‰). These and other pioneering investigations highlighted key differences in products of plant metabolism (see Section 1.3.3 for further details).

Microbial products which are a major aquatic source of autochthonous and certain diagenetic processes can also give rise to characteristic isotopic characteristics. As diagenesis proceeds the more biologically stable components (e.g., lignin; Benner et al., 1987) will increase in concentration and this is often reflected isotopically (Macko et al., 1991). For example, the $\delta^{13}C$ preservation of NOM or fractions/components thereof may indicate source water persistences or recalcitrance to treatment (Lichtfouse et al,. 1998).

1.3.2.2 Isotopic Fractionation in Microorganisms

Autotrophic organisms generally utilise CO_2 or HCO_3^- as their energy source. These primary sources can be isotopically distinguished. Aquatic autotrophs utilise dissolved CO_2 which is depleted in ^{13}C relative to atmospheric CO_2 by 1 ‰ (Wendt, 1968) or HCO_3^- which is

enriched in ^{13}C relative to dissolved CO_2 by 8 ‰ (Mook et al., 1974). Symbiotic methanogen and methanotrophic communities which can also occur in anoxic freshwaters give rise to very distinctive stable isotopic values (Whiticar et al., 1986). $\delta^{13}C$ isotopic shifts in the range -55 ‰ to -90 ‰ have been observed for organisms that generate methane (methanogens) or use methane (methanotrophs) as their carbon source (Summons et al., 1994; Jahnke et al., 1999; Birgel et al., 2006; Birgel & Peckmann, 2008).

The isotopic compositions of heterotrophic organisms are similarly influenced by their feeding behaviour. DeNiro and Epstein (1978) reported a relative enrichment in $\delta^{13}C$ of 1 ‰ to 1.5 ‰ per trophic level. As aquatic microorganisms decay and die, their relatively ^{13}C enriched detrital material sinks through the water column and is utilised by other organisms. It has been demonstrated that the $\delta^{13}C$ value of sterols, representing the primary diet of crustaceans is preserved (i.e., no isotopic fractionation) in the cholesterol biomarker of the crustaceans (Grice et al., 1998). Whilst the effect of trophic systems on stable isotopic phenomena has been the focus of several studies (e.g., Freeman et al., 1991, Grice et al., 1998), the stable isotopic characteristics of aquatic NOM generally remains limited.

1.3.3 Background to Analytical Technique

1.3.3.1 Bulk Analysis

Elemental Analyser-Isotope Ratio Mass Spectrometry (EA-irMS) measures the bulk isotopic analysis of whole samples or their fractions. Bulk stable carbon isotope analysis has been used to further the understanding of biogeochemical cycling in aquatic systems. For example, Guo et al. (2003) identified differences in the bioavailability of different size fractions of organic matter in riverine systems by measuring the $\delta^{13}C$ of Particulate Organic Matter (POM), Colloidal Organic Matter (COM) and DOM fractions from the Chena River in Alaska. The POM ($\delta^{13}C$ = -29.6 ‰) was more depleted in ^{13}C than the COM ($\delta^{13}C$ = -27.5 ‰), which was in turn more ^{13}C depleted than the DOM (-16.9 ‰). In the same study, a lower contribution of polysaccharides with decreasing MW (reflecting increased biodegradation) was similarly identified by Py-GCMS of the fractions. Guo et al. (2003) attributed these effects to the preferential decomposition of the lighter ^{12}C isotope with decreasing particle size.

Bulk isotopic values have also been measured for solutions such as DOC. This requires the use of a Total Organic Carbon (TOC) analyser and an adapted irMS to measure CO_2 produced via high temperature wet-oxidation methods (Bouillon et al., 2006). TOC-irMS can sequentially provide an isotopic ($\delta^{13}C$) and concentration (ppm C) measure of Dissolved Inorganic Carbon (DIC) and DOC of solution samples. DOC $\delta^{13}C$ values were utilised by Amiotte-Suchet et al. (2007). Comparing variations in the $\delta^{13}C$ of soil solute and stream DOC to that of SOM, Amiotte-Suchet et al. (2007) found that the δ^{13} DOC value of a stream located under deciduous vegetation was 1 ‰ to 2 ‰ more depleted in ^{13}C than streams located under other vegetation types (e.g., 0.5 ‰ for coniferous). Deciduous stream DOC was also found to be more depleted in ^{13}C than the adjacent SOM, an affect attributed to preferential use of ^{12}C by soil microbiota, resulting in enrichment in ^{13}C of the remaining SOM (Lichtfouse et al., 1998) which produces ^{13}C depleted DOC compared to the corresponding SOM. DOC is relatively more depleted in ^{13}C in soils under deciduous vegetation than under coniferous vegetation as ^{13}C enrichment of SOM in deciduous species is more pronounced (Agren et al., 1996).

Carbon isotopic values of DIC, POC and DOC were used by Kritzberg et al. (2006) to compare the relative contributions of autochthonous and allochthonous carbon sources to lake bacterial growth. Kritzberg et al. (2006) manipulated Lake DIC by adding $NaH^{13}CO_3$ to two forested lakes daily for 35 days. One lake was fertilised (with N and P) to maximise primary production, the other lake remained in a natural state of low productivity. Bacterial biomass cultivated *in situ* was used to examine and determine $\delta^{13}C$ values. The resulting $DIC^{13}C$ affected the $PO^{13}C$ (Particulate Organic Carbon) reflecting a ^{13}C enriched phytoplankton community. In the fertilised lake the increase in $POC^{13}C$ directly followed the $DIC^{13}C$ increase, whereas in the non-fertilised lake the POC enrichment was half (*Cf*. fertilised). POC also became relatively more ^{13}C enriched *Cf*. bacteria during the experiment. From these findings bacterial biomass was estimated as 45 % allochthonous carbon in the fertilised lake and 75 % of bacterial biomass in the non-fertilised lake, showing that both autochthonous and allochthonous carbon sources contribute to lake bacterial growth and the utility of ^{13}C tracer studies to follow carbon dynamics in aquatic systems.

Bulk ^{13}C isotopic data was also integrated with the molecular data from the spectroscopic approaches of ^{13}C-NMR and Py-GCMS to study the DOC isolated from four estuarine systems of varying salinity (van Heemst et al., 2000). Whilst the molecular data provided

negligible differentiation of the DOC samples, their $\delta^{13}C$ values showed a progressive increase from -27.8 ‰ to -25.4 ‰ with increasing salinity, a relationship which suggests the mixing of river DOC with marine DOC of comparable ages. The pyrolysis analysis showed no evidence of unaltered polysaccharides, proteins and lipids. This implied a high proportion of refractory organic matter, which typically is ^{13}C depleted relative to the source DOC because ^{13}C-enriched material such as carbohydrates (present in source DOC) are preferentially consumed by microorganisms.

Whilst bulk $\delta^{13}C$ analysis has frequently added some value to the study of DOC, this measure of the average $\delta^{13}C$ signal of such complex organic materials does miss the detail that the unique isotopic value of source specific compounds can provide (Silfer et al., 1991). The same or similar organic species from different sources can have vastly different isotopic values (Grice & Brocks, 2011).

1.3.3.2 Compound Specific Isotope Analysis

CSIA can allow measurement of the stable carbon isotopic composition of organics within complex mixtures. A schematic of a typical $^{12}C/^{13}C$ GC-irMS set-up is presented in Figure 1.4.

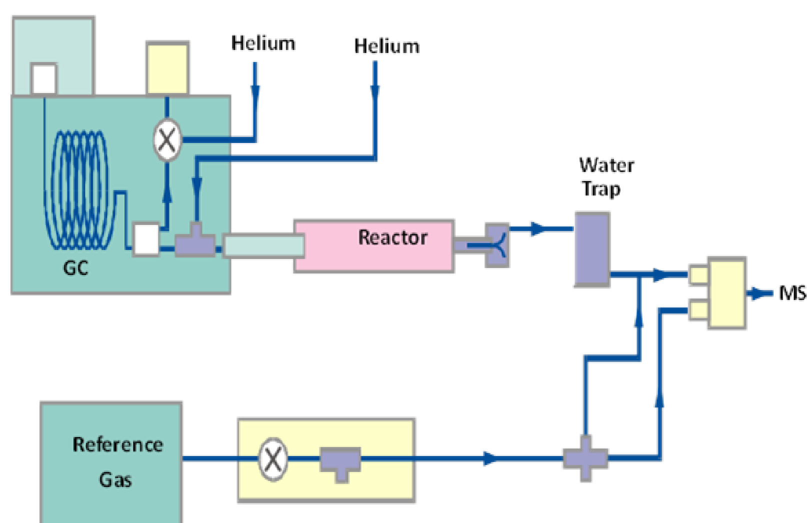


Figure 1.4 Schematic of $^{12}C/^{13}C$ GC-irMS instrumentation

The instrumental set-up for CSIA involves the interfacing the GC to an irMS via a combustion reactor (Boutton, 1991). The $^{12}C/^{13}C$ GC-irMS method was pioneered by Matthews and Hayes (1978) and several reviews provide detailed accounts of the development of this technology (Hayes 1993 & 2001; Summons et al., 2008a&b; Grice & Brocks, 2011).

Accurate CSIA measurements require baseline GC because $^{13}CO_2$ elutes chromatographically earlier than the $^{12}CO_2$ (Meier-Augenstein, 2002; Blessing et al., 2008). CSIA of organic sediments or DOC via GC-irMS can provide more detailed $\delta^{13}C$ data than bulk $\delta^{13}C$ values. Like the occurrence of certain biomarkers, the stable isotopic value of biochemical products may also be diagnostic of particular organic precursors.

The $\delta^{13}C$ data for a number of biomarkers measured in a single CSIA analysis can provide information about multiple environmental inputs and processes (Grice & Brocks 2011). Several compounds with characteristic isotopic values useful for defining the origin and processing of aquatic NOM are shown in Figure 1.5. Isotopic differences are found in the biochemical constituents of all plants species (Brugnoli & Farquhar, 2000), as the typical C3 plant data shows (Fig. 1.5).

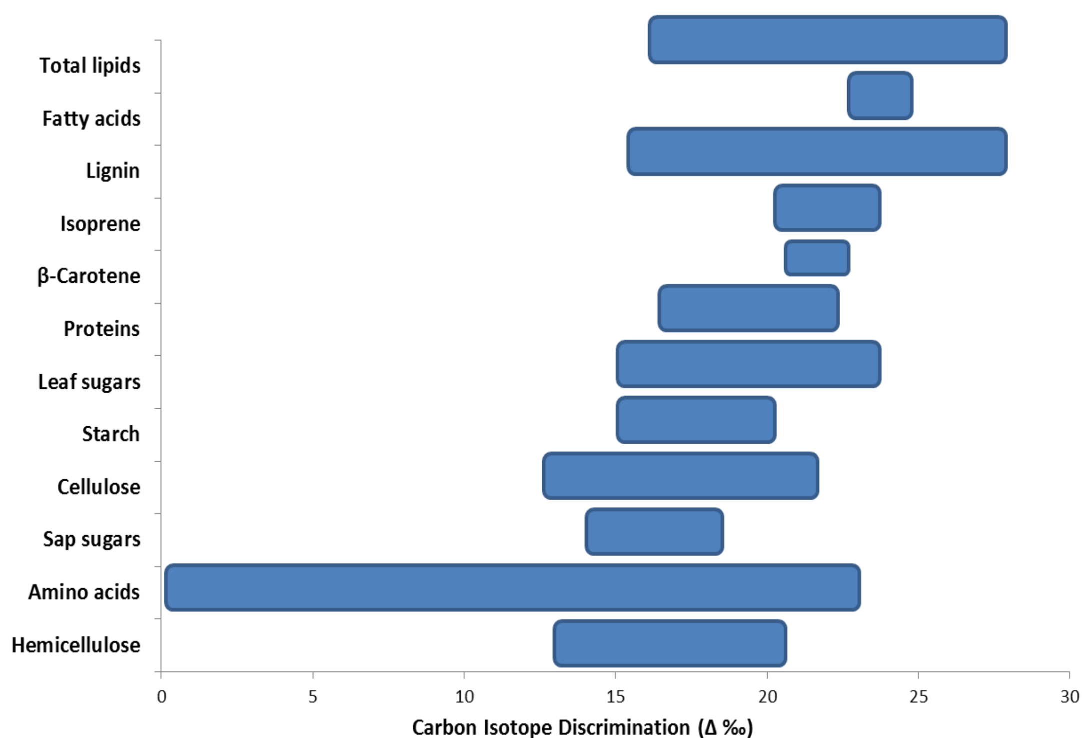


Figure 1.5 The carbon isotope range of the main biochemical classes within a C3 plant (assumed bulk biomass $\delta^{13}C$ value of 19 ‰; Brugnoli & Farquhar, 2000).

Isotopic variance between different biochemicals arises from isotopic fractionation at specific branch points in photosynthesis, biosynthesis and metabolism. A comprehensive review by Hayes (2001) provides details of the fractionation effects of major biosynthetic processes and a schematic overview from Hayes (2001) illustrating the metabolic

relationships of these compounds is shown in Figure 1.6. A more detailed review of algal lipids has been provided by Schouten et al. (1998).

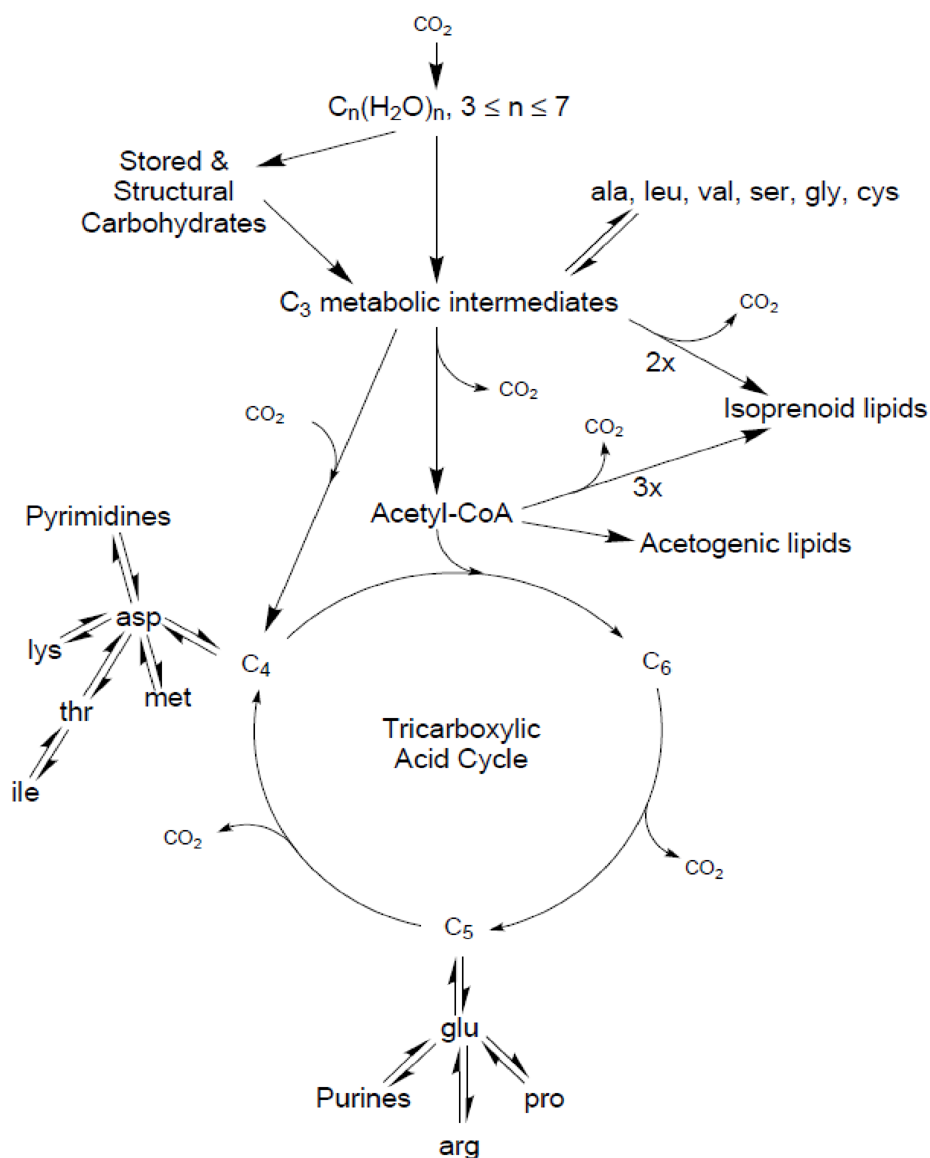


Figure 1.6 Metabolic relationships between major plant biochemicals (from Hayes, 2001). Abbreviations as follows: Ala = alanine, leu= leucine, val = valine, ser = serine, gly = glycine, cys= cysteine, asp = aspartic acid, lys =lysine , thr = threonine, ile = isoleucine, met = methionine, glu = glutanic acid, arg = arginine, pro = proline

The $\delta^{13}C$ values of microbial lipid biomarkers such as bacteriohopanes and isoprenoidal lipids preserved in sediments or present in petroleum have been used in the reconstruction of palaeo-environments (e.g., Grice et al., 2007; Grice, 2001; Grice & Brocks, 2011) and investigations of microbially-mediated biogeochemical processes (Freeman et al., 1990; Summons et al., 1994; Meckenstock et al., 2004; Rolle et al, 2010). There have also been

many applications of CSIA to extant environments. Several examples include investigations into soil microbial residue stabilisation (Glaser & Gross, 2005); the microbial activity of landfill leachate DOC (Mohammadzadeh et al., 2005) sources and biodegradability of ground water contaminants (Hunkeler et al., 2008) and evaluating the fate of biochemical constituents in the dung of grassland ecosystems (Dungait et al., 2008 a & b, 2009, 2010).

1.3.3.3 Products of Primary Metabolism - Simple Sugars and Carbohydrates

The cycling of carbohydrates is particularly important in understanding the flux of organic carbon in aquatic environments. Carbohydrates are generally susceptible to mineralisation (Degens et al., 1968), and some 'labile' species (i.e., simple sugars) are preferentially utilised by microbes during early diagenesis within the water column. Despite the structural and behavioural complexity of carbohydrates, biosynthetic and diagenetic features may be illuminated by stable isotopic signatures.

Macko et al. (1989) investigated the isotopic content of individual carbohydrates isolated from extant microorganisms and sediments, finding their $\delta^{13}C$ values to be indicative of isotopic fractionations that occur during biosynthetic substrate utilisation and the metabolism of carbon. Carbohydrates produced by microbial reworking (i.e., non-indigenous carbohydrates) were particularly depleted in ^{13}C. Xylose produced by bacteria or fungi, for example, was 8 ‰ more depleted than xylose of the bulk biomass (peat) or mannose (an indigenous carbohydrate). Furthermore, ^{13}C depletion typically increased with depth (Macko et al., 1991) as new carbohydrate material was increasingly produced (Macko et al., 1986 & 1987). More recently, Teece and Fogel (2007) showed $\delta^{13}C$ values of the individual sugars isolated from algae, cyanobacteria, terrestrial plants, bacterial cultures, and marine invertebrates, relative to bulk carbon isotopic compositions varied by as much as 8 ‰. They also showed that glucose produced by heterotrophic organisms was consistently enriched in ^{13}C relative to all other monosaccharides and proposed that this step may be a factor controlling the progressive ^{13}C enrichment through the trophic levels of an aquatic foodweb. This enrichment (resulting from the loss of $^{12}CO_2$), however, is only applicable to bulk biomass values—CSIA of sterol fractions isolated from the copepod *Temora longicornis* show that cholesterol retains the $\delta^{13}C$ value of the algal diet (Klein Breteler et al., 2002).

1.3.3.4 Products of Secondary Plant Metabolism

1.3.3.4.1 Differences between Primary and Secondary Products

Metabolic processes can convert carbohydrates and other primary units into more complex biochemicals such as proteins, nucleic acids and their associated cellular lipids. The fructose-1,6-biphosphate aldolase reaction (Gleixner et al*.*, 1993; Gleixner & Schmidt, 1997) and fractionation effects associated with metabolite transport (Brugnoli & Farquhar, 2000) lead to the ^{13}C depletion of the secondary metabolites. The ^{13}C depletion generally increases with progression of the cycle and secondary processing (Blair et al*.*, 1985; Sternberg et al*.*, 1986; Benner et al*.*, 1987; Coffin et al*.*, 1990). The $\delta^{13}C$ values of secondary metabolites (described below) can span a large range of values.

1.3.3.4.2 N-Products (Proteins and Amino Acids)

The $\delta^{13}C$ values of amino acids span the range of 6–23 ‰ (Abelson & Hoering, 1961). This large range has been attributed to the different biosynthetic behaviours of amino acid families (shown in Fig. 1.6). A detailed account of the stable isotopic characteristics of amino acids was presented by Hayes (2001).

1.3.3.4.3 Lipids

The isotopic fractionations of lipids are extremely complex (Hayes, 2001). Generally, lipids reflect a significant ^{13}C depletion (5–10 ‰ relative to bulk biomass) due to secondary fractionation associated with the enzymatic (pyruvate dehydrogenase) decarboxylation of pyruvic acid (De Niro & Epstein, 1977; Melzer & Schmidt, 1987). A similar explanation involving pyruvate dehydrogenase and Acetyl-Co-A accounts for the high carbon isotope discrimination observed in β-carotene and isoprene shown in Figure 1.5 (Sharkey et al., 1991).

1.3.3.5 Challenges for Compound Specific Isotope Analysis

CSIA can potentially help understand the biological origins and transformations of NOM. However, several important operational constraints need to be satisfied. Any *derivatisation* methods used to assist the GC resolution of structural polarity must take into account the effect of the derivative on $\delta^{13}C$ values. Ideally the derivative introduces only a small number of carbon atoms (Rieley, 1994) which have a systematic effect on the measured $\delta^{13}C$ which can be corrected for (e.g., N-O-bis (trimethylsilyl)trifluoroacetamide (BSTFA); Jones et al., 1991)

New offline thermal degradation procedures such as catalytic HyPy (Love et al., 2005) can also reduce the functionality of some NOM structures making them more amenable to GC-irMS (see Chapter 4). Offline pyrolysis can also be combined with additional preparative procedures – e.g., column chromatography; molecular size/sieve separations; thin layer chromatography (TLC); liquid chromatography (LC); gel permeation chromatography - to produce fractions of reduced complexity. This is an important advantage given the high GC resolutions needed for accurate $\delta^{13}C$ measurement by GC-irMS. Relatively large sample quantities would need to be pyrolysed however, as GC-irMS is less sensitive than GC-MS. Depending on the mode of injection onto the GC and the nature of the component(s) of interest, typical analyte concentration requirements for $\delta^{13}C$ measurement range from 50–300 ng μL^{-1} (Schmidt et al., 2004) although this continues to decrease as instrument sensitivity increases over time (Grice & Brocks, 2011).

1.3.3.6 Summary: - Stable Isotope Analysis of NOM

In brief, stable isotope analysis of NOM, particularly at the compound specific level can help:

- Distinguish the contributions of different organic precursors (Klein Breteler et al., 2002a&b).
- Monitor microbial activity (Kuypers et al., 2001; Hinrichs et al., 2003) and the dynamics of other major precursors of NOM.
- Understand the diagenetic processing (e.g., biodegradation, humification) of aquatic NOM (Lu et al., 2003).

CSIA can help resolve major NOM sources including C4, C3 or CAM plants or algogenic material. CSIA will also help study biological processes, including the biomass which can foul the membranes of water treatment plants. The metabolism of carbon during biochemical syntheses is reflected by characteristic stable carbon isotopic fractionations (e.g., microbial carbohydrate products are typically ^{13}C depleted; Macko et al., 1990).

However, several analytical challenges confront the reliable application of CSIA to NOM, and the present study addresses some of these. The overarching aim of this research project was to separate NOM into fractions appropriate for the measurement of biomarker $\delta^{13}C$ profiles by GC-irMS. A range of potentially useful preparative approaches were explored, including chemical and thermal extraction methods which aim to isolate,

concentrate or derivatise specific structural moieties of NOM. The effect of these steps on the isotopic integrity of the fractions obtained and individual species within was also investigated.

1.4 Aims and Objectives of Present Research

The aim of this research project is to investigate the development and optimisation of several stable isotope analysis methods for analysing the compositional and structural character of NOM, with a particular focus on isotopic signatures at the compound specific level – i.e., biomarkers of NOM from simulated (i.e., plant leachates) and real (i.e., Harding, Boganup) source waters, water treatment (membrane biofoulants) and distribution systems.

The objectives of the research are outlined below:

- Develop sample treatment methods – SPME targeting VOC/SV (Chapter 2), extraction and derivatisation of methyl ester PLFAs (Chapter 3); and aliphatic and aromatic solvent fractions of pyrolysates (Chapter 4) to make aquatic NOM amenable to GC-irMS analysis and test the utility of these new CSIA methods for characterising NOM.

- Apply the CSIA techniques developed to NOM materials representative of humic, algogenic and biofilm NOM. The selected sample suite also allows an investigation of seasonal and spatial dynamics of stable isotopic values of organic biomarkers.

- Practically assess how the application of these CSIA can assist structural and compositional data from established molecular analyses (e.g., HPSEC, Py-GCMS, thermochemolysis-GCMS, and ^{13}C-NMR spectroscopy) and bulk δ^{13}C data to determine the main precursors of DOC from simulated (plant leachates) and real (Bolganup, Harding) source waters (Chapter 5), as well as helping to monitor the aquatic fate of organic constituents (e.g., diagenetic and biodegradative processes).

Chapter 2

Characterisation of Volatile and Semi-Volatile Components of DOC

2.1 Overview

Naturally occurring Volatile Organic Compounds (VOCs) and Semi-Volatile compounds (SVs) from DOC derived from plant leachates were investigated for the first time using a combination of SPME (for sample introduction onto the GC) followed by CSIA. The SPME-CSIA method was initially optimised for extraction time; desorption time; and concentration using water soluble standard compounds (i.e., thymol, eucalyptol, myristic acid, methyl palmitate and methyl stearate) of known $\delta^{13}C$ values as well as the organic-rich leachate of Marri leaves. These five standard compounds are representative of major compound classes present in DOC. Potential 'isotopic fractionation' using SPME as a method for sample introduction was examined by comparing $\delta^{13}C$ values of the standards measured by EA-irMS and conventional liquid CSIA with those determined by SPME-CSIA.

The leachates of four contrasting vegetation types, covering two different pathways of CO_2 fixation, were also used in the development and assessment of the SPME-CSIA method. These comprised Marri (a C3 plant), Maize, Sorghum and Spinifex (all C4 plants). These are significant Western Australian plants, occurring in close proximity to some natural water source(s) or are commercially important crops.

2.2 Background

2.2.1 Volatile and Semi-Volatile Components of DOC

Volatile, low MW components of DOC (e.g., monoterpenes) are referred to as VOCs. Slightly less volatile products such as longer chain fatty acids and their esters are referred to as SVs. Most of these components can be isolated in the neutral HPI fraction of DOC (Thurman, 1985; Zander et al., 2007).

Isolation of the HPI fractions (described in Leenheer, 1981; Aiken et al., 1992; Croué et al., 1998; Findlay & Sinsabaugh, 2003) typically involves laborious sequential extractions and

the yields can be quite low (<20 %, South Platte NOM; Croué et al., 1998). As a consequence, relatively little is known about the compositions of VOCs and SVs in natural waters (Thurman, 1985).

Many VOCs can also occur in complex polymeric forms derived via diagenetic processes. Thus, the molecular and isotopic compositions of VOCs may yield useful information about the diagenetic behaviour of aquatic organics as well as provide insight into active organic precursors.

Many different and complimentary analytical techniques have been used to examine the VOC/SV composition of source waters (see Sections 2.2.3 and 2.2.4). This chapter investigates the potential use of SPME-CSIA as an analytical tool to provide additional stable isotopic information about the source(s) and characteristics of VOCs and SVs in natural waters.

2.2.2 Significance of VOCs and SVs in Drinking Water

The occurrence of VOCs and SVs in drinking water sources is a source of public health concern (Irace-Guigand & Aaron, 2004). Some VOCs (e.g., benzene, MTBE) are known or suspect human carcinogens (USEPA, 1996; NTP 2011). Their environmental occurrence may arise from natural or anthropogenic sources and analyses to confirm their source in aquatic systems is a subject of active research. Tightening legislation and a growing awareness of emerging pollutants combined with the possible need to recycle wastewater as an alternative water source has added to the impetus for more effective VOC analyses. The detection and monitoring of environmental contaminants has been a driving force for establishing many new and novel analytical techniques, including previous application of SPME coupled with CSIA (Dias & Freeman, 1997; Palau et al., 2007; Shouakar-Stash et al., 2009).

As a consequence of their high volatility and the trace concentrations (in the ng L^{-1} and µg L^{-1} range) at which SV and VOCs typically occur in surface waters (Demeestere et al., 2007), they are difficult to detect by traditional analytical methods. For accurate CSIA relatively high concentrations of analytes are required (typically 50–300 ng µL^{-1}) necessitating the use of pre-concentration steps (Schmidt et al., 2004). All sample preparation steps relating to concentration, extraction and separation are potentially prone to 'isotopic fractionation' (Meier-Augenstein et al., 1996; Dias & Freeman, 1997; Slater et

al., 1998; Schmitt et al., 2003; Zwank et al., 2003). As such, the development of methods to measure the stable isotopic values of VOCs and SVs of DOC also requires a detailed evaluation of any associated 'isotopic fractionation'.

2.2.3 Analysis of VOCs and SVs in Aquatic DOC

Analytical methods have been developed to access the volatile fraction of the DOC without going through laborious concentration, purification and fractionation steps (Thurman, 1985). This typically involves membrane filtration, selective adsorption/desorption of different DOC fractions using pH adjustment and various eluents with non-ionic exchange resins (XAD-8 and XAD-4) followed by cation exchange (MSC-1H resin) and lyophilisation, (detailed in Leenheer, 1981; Croué et al, 1998). In a more recent study Parnis and Brooks (2001) used liquid-liquid extraction (LLE) to compare SV profiles of water samples from catchments of the Campaspe River, Victoria. They identified 25 compounds, with concentrations ranging from 1 to 39 mg L^{-1}, covering several common chemical classes including fatty acids, esters, amides and hydrocarbons. A distinct seasonal effect associated with floodwaters was observed, with SV concentrations increasing with runoff, and a strong influence of catchment land type, with the greatest SV concentrations being found in native forest catchments. SPME-GCMS holds great potential for monitoring DOC. Zander et al. (2007) showed that SPME-GCMS was useful for detecting many VOC and SV compounds (in some cases >100) in natural waters from the Riverina of southern New South Wales, and identified that their distributions showed a relationship with geographical location and season(s).

2.2.4 Molecular and Stable Isotope Analysis of VOCs and SVs in Drinking Water

LLE is the most commonly used method of VOC detection in drinking water sources (Kostopoulou et al., 2000; Nikolaou et al., 2002; Golfinopoulos et al., 2003). The main drawbacks of this method include a requirement for large volumes of solvent and the frequent formation of emulsions at the solvent interface (particularly with carboxylic acids and other polar compounds, Lacorte et al., 2003). As such the popularity of this method has waned with the emergence of alternative solvent-free methods offering greater analyte extraction efficiency and rapid preparation times (Rezaee et al., 2006).

Solid Phase Extraction (SPE), requires only small volumes of solvent applied to a suitably packed cartridge and minimises the formation of emulsions (Ridal et al., 1997). It has been used with GCMS analyses in several studies to investigate VOCs and SVs in water samples (Kostopoulou et al., 2000; Mottaleb et al., 2004) but few applications of SPE with CSIA have been reported. SPE was used to help measure the δ^{13}C of trinitrotoluene in groundwater (Coffin et al., 2001) but this study did not address the potential 'isotopic fractionation' caused by the SPE cartridge. Benbow et al. (2008) developed a SPE-CSIA method which required 'isotopic fractionation' corrections ranging between 1–1.8 ‰, depending on structure. The 'isotopic fractionation' observed was attributed to variable interactions of the analytes with the SPE cartridge and/or incomplete elution from the SPE cartridge (Benbow et al., 2008).

Headspace (HS) Analysis targets the partitioning of VOCs between the headspace and sample solution, with a subsample removed from the former and injected directly into a GC. This technique has been applied extensively to the study of VOC contaminants in water samples (Hino et al., 1998; Kostopoulou et al., 2000; Golfinopoulos & Nikolaou, 2001; Nikolaou et al., 2002; Golfinopoulos et al., 2003; Safarova et al., 2004).

HS injection has been combined with CSIA to study VOC contaminants in groundwater and has been shown to be successful for some compounds, specifically LMW chlorinated hydrocarbons and toluene (Morrill et al., 2004). Smallwood et al. (2001) used HS-CSIA to show the δ^{13}C of methyl-tertiary-butyl ether (MTBE) was 4.6–2.7 ‰ more ^{13}C enriched relative to the bulk δ^{13}C. This isotopic differential was ascribed to preferential vaporisation of the heavier isotope in MTBE. The sensitivity (200 µg L^{-1}) and precision over a range of MTBE concentrations was found to be relatively low (average standard deviation of 1.6) and the technique was deemed not suitable for the analysis of groundwater samples.

Purge and Trap (P & T) combines extraction, concentration and sample introduction techniques. A solid trap is used to remove the VOCs from the sample matrix via an inert gas flow (Lara-Gonzalo et al., 2008). It has been widely used to analyse VOCs of surface (Miermans et al., 2000; Golfinopoulos et al., 2001; Nikolaou et al., 2002; Lara-Gonzalo et al., 2008) and ground (Arthur et al., 1992b; Wu & Fung, 2002; Moran et al., 2004) waters.

The high analyte concentrations collected by P & T can meet the detection limits of CSIA (Bergamaschi et al., 1999; Smallwood et al., 2001; Zwank et al., 2003; Jochmann et al.,

2006). The $\delta^{13}C$ analysis of MTBE using a P & T sample concentrator interfaced to a GC-irMS was reported to be precise and reproducible to concentrations of 15 µg L^{-1} (Smallwood et al., 2001). The $\delta^{13}C$ of MTBE measured by P & T was 0.7 ‰ more ^{13}C enriched than the bulk $\delta^{13}C$ value. This isotopic differential was much lower than that of the HS analysis as the problematical transfer from aqueous to vapour phase was minimised in P & T (Smallwood et al., 2001). The slightly heavier $\delta^{13}C$ values of the MTBEs were attributed to incomplete transfer from the aqueous phase to the GC (Smallwood et al., 2001). Similarly, Zwank et al. (2003) investigated 'isotopic fractionation' from P & T-CSIA on organic contaminants at concentrations of 5–60 µg L^{-1} in environmental samples finding minimal fractionation of LMW, non-polar VOCs (e.g., Benzene, Toluene, Ethylene and Xylenes; BTEX, chloroethanes and trimethyl benzenes).

Solid Phase Micro Extraction (SPME) is another pre-concentration technique which has been used successfully for VOC and SV analysis for a range of organic samples (Llompart et al., 1998; Zander et al., 2005; Zander et al., 2007; Bowerbank et al., 2009). Examples of SPME-GCMS application include:

- Food and beverage products: provenancing, detection of contaminants, quality control of aroma and flavour (Steffen & Pawliszyn, 1996; Kataoka et al., 2000; Aguilar-Cisneros et al., 2002; Pinho et al., 2002; Giordano et al., 2003; Carrillo & Tena, 2006; Altaki et al., 2007).
- Environmental analysis: air (Koziel & Pawliszyn, 2001), wastewater (Abalos et al., 2000; Francioso et al., 2010), water (Porschmann et al., 1998; Kopinke et al., 1999; Minicola et al., 2001; Ouyang & Pawliszyn, 2006b; Qin et al., 2009), soil (Moder et al., 1999) and sediments (Millan & Pawliszyn, 2000).
- Natural products: e.g., essential oils (Cornu et al., 2001; Zini et al., 2001; Zini et al., 2002; Zini et al., 2003; Liu et al., 2004; Cai et al., 2007; Stashenko & Martínez, 2007).
- Forensic analysis of biological materials: urine, blood and milk e.g., biomonitoring of anthropogenic exposure (DeBruin et al., 1998) and *in vivo* sampling of model drugs in plasma (Vuckovic et al., 2009).

Organic compounds in aqueous samples have been analysed successfully by several different applications of SPME, including direct immersion (D-SPME) and headspace analysis (HS-SPME) which are the two most common methods of sample introduction.

Derivatisation techniques combined with SPME are becoming more popular and have been applied to the analysis of phenols (Buchholz & Pawliszyn, 1993; Buchholz & Pawliszyn, 1994), aromatic amines (Müller et al., 1997; DeBruin et al., 1998), aldehydes (Schmarr et al., 2008) and organo-metallic compounds in water (Millan & Pawliszyn, 2000). On-site analyses are also emerging as technology improves (Schmarr et al., 2008; Risticevic et al., 2009; Sanchez-Prado et al., 2009; Vuckovic et al., 2009).

In drinking water research, SPME-GCMS has been routinely used to analyse DBPs (Valente et al., 1998; San Juan et al., 2007; Niri et al., 2008) and has been particularly important for the measurement of emerging DBPs such as nitrosamines (Pan & Pawliszyn, 1997; Grebel et al., 2006; Mhlongo et al., 2009). It has also been used for monitoring taste and odour compounds (Lloyd et al., 1998; McCallum et al., 1998; Bao et al., 1999; Watson et al., 2000; Lin et al., 2002; Sung et al., 2005; Bagheri et al., 2007). SPME-GCMS investigations of natural source water inputs have been limited—two recent studies of the River Red Gum dominant DOC of the Murray-Darling river system (Zander et al., 2005, 2007) however, showed it was capable of identifying detailed molecular DOC signatures of riverine vegetation.

SPME has been successfully combined with CSIA. Studies include the detection of:

- Toluene, methylcyclohexane, hexanol, acetic, propionic, and valeric acids in aqueous media (Dias & Freeman, 1997).
- Diacetyl and acetoin (flavour compounds produced by lactic acid bacteria) in aqueous solutions (Goupry et al., 2000).
- Organic contaminants in groundwater (Hunkeler & Aravena, 2000).
- Vinyl chloride in contaminated aquifers (Shouakar-Stash et al., 2009).
- Carboxylic acids in human plasma (Ferchaud-Roucher et al., 2006).
- Gasoline range hydrocarbons in oils (Harris et al., 1999).

The SPME technique has shown little evidence of any ‘isotopic fractionation’ (described in Section 2.2.5.3). Thus, it holds promise for measuring the stable isotopic composition of aquatic VOCs and SVs.

2.2.5 SPME-GCMS and CSIA of aquatic VOCs

2.2.5.1 Analytical Background

Originally developed to address the need for rapid sample preparation (Arthur & Pawliszyn, 1990), SPME is an efficient method for analysing volatile organic substances in complex matrices at trace (i.e., ng L^{-1}) concentrations (Boyd-Boland & Pawliszyn, 1996). Combining sample preparation, extraction, concentration and sample introduction, SPME is now a common analytical method. It has also proved capable of on-site analysis (Ouyang & Pawliszyn, 2006a). SPME technique involves the exposure of a fibre coated with an extracting phase for an optimised equilibration time, during which, compounds partition between the sample matrix and fibre coating (Lord & Pawliszyn, 2000). The analytes which have adsorbed to the extraction phase fibre can then be thermally desorbed for analysis such as in the vaporisation injector of a GC.

2.2.5.2 SPME-GCMS Optimisation

SPME parameters need to be optimised for each new sample. Several important parameters are considered below:

Sample mode selection

D-SPME is appropriate for analytes of medium to low volatility whereas HS-SPME is more suited to dissolved analytes of high to medium volatility. HS-SPME protects the fibre and allows experimental parameters such as pH to be varied without damaging the fibre (Pawliszyn, 1997). Equilibration times are shorter for HS-SPME of VOCs. The technique, however, has proved ineffective for extracting polar analytes from aqueous samples (Pawliszyn, 1997).

Selection of fibre coating

Different fibre coatings have been specifically developed for specific materials including environmental samples. The types of samples targeted by several commercially available fibres are listed in Table 2.1.

The main analytical parameters are fibre coating polarity and adsorption or absorption modes of analyte retention (Pawliszyn, 1997). Fibres with liquid phase coatings (polyacrylate, PA, and polydimethylsiloxane, PDMS) function primarily by absorption.

Adsorbent fibres trap analytes in a porous matrix where they remain until thermally desorbed or chemically displaced. Analytes are retained on the basis of size as they migrate in and out of the pores of the fibre (Shirey, 1999). The rate of pore migration determines the degree of extraction and can be influenced by analyte size, the affinity of analytes towards the polarity of the fibre coating and thickness (Shirey, 1999). Composite fibres (e.g., DVB/PDMS and DVB/CAR/PDMS) combine the functionality of both absorbent and adsorbent fibres and may have the additional advantage of divergent polarities. Such fibres have been reported to be particularly effective at extracting LMW compounds (Shirey, 1999).

Table 2.1 Commercially available fibres and their recommended uses (Mills & Walker, 2000; Mullett & Pawliszyn, 2003).

Primary mechanism	Fibre coating	Polarity	Recommended uses
Absorption	Polydimethylsiloxane (PDMS)	Non-polar	Volatiles
	Polyacrylate (PA)	Polar	Polar semi-volatiles
Adsorption	PDMS/divinylbenzene (DVB)	Bi-polar	Polar volatiles
	Carboxen (CAR)-PDMS	Bi-polar	Gases and volatiles
	DVB-PDMS-CAR	Bi-polar	Odours and flavours

2.2.5.3 SPME-CSIA Considerations and Optimisation

'Isotopic fractionation' may occur with SPME by equilibrium partitioning or preferential thermal desorption of analytes on the GC during SPME desorption. Composite fibre coatings have not been widely used for CSIA applications and little has been done on aqueous samples. In one of the few CSIA studies with composite fibre coatings, Goupry et al. (2000) used PDMS/DVB and CAR/PDMS fibres to reliably determine $\delta^{13}C$ values of the flavour compounds acetoin and diacetyl in an aqueous fermentation media, reporting a consistently small fractionation of 0.4 ‰ for both fibres. Palau et al. (2007), after a systematic study of the 'isotopic fractionation' effects associated with the CAR/PDMS fibre analysis of multiple VOCs in aqueous matrices via D-SPME and HS-SPME CSIA, showed minimal carbon isotopic fractionation (<1 ‰) of chlorinated ethenes. Likewise, Gray et al. (2002) reported negligible fractionation (<1 ‰) of MTBE from water using SPME-CSIA (CAR/PDMS). Dias and Freeman (1997) reported similarly negligible 'isotopic fractionation' of hydrophobic compounds extracted by a non-polar PDMS fibre, although some organic acids reflected a slight depletion in ^{13}C (<1.5 ‰) due to mass dependent kinetic shifts upon

dissolution of each analyte into the organic phase.

2.2.5.4 Research Focus

SPME has been used for successful DOC analysis of VOCs/SVs including phenols, fatty acids, fatty acid methyl esters and terpineoles (Volk et al., 1997; Parrish, 1999; Zander et al., 2005). Zander et al. (2007) provided the first dedicated molecular investigation of DOC by SPME to identify the main organic precursors of source waters. DOC has not previously been analysed by SPME-CSIA and the present research aims to address whether this is a viable analytical opportunity. Several analytical aspects of the Zander et al. (2005, 2007) and Dias and Freeman (1997) SPME based molecular studies formed the basis for the present SPME-CSIA study of the stable isotopic characteristics of SVs/VOCs of source waters DOCs. Such isotopic data may provide new insight(s) into not only the main source inputs of DOC (see Chapter 5), but also water system transportation and the pathways and magnitude of natural alteration processes (e.g., biodegradation).

2.3 Experimental

2.3.1 Chemicals and Glassware

Details of reagents used in the study are listed in Table 2.2. All glassware was heated at 500°C for at least 6 h prior to use.

Table 2.2 Chemicals and standards used in SPME-CSIA development.

Chemical	Grade	Supplier
Solvents		
acetone	ChromAR HPLC	Mallinckrodt
n-hexane	UltimAR	Mallinckrodt
methanol	ChromAR HPLC	Mallinckrodt
Standards		
1,8-cineole (eucalyptol)	Puriss terpene standard for GC	Fluka
thymol	≥ 99.5 % purity	Sigma
myristic acid	≥ 99 % purity	Sigma
methyl palmitate	≥ 99% purity	Sigma
methyl stearate	≥ 99% purity	Sigma
General		
sodium chloride	AR	BDH
hydrochloric acid	37 % w/v	Fluka
sodium azide	SigmaUltra	Sigma-Aldrich
phosphoric acid	85 % w/v	BDH
persulfate oxidser solution	98 % w/v	Sigma-Aldrich
potassium hydrogen phthalate	98 % w/v	Aldrich

2.3.2 Sample Collection and Preparation

2.3.2.1 Sample Collection

Marri

Green leaves of *Corymbia calophylla* trees were collected, specifically for this study, from two locations on the Darkin River, an inflow of the Mundaring Reservoir Catchment (Western Australia) in mid-September 2008. Woody stems were removed using a scalpel blade.

Spinifex

Blades of *Triodia basedowii*, commonly referred to as Spinifex, were sampled from plants in the Pilbara region (Western Australia) in mid-July 2008.

Sorghum and Maize

Sorghum (*Sorghum bicolour*) and Maize (*Zea mais*) samples were germinated from seed at the Research School of Biological Sciences (ANU). Growth conditions were described by Grice et al. (2008). Plant material was provided separately as a finely ground powder.

2.3.2.2 Sample Preparation

The Marri leaves and Spinifex blades were air dried for seven days. The dried vegetation was ground to a fine powder using an IKA Labortechnik A10 mill (IKA laboratory Equipment) and stored in annealed glass vessels in the dark at room temperature. All ground vegetation samples were coned and quartered to ensure a homogenous sample of representative source material with no systematic bias (IUPAC, 1997).

2.3.3 Preparation of Standard Solutions

Five standard compounds (Table 2.2) were selected to represent common biochemical precursors of DOC such as terpenoids, phenolic compounds (Zander et al., 2005) and fatty acids (Parrish, 1999). Eucalyptol is representative of terpenoids; thymol of phenolics, myristic acid of a free fatty acid, and methyl palmitate and methyl stearate, of fatty acid methyl esters. Stock solutions of each standard were prepared at concentrations of 1 mg mL^{-1} in methanol or 9:1 (v/v) methanol: acetone (depending on compound solubility). These standards were stored frozen (-20°C). A standard mixture of the five compounds was prepared at a concentration of 0.010 mg mL^{-1}. The five component standard mixture

(150 µL, 100 ng µL^{-1}) was added to 15 mL of extraction matrix immediately before GCMS analysis, functioning as an internal standard. The recovery and GC resolution of the standard components, both individually and as a standard mixture, were used to initially assess the suitability of the fibres for SPME-GCMS analyses. For CSIA, a more concentrated solution of standard mixture (150 µL, 500 ng µL^{-1}) was used.

2.3.4 Preparation of Leachate Solutions

2.3.4.1 Initial Leachate Solutions (100 min)

Duplicates of the Marri leachate solutions (1 L, 1000 mg L^{-1}) were prepared in Milli-Q water. The mitochondrial inhibitor, sodium azide (NaN_3, 0.25 mM), was added to one aliquot to inhibit microbial activity. A control comprising a blank solution containing just Milli-Q water was also prepared. Immediately following immersion of the vegetation, subsamples (3 x 20 mL) were taken for DOC analysis (nominal zero time), these samples were diluted) with Millli-Q water (1:200 for Marri, Maize and Sorghum 150 Day leachates and 1:100 for all other leachates prior to DOC analysis. After leaching (100 min), 48 aliquots (15 mL) were sub-sampled and transferred into 20 mL capacity screw cap SPME vials. Half were used for SPME-GCMS method development and half for CSIA method development.

Samples for CSIA were stored frozen for up to 90 days. To check whether there was any 'isotopic fractionation' associated with the freezing event, three additional vials of the Marri and an inhibitor were chilled (not frozen) and stored until analysed. As summarised in Table 2.3, different treatments were prepared for the leachate solutions, the working standard and the procedural blank, to separately investigate potential contamination, matrix effects or extraction efficiency. Automated SPME-GCMS were conducted in triplicate.

Table 2.3 Treatment matrix of investigated controls during preliminary method development.

Treatment	Marri & NaN_3	Marri	Standard mix	Blank
Blank	3	3	3	3
Salt	3	3	3	3
pH	3	3	3	3
Salt & pH	3	3	3	3
Thymol	3	3	3	3
Thymol & salt	3	3	3	3
Thymol & pH 2	3	3	3	3
Thymol & salt & pH 2	3	3	3	3

NaN_3 = Sodium azide (microbial inhibitor)

For the salt treatment, NaCl 4.5 g (i.e., saturation) was used—recommended by Zander et al. (2005). For acid treatment, the vial contents were adjusted to pH 2 using 0.1 M HCl. Triplicate sub-samples (20 mL) were removed for DOC analysis (nominal time 100 min). For thymol treatment, 150 µL of thymol standard (100 ng μL^{-1}) was added immediately prior to analysis to eliminate the potential for interaction with leachate analytes during storage.

2.3.4.2 Main Leachate Solutions (150 days)

Two batches of leachate solutions were prepared at concentrations of 10 mg mL^{-1} for each of the four homogenised source materials. Tests were conducted in 2 L capacity glass jars with screw caps. A 2.5 mM aqueous solution (2 L) of sodium azide was used for the microbially-inhibited leachates. Milli-Q water (2 L) was used for leachates without microbial inhibition (used in Chapter 3).

The Milli-Q water used had been filtered through a 0.2 µm LC134 point-of-use filter (ELGA LabWater) to remove residual biologically active contaminants. Samples were loosely capped and kept out of direct sunlight for 150 days. In order to maintain the aerobic environment, caps were removed and samples swirled most days, no more than three days apart.

After 150 days, leachate samples were filtered through a binder-free 90 mm diameter GF/F filter (nominal pore size 0.7 mm) which had been pre-combusted at 450°C for 4 h. Filters were kept frozen (-20°C) until analysed. Main DOC samples were taken at 0, 24, 48, 72, 150 and 3600 (i.e., the end) hour treatment times. Leachate was sampled by withdrawing duplicate 20 mL aliquots with a pipette and passing through a pre-washed nylon membrane filter (Pall Acrodisc, 0.45 µm).

2.3.5 Analysis of Leachate Solutions

2.3.5.1 DOC Measurement

Pre-washed nylon membranes (Pall Acrodisc, 0.45 µm) were used to filter the leachates prior to analysis. Diluted leachates (1:200 for Marri, Maize and Sorghum 150 day leachates and 1:100 for all other leachates) were prepared by combining 1mL of filtered extract with Milli-Q water in a total volume of 200 mL or 100 mL as appropriate to the solution. The diluted leachate (40 mL) was analysed with a Shimadzu Total Organic Carbon Analyser (TOC-Vws) using a Non-Purgable Orga nic Carbon (NPOC) method. The NPOC parameters

included 3–5 injections of 2.5 mL of sample (with a maximum standard deviation of 0.05 mg L^{-1}). Samples were run in duplicate, and Milli-Q water and standard controls were frequently run to cross check instrument calibration. Standard solutions were prepared from a stock solution made by dissolving dried potassium hydrogen phthalate (2.125 g) in Milli-Q water (1 L), giving a nominal concentration of 100 mg organic carbon L^{-1}. This stock solution was serially diluted with Milli-Q water to produce solutions of 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 mg organic carbon L^{-1} for construction of calibration curves.

2.3.5.2 Bulk Isotope Analysis

Bulk stable carbon isotope analyses of the five standards (eucalyptol, thymol, myristic acid, methyl palmitate and methyl stearate) and plant biomass were conducted with a Micromass IsoPrime isotope ratio MS (GV instruments) interfaced to a EuroEA3000 elemental analyser.

Average and standard deviation values were obtained from at least three analyses. Ground vegetation (or standard) samples (0.02–0.05 mg) were weighed into tin capsules, folded, compressed and placed in an autosampler. Samples were dropped into a combustion reactor (1025°C) and subjected to flash combustion. Combustion products were swept by a flow of Ultra High Purity (UHP) He to a chromium oxide oxidation catalyst. Oxidised products were subsequently purified by reduction in a reactor containing copper granules (650°C) then passed over a magnesium perchlorate filter to remove water. The remaining CO_2, together with N_2 and SO_2 (if present) were then chromatographically separated (3 m column, Poropak Q), before passage through a thermal conductivity detector to the irMS.

The isotopic compositions were calculated by integration of the ion currents obtained from measuring *m/z* 44, 45 and 46 CO_2 peaks. The $\delta^{13}C$ composition was reported relative to a pulse of CO_2 reference gas of known $\delta^{13}C$ content. Isotopic compositions were recorded in the delta notation (δ) in per mil (‰) relative to the V-PDB.

2.3.5.3 SPME-GCMS Analysis

All SPME fibres used were purchased from Supelco. A sample holder (part number 57330-U) was used for all manual injections. New fibres were conditioned following the manufacturer's recommendations.

Triplicate aliquots (15 mL, 0.01 mg mL^{-1}) of the standard mix were analysed with each fibre. Initially, samples were extracted for 20 min at 40°C and desorbed for 2 min at the maximum working range of the fibre coating (following the method of Zander et al., 2005). However, a 45 min extraction and 5 min desorption time was found to be better for CSIA (Section 2.4.1.3). Fibres were desorbed in the injector port at either 250°C or 280°C for PDMS and PA coatings, respectively (Nb., these were the maximum specified working temperature of the fibres). Automated extractions were also performed at 40°C which was maintained in an incubator (Gerstel).

GCMS analysis was performed on a HP5973 (Hewlett Packard) mass-selective detector (MSD) interfaced to a HP6890 GC fitted with a 60 m × 0.25 mm i.d. fused silica column coated with 0.25 µm 5 % phenyl-methylpolysiloxane (DB5, Agilent J&W). UHP He was used as carrier gas at a constant flow rate of 1 mL min^{-1}. A Gerstel MPS2 autosampler was used for initial SPME-GCMS injections. Samples were injected (split/splitless injector) using pulsed-splitless mode (207 kPa, 30 s). The GC injector temperature was set at 250 or 280°C for PDMS and PA fibres, respectively. The temperature of the MS transfer line was kept at 280°C. The MS analyses were performed using 70 eV electron impact in a full scan mode, with a mass range of 50–600 amu. The GC oven was programmed to increase from an initial temperature of 50°C at a rate of 20°C min^{-1} to 100°C where it was held for 15 min. The temperature was then increased again at 20°C min^{-1} to 135°C, thereafter the temperature increase was 1°C min^{-1} to 155°C (held isothermal for 5 min) then 6°C min^{-1} to a final temperature of 300°C (held isothermal for 10 min).

Minor products exclusive to one vegetation type were not used in the comparison of sample data. The major compounds were tentatively identified—sometimes only to the level of compound class. MS data were correlated with previously published data and MS libraries (NIST 05 and Wiley 275). Peak identifications were also based on correlation of GC elution position where possible.

2.3.5.4 SPME-CSIA

CSIA was performed using a Micromass IsoPrime isotope ratio MS (GV instruments) interfaced to a HP 6890 GC, operated at constant flow (1 mL min^{-1}) of He carrier gas and fitted with a 60 m × 0.25 mm i.d. fused silica column coated with 0.25 µm 5 % phenyl-methylpolysiloxane (DB5, Agilent J&W). The same GC temperature program described for SPME-GCMS (Section 2.3.5.3) was used.

Chromatographically separated compounds were combusted to CO_2 in the furnace (850°C) using copper oxide pellets (Elemental Microanalysis Ltd.). Cryogenic trapping via liquid nitrogen (BOC Gases, -100°C) removed the water formed during combustion. The $\delta^{13}C$ values were calculated upon integration of the *m/z* 44, 45, and 46 ion currents of the CO_2 from baseline resolved compounds, and are reported relative to pulses of a CO_2 reference gas of known $^{13}C/^{12}C$ content (BOC Gases) calibrated against the V-PDB. The $^{13}C/^{12}C$ content was monitored daily via the analysis of a mixture of reference compounds. Average $\delta^{13}C$ values and standard deviations were calculated from typically 2 or 3 analyses. Isotopic compositions are given in the standard delta notation (δ) in per mil (‰) relative to the V-PDB, as described in Chapter 1.

2.4 Results

2.4.1 Initial Optimisation of SPME Method for $\delta^{13}C$ Measurement

2.4.1.1 Fibre Coating Selection

The method used follows that reported by Zander et al. (2005 & 2007). These researchers investigated SVs and VOCs of River Red Gum leachate using a PDMS fibre coating and several other types of vegetation using a PA fibre coating (Zander et al., 2007). To determine the most suitable fibre for SPME-CSIA, both fibre coatings (detailed in Table 2.4) were tested.

Table 2.4 Fibre coatings tested in preliminary SPME analyses.

Fibre Composition	Part No.	Primary	Preconditioning treatment
PDMS 100 µm	57300-U	Absorbent	250°C for 30 min
PA 85 µm	57304	Absorbent	300°C for 2 hrs

New fibres were used and preconditioned according to the manufacturer's instructions. The analytical conditions used for fibre assessment are as described in detail in Section 2.3.5. Briefly, a five component standard mix (eucalyptol, thymol, myristic acid, methyl palmitate and methyl stearate) was analysed with 15–20 min adsorption and 2 min desorption times, which were the initial conditions for SPME GCMS utilised by Zander et al., (2005). The analyses were conducted in triplicate, and expressed as percent of the value obtained with that determined by direct liquid injection (shown in Fig. 2.1).

The PA fibre (*Cf*. PDMS fibre) showed marginally higher extraction recovery of eucalyptol and thymol, and slightly lower recovery of myristic acid and the two methyl esters. Both fibres showed relatively low recovery of myristic acid. Others have reported inefficient recovery of moderate carbon number fatty acids (Parrish 1998; Pfannkoch et al., 2003; Zander et al., 2007; Zander, 2008) and which has been attributed to low solubility (globule formation) and adhesion to the walls of the extraction vessel. Free fatty acids are poorly resolved and often exhibit peak tailing with non-polar columns, due to the structural polarity of the carboxylic group and its propensity for forming hydrogen bonds (Porschmann et al., 1987).

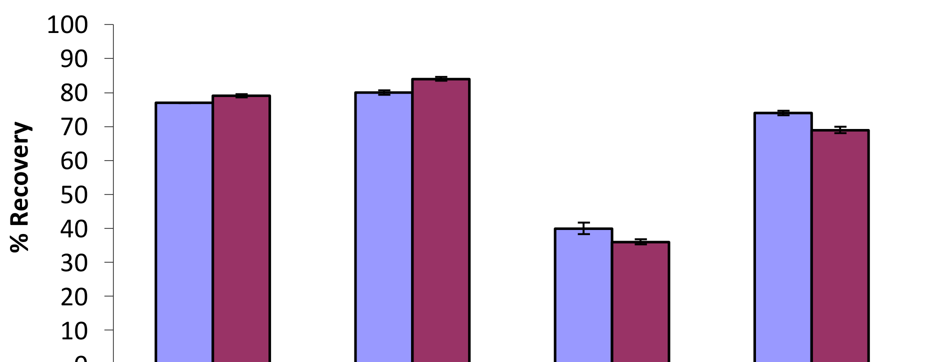


Figure 2.1 SPME-GC recovery of the five components of the standard mixture using the PDMS and PA fibre coatings. Data are the mean of triplicate analyses and error bars represent standard deviation.

The wide range of functional groupings, polarities and MW of compounds present in DOC necessitated a compromise in extraction conditions. Since non-bound fatty acids rarely occur in aquatic systems (Parrish, 1998; Zander et al., 2005; Zander, 2008), the low yields and variable response factors were accepted for the purposes of the present study. The basic method developed for SPME-GCMS by Zander et al. (2005 & 2007) which led to the

detection of 100s of leachate products (with River Red Gum) was therefore maintained. Both fibres were subsequently trialled on Marri leachates.

2.4.1.2 Preliminary Tests on Marri Leachate (100 min)

The PA and PDMS fibres provided quite different product profiles (Fig. 2.2) for the Marri leachate (1000 g L^{-1} leached for 100 min). A comprehensive list with MS information and source assignments in Table 2.5.

The range of products and functional groupings detected were comparable with those reported by Zander (2008). Of the five phenolic products detected the two most prominent (**84** and **88**) were likely derived from anthropogenic sources and the other three (**26, 32, 74**) attributable to lignin (Table 2.5). Of the latter, 4-hydroxy-1-propenyl-2-methoxyphenol (**74**), previously detected from *Eucalyptus* leaves (Yokoi et al., 1999; Zander, 2008), was the most abundant. Lignin can comprise up to 27 % of the total organic carbon content of the litter from some *Eucalyptus* leaves (Ribeiro et al., 2002).

Functionalised aromatics, terpenoids, furans and ketones were detected (although, terpenoid products were not nearly as prominent as in the Zander led studies (2005, 2007, 2008) of DOC from River Red Gum catchment. Many of the aromatics detected in the Marri leachate can be assigned a lignin source—e.g., methoxy aromatics (**70, 84**) and the ketone 1-phenyl-1-propanone (**11**; Nelson et al., 1987).

A diverse range of N-products including pyrazines, amino acids, and nucleoside bases were also detected (Table 2.5). Minor products included carbohydrate derived (Christy et al., 1999; Croué, 2004) furans (**12, 57, 67**), aldehydes (**18**) and alcohols (**86**). These volatiles are common to *Eucalyptus* leaves (Conde et al., 1995; Bauer et al., 2001).

Method Development

Consistent with previous research, the PA fibre coating extracted a greater proportion of phenols (Buchholz & Pawliszyn, 1993; Buchholz & Pawliszyn, 1994; Llompart et al., 1998; Penalver et al., 2002) whilst the PDMS fibre extracted terpenes, fatty acids and fatty acid esters more effectively (Pawliszyn, 1997; Cornu et al., 2001; Zabaras & Wyllie, 2001; Zander et al., 2005). Given their complementary performance, SPME-GCMS analysis of all 150 day leachates was subsequently conducted with both fibres (refer Section 2.4.2.2).

In early investigations (data not shown), an internal standard (thymol, 10 µg L^{-1}) was added to SPME vials prior to GCMS analysis to assist quantification. However, the recovery of this standard proved highly variable, consistent with the variable internal standard recovery in other studies (Kopinke et al., 1999; Zander et al., 2007). Kopinke et al. (1999) ascribed such variation to the unpredictable binding of fine particulate matter or chemical interaction/interferences from humic-like substances. Without a reliable standard, absolute analyte concentrations were not measured, limiting the analysis to a semi-quantitative level.

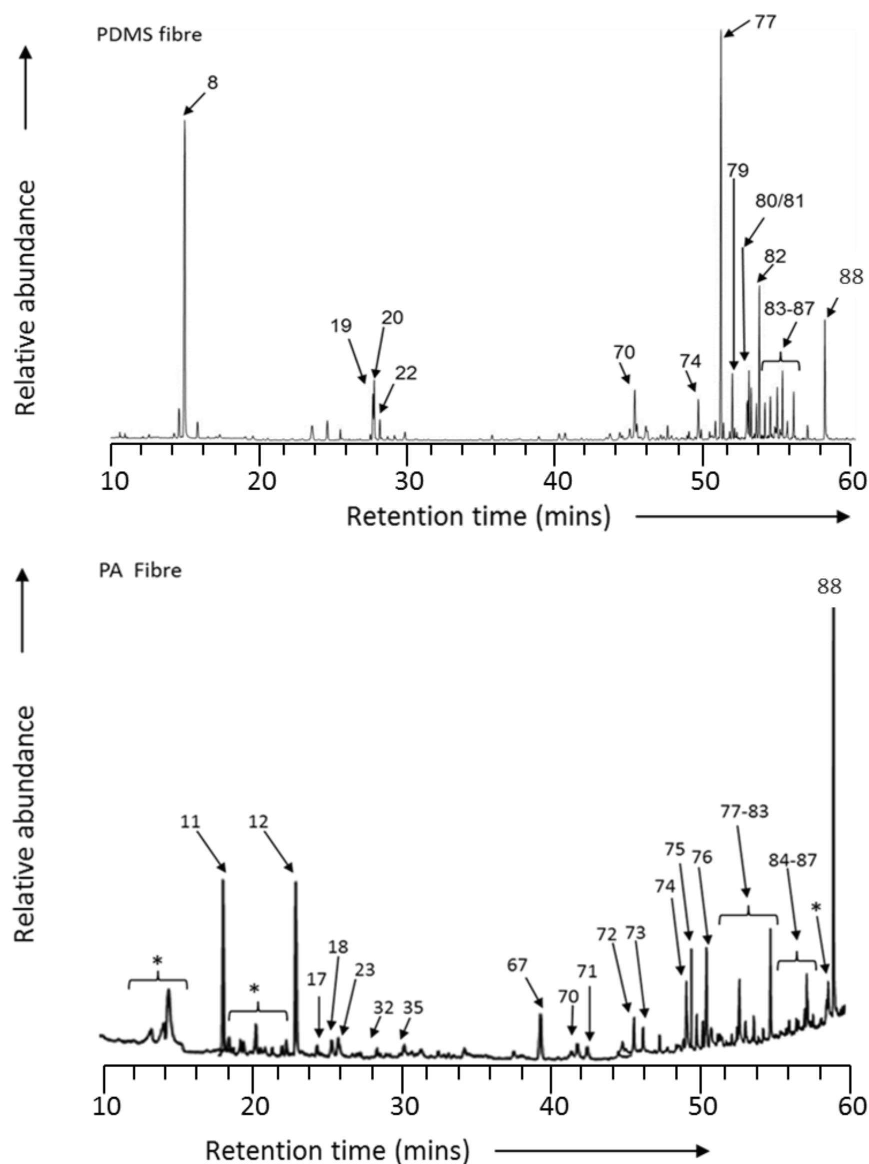


Figure 2.2 Partial TIC (RT window 10 to 60 min) from SPME-GCMS of the 100 min Marri leachate using PDMS fibre (top) and PA fibre (bottom). Peak assignments correspond to products listed in Table 2.5. * = Poorly resolved/ not identified.

Table 2.5 Consolidated list of all identified products (some tentative) from SPME-GCMS analysis of the Marri, Sorghum, Maize and Spinifex leachates (150 day), including peak number, retention time (RT), identification details, mass/charge ratio (*m/z*) of four main MS ions, molecular weight (MW), product class and likely precursors.

Peak No	RT (min)	Peak i.d.	*m/z*	MW	Product Class	Precursor	Vegetation
1	6.87	2,4-dimethyl-3-pentanone	43, 71, 41, 114	114	Ketone	Carb.	Marri
2	7.02	hexanal	44, 56, 72, 100	100	Aldehyde	Carb.	Maize
3	7.53	3-methyl-butanoic acid	60, 43, 41, 87	102	Acid	Carb.	Sorghum
4	8.11	3-hexen-1-ol	67, 55, 82, 100	100	Alcohol	Lipid	Marri
5	11.16	camphene	93, 121, 91, 136	136	Terpenoidal	Lipid	Marri
6	11.61	phenol	94, 66, 65, 96	94	Phenolic	Non-specific	Maize
7	14.37	*o*-cymene	119, 134, 117, 115	134	Terpenoidal	Lipid	Marri
8	14.95	eucalyptol	81, 108, 139, 154	154	Terpenoidal	Lipid	Marri, Spinifex
9	18.84	*p*-cymene	119, 134, 117, 115	134	Terpenoidal	Lipid	Marri, Spinifex, Sorghum
10	18.68	2-methoxyphenol	109, 124, 81, 77	124	Phenolic	Lignin	Marri
11	19.54	1-phenyl-1-propanone	105, 77, 134, 51	134	Ketone	Carb.	Marri
12	23.07	2,3-dihydro-benzofuran	120, 91, 39, 65	120	Furan	Carb.	Marri
13	23.30	benzoic acid	105, 122, 77, 51	122	Acid	Lipid	Marri, Maize, Spinifex
14	23.44	octanoic acid	60, 73, 101, 85	144	Acid	Lipid	Marri, Spinifex
15	23.53	4-ethylphenol	117, 122, 77, 119	122	Phenolics	Lignin	Marri
16	23.82	pinocarvone	108, 81, 150, 135	150	Terpenoidal	Lipid	Marri, Sorghum
17	23.96	2-phenyl-propanedioic acid	91, 92, 77, 136	180	Acid	Lipid	Sorghum
18	24.12	4-oxnononal	57, 71, 85, 99	156	Aldehyde	Carb.	Marri

19	24.21	borneol	110, 139, 121, 154	154	Terpenoidal	Lipid	Marri
20	24.31	*iso*-borneol	110, 139, 121, 154	154	Terpenoidal	Lipid	Marri
21	24.43	terpinen-4-ol	111, 93, 86, 154	154	Terpenoidal	Lipid	Marri
22	24.56	*p*-cymen-8-ol	135, 132, 117, 91	150	Terpenoidal	Lipid	Marri
23	24.74	nonanoic acid	60, 73, 115, 129	158	Acid	Lipid	Marri
24	24.89	α- terpineol	93, 121, 136, 81	154	Terpenoidal	Lipid	Marri
25	26.09	benzene-acetic acid	91, 136, 92, 65	136	Acid	Carb.	Sorghum
26	26.28	3-methyl-4-*iso*propyl phenol	134, 91, 77, 119	150	Phenolic	Lignin	Maize
27	26.36	4-phenyl-2-butanone	105, 148, 91, 77	148	Ketone	Carb.	Marri, Maize, Spinifex
28	27.25	*p*-ethyl guaiacol	137, 152, 122, 91	152	Phenolic	Lignin	Marri
29	27.32	thymol	135, 150, 115, 91	150	Terpenoidal	Lipid	Marri, Spinifex
30	27.65	vitispirane	149, 192, 107, 177	192	Terpenoidal	Lipid	Marri, Spinifex
31	27.86	2-methyl-5-(1-methylethyl)-phenol	135, 150, 107, 91	150	Terpenoidal	Lipid	Marri
32	28.30	2-methoxy-4-vinylphenol	135, 150, 107, 77	150	Phenolic	Lignin	Marri, Sorghum, Maize, Spinifex
33	28.34	2-methoxy-3-ethylpyrazine	138, 123, 107, 95	138	N-product	Unspecific N source	Marri, Spinifex
34	28.62	hydrocinnamic acid	91, 104, 150, 77	150	Acid	Lignin/Tannin	Marri , Spinifex
35	28.92	*n*-decanoic acid	60, 73, 129, 172	172	Acid	Lipid	Spinifex
36	29.38	4-hydroxy-benzaldehyde	121, 122, 93, 65	122	Aldehyde	Carb.	Sorghum, Spinifex
37	29.39	2-methoxy-4-(2-propenyl) - phenol	164, 103, 131, 149	164	Phenolic	Lignin	Spinifex
38	29.65	1,1,6-trimethyl-1,2-dihydro naphthalene	157, 142, 172, 115	172	Aromatic	Non-specific	Marri, Spinifex
39	29.99	5-hydroxy-5-methyl-3,4-benzo pyrrolizidine	190, 105, 147, 175	190	N-product	Unspecific N source	Marri, Maize, Spinifex
40	31.25	dodecanoic acid methyl ester	74, 87, 55, 214	214	Ester	Lipid	Spinifex
41	31.31	1,1-diethylpropyl-benzene	91, 105, 147, 176	176	Aromatic	Unspecific (poss. lignin)	Sorghum, Spinifex
42	31.35	4-hydroxy-3-methoxybenzonitrile	106, 134, 63, 149	149	N-product	Unspecific N source	Spinifex

43	31.47	1,6-dimethylnapthalene	156, 141, 153, 128	156	Aromatic	Lignin	Marri, Sorghum, Spinifex
44	31.85	unidentified N-Product 1	55, 67, 84, 126	126	N-product	Unspecific N source	Marri
45	31.93	2-aminoresorcinol	125, 67, 79, 108	125	N-product	Unspecific N source	Marri
46	32.03	hexadienoic acid ethyl ester (unspecified isomers)	95, 67, 97, 41	140	Ester	Lipid	Marri, Maize, Sorghum
47	32.09	megastigmatrienone (C_{13} nor-isoprenoid)	190, 148, 175, 133	190	Terpenoidal	Lipid	Marri
48	32.21	hexadienoic acid ethyl ester (unspecified isomers)	95, 67, 97, 41	140	Ester	Lipid	Marri, Maize, Sorghum
49	32.23	unidentified N-Product 2	32, 78, 106, 133	133	N-product	Unspecific N source	Marri
50	32.40	pentamethyl-2,3-dihydrobenzofuran	190, 131, 147, 175	190	Furan	Carb.	Spinifex
51	32.42	4-acetyl-1,1,3,6-tetramethyl-4,5,6,7,8,8-hexahydro-2-benzopyran	190, 175, 91, 43	190	Pyran	Anthro.	Marri
52	32.58	4-hydroxybenzoic acid	138, 94, 79, 66	138	Acid	Lignin/Carb.	Marri
53	32.89	2,5 *bis* 1,2-dimethylethylphenol	57, 206, 191, 32	206	Phenolic	Anthro.	Spinifex
54	32.94	1,2,3,4 tetrahydro-1,5-dimethyl-napthalene	132, 145, 160, 117	160	Aromatic	Non-specific	Marri, Spinifex
55	33.21	3-ethoxy-4-methoxy phenol	97,125, 140,168	168	Phenolic	Lignin	Marri, Maize, Spinifex
56	33.71	4-*iso*-propyl-2,5-dimethoxybenzylacetate	252, 237, 209, 81	252	Ester	Lignin/carb.	Marri
57	33.89	5,6,7,7 -tetrahydro-4,4,7 trimethyl-benzofuranone	180, 111, 137, 67	180	Furanone	Carb./Lipid	Sorghum, Spinifex
58	34.74	2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid, *iso*-butyl ester	71, 111, 159, 243	286	Ester	Carb./Lipid	Marri
59	35.09	viridiflorol	161, 175, 189, 204	204	Terpenoidal	Lipid	Marri
60	35.29	dodecanoic acid, 1-methylethyl ester	102, 200, 201, 183	201	Ester	Lipid	Marri

61	35.48	1,6-*bis* (1,1-dimethyl ethyl)-4-(1methylpropyl)phenol	262, 51, 233, 247	262	Phenolic	Anthro.	Marri
62	35.54	6-methoxy-1-phenyl-3,4-dihydronapthalene	236, 165, 166, 69	236	Aromatic	Lignin	Marri
63	35.95	cadinene	91, 62, 120, 204	204	Terpenoidal	Lipid	Marri
64	36.53	3,5,5,-trimethyl-4-(3-oxobutyl) 2-cyclohexen-1-one	109, 135, 151, 208	208	Ketone	Carb.	Marri
65	38.65	4-hydroxy-3,5,6-trimethyl-4-(3-oxo-1-butenyl)-2-cyclohexen-1-one	55, 124, 166, 222	222	Ketone	Carb.	Marri
66	39.00	*iso*-propyl myristate	102, 60, 228, 211	270	Ester	Lipid	Marri, Maize, Spinifex
67	39.18	tetrahydro--trimethyl-1-benzofuranone (unknown isomers)	180, 111, 137, 67	180	Furan	Carb.	Sorghum, Spinifex
68	40.33	benzoic acid - 2,4-dihydroxy-(3-diethylamino-1-methyl) propyl ester	32, 207, 255, 281	281	Ester	Carb./amino sugar	Marri
69	40.49	*n,n*-dimethyl-1-hexadecanamine	58, 59, 269, 43	270	N-product	Protein	Marri
70	41.05	4-methyl-2,5-dimethoxybenzaldehyde	180, 137, 77, 165	180	Aromatic	Lignin	Maize
71	41.55	4-amino-2,6-dihydroxypyrimidine	43, 55, 99, 127	127	N-product	Nucleoside	Spinifex
72	45.81	benzophenone	105, 77, 182, 152	182	Ketone	Carb.	Marri
73	47.12	methyl 7-(2-furyl) heptanoate	53, 81, 95, 210	210	Furan	Carb.	Marri
74	49.91	4-hydroxy-1-propenyl-2-methoxyphenol	103, 124, 137, 180	180	Phenolic	Lignin	Marri
75	50.57	tetradecanoic acid	73, 129, 185, 228	228	Acid	Lipid	Marri, Maize, Spinifex
76	51.20	6-methoxy-2-benzoxazoline	80, 106, 150, 165	165	N-product	Protein/nucleoside	Marri
77	51.50	indole-carboxaldehyde (unspecified isomers)	144, 145, 116, 89	145	N-product	Amino sugars	Marri
78	51.93	indole-3-carboxaldehyde (unspecified isomers)	144, 145, 116, 89	145	N-product	Amino sugars	Marri

79	52.84	8-phenyloctanoic acid	91, 92, 98, 220	220	Acid	Lipid	Marri, Maize
80	53.16	pentadecanoic acid	129, 199, 144, 242	242	Acid	Lipid	Marri
81	53.32	3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid	194, 179, 133, 105	194	Acid	Carb./Lignin	Marri
82	54.85	N-product A	121, 134, 180, 195	195	N-product	Protein/ nucleoside	Marri
83	55.31	*n*-hexadecanoic acid	73, 129, 256, 213	256	Acid	Lipid	Marri
84	56.98	2,4-isopropylidenedi-phenol	119, 134, 213, 228	228	Phenolic	Lignin	Marri
85	57.12	2,4-diethoxy-5-methoxy-1-(2-propenyl)benzene	236, 179, 165, 91	236	Aromatic	Lignin?	Marri
86	57.53	3,7,11-trimethyl-2,6,10-Dodecatrien-1-ol	69, 81, 92, 136	222	Isoprenoidal (sesquiterpene) alcohol)	Lipid	Marri
87	59.04	3-ethyl-3,5,5-trimethyl-cyclohexanone	105, 120, 133, 232	232	Ketone	Carb.	Marri
88	59.38	4,4-(1-methylethylindine)bis-phenol	228, 213, 99, 135	228	Phenolic	Anthro.	Marri

2.4.1.3 Further Technique Refinement for Stable Isotope Measurement

High concentrations of analytes are required for accurate CSIA (50–300 ng μL^{-1}) necessitating the use of pre-concentration steps (Schmidt et al., 2004). Extension of the method described in Section 2.3.5.3, was therefore required, with the introduction of longer adsorption (45 min) and desorption (5 min) times, as well as the use of multiple (4) SPME fibres to maximise analyte concentration. Concentration procedures, like other preparation steps (e.g., extraction, fraction separation) are potentially prone to 'isotopic fractionation' (Meier-Augenstein et al., 1996; Dias & Freeman, 1997; Slater et al., 1998; Schmitt et al., 2003; Zwank et al., 2003) which also required evaluation.

Single Injections

Initial SPME-CSIA analysis was conducted on a mixture of the five pure standards (eucalyptol, thymol, myristic acid, methyl palmitate and methyl stearate), using a 45 min absorption time (required to meet the detection limits required for CSIA). These values were compared to separate analysis of the five pure standards by both traditional solution injection CSIA and EAirMS (Table 2.6).

Table 2.6 $\delta^{13}C$ values of standard compounds measured by SPME analysis of a mixture of the standards; and separate liquid injection irMS and EA-irMS of the pure compounds. Data is the average of $^{(n)}$ replicates.

Standards	$\delta^{13}C$ ‰ EA-irMS	$\delta^{13}C$ ‰ liquid CSIA	$\delta^{13}C$ ‰ SPME	
			PDMS fibre	PA Fibre
eucalyptol	n/a	-26.9 ± 0.31$^{(4)}$	-26.8 ± 0.22$^{(2)}$	-26.6 ± 0.54$^{(2)}$
thymol	-26.1 ± 0.06$^{(2)}$	-26.8 ± 0.25$^{(4)}$	-26.1 ± 0.07$^{(2)}$	-27.0 ± 0.30$^{(2)}$
myristic acid	-28.9 ± 0.04$^{(2)}$	-30.2 ± 0.64$^{(3)}$	-30.7 ± 0.41$^{(2)}$	-30.1 ± 0.61$^{(3)}$
methyl palmitate	-29.5 ± 0.07$^{(2)}$	-31.1 ± 0.56$^{(4)}$	-29.1 ± 0.23$^{(2)}$	-31.7 ± 0.59$^{(3)}$
methyl stearate	-29.4 ± 0.23$^{(2)}$	-30.7 ± 0.11$^{(4)}$	-28.8 ± 0.49$^{(2)}$	-31.1 ± 0.66$^{(2)}$

Isotopic fractionation with 45 min absorption/2 min desorption times were examined; the high volatility eucalyptol prevented its reliable analysis by EA-irMS. SPME-CSIA with the PA fibre showed generally similar (<0.6 **‰**) $\delta^{13}C$ values to the standards measured by traditional solution injection CSIA.

THE PDMS fibre showed slightly more divergent $\delta^{13}C$ values (e.g., $\Delta\delta^{13}C$ methyl stearate = 1.9 **‰**). Similar variations in $\delta^{13}C$ values of up to 1.5 ‰ have previously been reported for organic acids analysed with a PDMS fibre (Dias and Freeman, 1997). The EAirMS $\delta^{13}C$ data

reflected consistently richer ^{13}C levels which can be attributed to the volatile nature of these compounds (Jancso & Van Hook, 1974).

Overall, the data in Table 2.6 indicate that the SPME-CSIA method used is sufficiently reliable with respect to precision and reproducibility, although more so with the PA fibre than the PDMS, providing stringent controls on salt concentration, pH, extraction time (i.e., 45 min), temperature and fill volume were maintained.

Multiple Injections

The previous experiments indicated that SPME-CSIA would greatly benefit from higher product concentrations than provided by a single SPME fibre, so multiple fibres were subsequently trialled. The operating software of the Gerstel SPME autosampler and Micromass GC-irMS were not compatible, therefore all SPME analyses on the GC-irMS were performed manually. A schematic of the lab set-up is shown in Fig. 2.3.

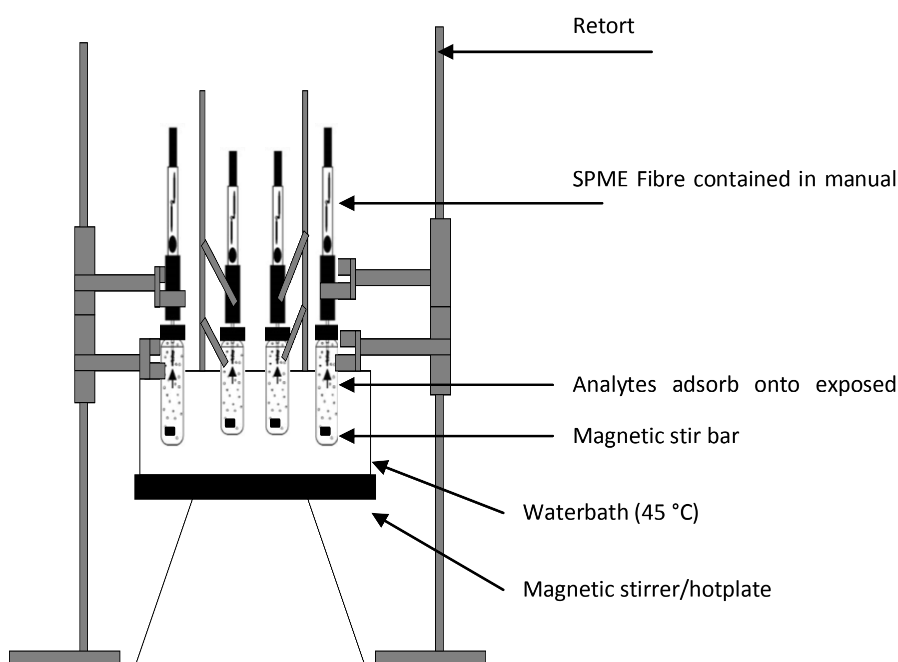


Figure 2.3 Multi-fibre SPME laboratory set-up for CSIA analysis.

Four fibres of the same type (e.g., PDMS or PA) were separately exposed to aliquots of the solution to be analysed, then subjected to sequential injection onto the GC and their combined analyte loads analysed. The products from the different probes were combined with a cryogenic trap, consisting of a loop at the front end of the GC column immersed in liquid nitrogen. Cryofocusing improved peak shape and resolution. Cryofocusing is a well-established method commonly used for SPME analysis and does not usually lead to any molecular fractionation (Arthur et al., 1992a).

Carryover was observed when multiple injections were trialled using the original two min desorption time (data not shown). As carryover can decrease the precision of $\delta^{13}C$ measurements, a longer five min desorption was used and all fibres were separately conditioned for 30 min at 250°C (PDMS) or 300°C (PA) after use (the supplier-specified pre-conditioning step). With these conditions no carryover was observed.

To assess whether any 'isotopic fractionation' occurred with this multi-fibre approach, the $\delta^{13}C$ values of the five standard mix measured by one PA fibre were compared with those from the multiple four PA fibre analysis. This test should also be representative of the PDMS fibre coating which functions with a similar absorption mechanism. No significant 'isotopic fractionation' was evident in the $\delta^{13}C$ measurement of the standards (Table2.7).

The standard deviations associated with multiple fibre injections for eucalyptol (0.8 ‰) and myristic acid (1.1 ‰) were higher than those for single fibre and liquid injections. Variations in SPME and solution injection values were highest for the eucalyptol analyte, probably due to its particularly high volatility. Peak tailing with SPME analysis of myristic acid was observed frequently (also observed during SPME-GCMS analysis and described in Section 2.4.1.1) and the likely cause of the higher variation seen in Table 2.6.

Table 2.7 $\delta^{13}C$ values from SPME-CSIA of five component standard mixture with single and multiple (4x) fibre injections (PA Fibre, 45 min absorption time, 5 min desorption time).

Standards	$\delta^{13}C$ ‰ 1 PA	$\delta^{13}C$ ‰ 4 PA	$\delta^{13}C$ ‰ Liquid	Δ Single	Δ Multi
eucalyptol	-26.6 ±0.54$^{(2)}$	-26.3 ±0.80$^{(2)}$	-26.9 ±0.31$^{(4)}$	0.3	0.6
thymol	-27.0 ±0.30$^{(2)}$	-27.2 ± 0.51$^{(3)}$	-26.8 ±0.25$^{(4)}$	0.2	0.4
myristic acid	-30.1 ±0.61$^{(3)}$	-30.2 ± 1.05$^{(2)}$	-30.2 ±0.64$^{(3)}$	0.1	0.0
methyl palmitate	-31.7 ±0.59$^{(3)}$	-32.0 ± 0.34$^{(3)}$	-31.1 ±0.56$^{(4)}$	0.6	0.3
methyl stearate	-31.1 ±0.66$^{(2)}$	-30.7 ±0.37$^{(3)}$	-30.7 ±0.11$^{(4)}$	0.4	0.0

$^{(n)}$ = number of replicates from which average value and standard deviation is determined.
Liquid = traditional liquid CSIA injection (originally presented in Table 2.6).
Δ = difference in $\delta^{13}C$ of standards determined by single and multiple SPME fibre desorptions.

A similar effect has been observed for the analysis of moderate carbon number fatty acids (Parrish 1998; Pfannkoch et al., 2003; Zander et al., 2007; Zander, 2008) which has been attributed to several factors, including low solubility (globule formation) and adhesion to the walls of the extraction vessel. Free fatty acids are poorly resolved using non-polar columns due to the structural polarity of the carboxylic group and consequent hydrogen bond formation (van Huyssteen, 1970; Kuksis, 1977; Porschmann et al., 1987).

2.4.1.4 Isotopic Characterisation of Marri Leachate (100 min)

Ten compounds in the 100 min Marri leachates were of sufficient concentration and resolution for $\delta^{13}C$ measurement (Table 2.8).

Table 2.8 $\delta^{13}C$ of products in the 100 min Marri leachate determined via SPME-CSIA using both the PA and PDMS fibre coatings. $^{(n)}$ =specifies the number of analyses (≥2) upon which value and standard deviation were based. **= Standard deviation is high due to low peak height, an inherent limitation of the SPME-CSIA analysis of DOC. This degree of error has been reported previously (Dias & Freeman, 1997). *= unresolved to baseline, value may be compromised.

Peak No.	ID	Group	$\delta^{13}C$ ‰
11	1-phenyl-1-propanone PA	Ketone (lignin)	$-34.4 \pm 0.4^{(2)}$
12	2,3-dihydro-benzofuran PA	Furan (lignin)	$-33.5 \pm 0.1^{(2)}$
17	2-phenyl-propanedioic acid PA	Acid (lipid)	$-32.9 \pm 0.1^{(2)}$
32	2-methoxy-4-vinyl phenol PA	Phenolic (lignin)	$-34.5 \pm 0.2^{(2)}$
35	decanoic acid PA	Acid (lipid)	$-34.9 \pm 0.9^{(2)**}$
74	4- hydroxy-1-propenyl-2-methoxyphenol PDMS	Phenolic (lignin)	$-34.7 \pm 0.7^{(2)**}$
79	8-phenyl octanoic acid PDMS	Acid (lipid)	$-34.5 \pm 0.1^{(2)}$
81*	3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid PDMS	Acid (Lignin)	$-40.7 \pm 0.3^{(2)}$
84	*n*-hexadecanoic acid PDMS	Acid (lipid)	$-36.2 \pm 0.5^{(2)}$
Bulk $\delta^{13}C$ value of Marri biomass -30.7 ‰			

The $\delta^{13}C$ values of all products measured (Table 2.8 and Fig 2.4) reflected ^{13}C depletion relative to the bulk $\delta^{13}C$ value of the biomass, and were in a range typical of the biochemicals constituents of C3 plants (Hobbie & Werner, 2004; Badeck et al., 2005; Bowling et al., 2008; Cernusak et al., 2009).

Lipid derived compounds were ^{13}C depleted relative to bulk by 1.9–8.8 ‰, consistent with other studies (Park & Epstein, 1961; Deines, 1980; Kennicutt et al., 1992; Ballentine et al., 1998; Boschker et al., 1999). The $\delta^{13}C$ values of lignin derived compounds varied from 3.7 ‰ to 10.0 ‰ more ^{13}C depleted than the bulk, also consistent with other studies (Wilson & Grinsted, 1977; Keppler et al., 2004; Preston et al., 2006; Dungait et al., 2007; Greule et al., 2009).

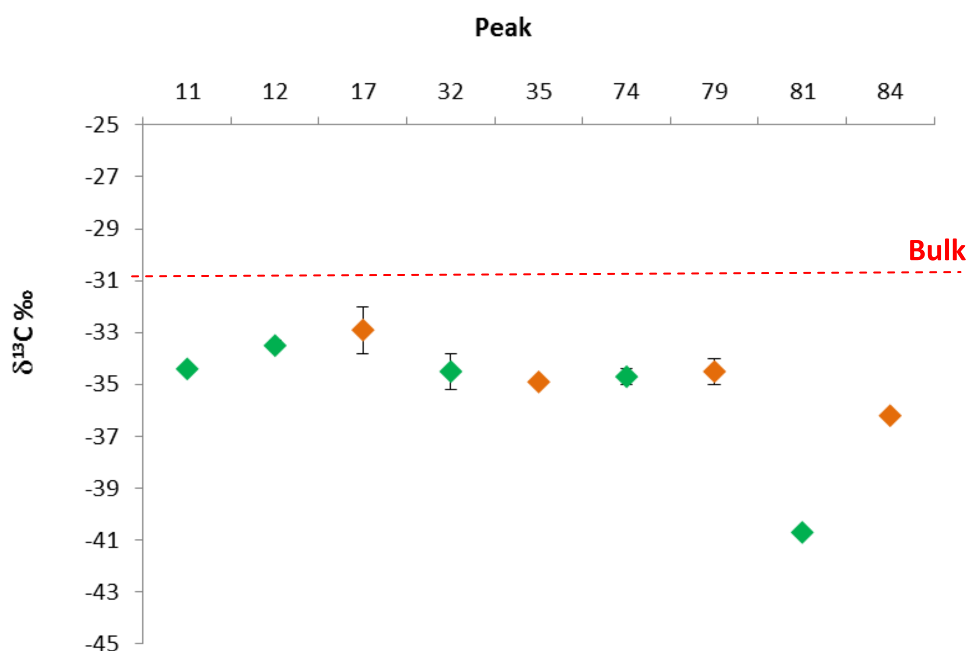


Figure 2.4 $\delta^{13}C$ values of products from the SPME-CSIA (PA or PDMS) of the 100 min Marri. Bulk Marri $\delta^{13}C$ value (30.7 ‰) indicated by red dashed line. Orange data points are representative of lipid-derived compounds and green data points of lignin products. Peak numbers correspond to products listed in Table 2.8.

2.4.2 Organic, Molecular and $\delta^{13}C$ Analysis of Plant Leachates

2.4.2.1 DOC Analysis

DOC values of the leachates at 'Day 0' (i.e., 5 min leaching) and 150 days are shown in Table 2.9. The Marri leachate showed the highest DOC values at Days 0 (760 mg L^{-1}) and 150 (1400 mg L^{-1}). The DOC values of the three C4 plant leachates were all considerably lower, with the Spinifex leachate showing the lowest initial (82 mg L^{-1}) and final DOC levels (145 mg L^{-1}).

Table 2.9 DOC values of the four plant leachates determined at beginning (5 min) and end of leaching period (150 days).

Sample	DOC concentration/ Timepoint	
	5 min (mg L^{-1})	Day 150 (mg L^{-1})
Marri	760	1400
Maize	190	1510
Sorghum	175	1195
Spinifex	82	145

2.4.2.2 Molecular Characterisation of Leachates (150 Day)

A comprehensive list of products with retention time (RT), mass spectral ion information and assigned class/precursor for the 150 day leachates is shown in Table 2.5.

Marri (C3-Plant)

Nor-terpenoids, the prominent products of this leachate, are ubiquitous plant derived aromatic compounds (Winterhalter & Rousseff, 2002). They are produced from the degradation of carotenoids such as β-carotene and xanthophylls (i.e., lutein and *neo*xanthin; Mordi et al., 1991; Winterhalter 1992) or released from bound glycosides formed by acid and enzymatic hydrolysis (Skouroumounis, et al., 1992; Mendes-Pinto, 2009).

Some of the C_{13} *nor*-terpenoids detected (e.g. vitispirane, **30**; and 1,1,6-Trimethyl-1,2-dihydro naphthalene, TDN, **38,** Table 2.5) have been identified in *Eucalyptus* impacted environments (Winterhalter & Rousseff, 2002; Versini et al., 2002; Bulleid, 2009; Mendes-Pinto, 2009). Megastigmatrienone (**47**) has also previously been detected by SPME-GCMS analysis of *Eucalyptus*-derived natural products (D'Arcy et al., 1997; Montenegro et al., 2009). The *nor*-terpenoids in the Marri leachate probably arise from glycol hydrolysis of cellular components of the Marri, promoted by the acidic pH of the SPME sample matrix.

Several of the hydroxy ketones (**64, 65**) detected in the Marri leachate are known to be precursors of *nor*-terpenoid related compounds (Knapp et al., 2002; Winterhalter & Rouseff, 2002) and have also previously been detected in acetone/water extracts of *Eucalyptus orelliana* (Yang et al., 2008).

Phenols (e.g., **6, 15, 26, 31, 61**) were also prominent Marri leachate products and are typically attributed to a lignin source, terpenoid-related precursors or anthropogenic origins. 3-Methyl-4-*iso*propylphenol (**26**) was present at early stages of leaching, as evidenced by the composition of 100 min leachate (Section 2.4.1.2). 2-Methyl-5-(1-methylethyl)-phenol (**31**) and 1,6-*bis* (1,1-dimethyl ethyl)-4-(1methylpropyl) phenol (**61**) were major constituents of water distilled *Eucalyptus camaldulensis* leaf oil (Akin et al., 2010), suggesting similar biosynthetic origin with other monoterpenes. 1,6-*bis* (1,1-dimethyl ethyl)-4-(1methylpropyl) phenol (**61**) has also been detected in *Eucalyptus*-products (Soria et al., 2009) but can also derive from other biological (e.g., *Larix gmelini*, Sun et al., 2010) and anthropogenic (Clark et al., 1991) sources.

Methoxy aromatics e.g., 4-ethyl phenol (**15**) and *p*-ethyl guaiacol (**28**) are diagnostic lignin structures (Bracewell et al., 1989; Saiz-Jimenez, 1993; Saiz-Jimenez; 1994; van Heemst et al., 1999; Guo et al., 2003) and were detected in the leachates of Marri (Table 2.5) but not from any of the C4 plants.

Other products of lower abundances include N-products (**33**), aromatic acids (**13, 34, 52**), alkyl (**60, 66**) and aromatic (**56**) esters and acetates (4-*iso*propyl-2,5-dimethoxybenzyl acetate, **56**) of varying source significance. LMW N-heterocyclic products (e.g., 2-methoxy-3-ethylpyrazine, **33**) are indicative of protein precursors (e.g., Bruchet et al., 1990). However, other products such as benzoic acid (**13**), hydro cinnamic acid (**34**) and 4-hydroxy benzoic acid (**52**) have many potential sources.

Sorghum, Maize and Spinifex (C4 Plants)

Leachates of all three C4 grasses showed a lower number of SPME products than the Marri leachate. Zander (2007) also reported a much lower number of SPME-GC peaks from the leachates of the *Poaceae* couch grass relative to River Red Gum. SPME-GCMS of the Maize leachate yielded 10 identifiable products (Table 2.5) spanning a range of chemical classes. Corresponding analysis of the Sorghum leachate yielded a quite different distribution of 11 main products comprising aromatics, terpenoids, furans and fatty acids (Table 2.5).

The Spinifex leachate showed the highest product concentrations of the leachates of the other C3 plants, comprising 24 products of relatively high abundance. These included lignin derived hydroxyl and methoxy aromatics.

A comparison of other plant leachate studies also provides evidence that Spinifex yields high amounts of lignin products (8.5–15.0 % for Spinifex *Triodia basedowii*; Islam & Adams, 1999) relative to Sorghum (8 %; Amaducci et al., 2000) or Maize (6.9 %; Amaducci et al., 2000). These results reflect the biochemical variance of C4 grasses. (Gutierrez et al., 1974; Kolattukudy, 1980; Hattersley & Browning, 1981) Conveniently from a CSIA viewpoint, most SPME products from the present plant leachates were well resolved by GC (apart from **29–30** and **36–37**).

2.4.2.3 Bulk Isotope Analysis of Raw Plants

The $\delta^{13}C$ values for all plants showed negligible variation before and after leaching (Table 2.10). The $\delta^{13}C$ values of the C3 Marri biomass, were within a -22 ‰ to -35 ‰ range (O'Leary, 1981; Ehleringer et al., 1991; Ehleringer & Monson, 1993) the biomass of the C4

plants within a range -9 ‰ to -17 ‰, which are typical value ranges for C3 and C4 plants, respectively (O'Leary, 1981; Hattersley, 1982; Krull et al., 2003).

Table 2.10 Bulk δ^{13}C properties of plants and their residues after 150 days of leaching. Values are the average of at least 2 replicates.

Sample	Before leaching δ^{13}C ‰	After leaching δ^{13}C ‰
Marri	-30.7 ± 0.12	-30.8 ± 0.14
Maize	-14.2 ± 0.07	-13.7 ± 0.17
Sorghum	-17.5 ± 0.25	-17.2 ± 0.14
Spinifex	-14.3 ± 0.21	-13.8 ± 0.10

The δ^{13}C value presently measured for Sorghum of -17.5 ‰ was slightly more depleted than the 10.9 ‰ to -14.4 ‰ range previously reported for Sorghum (Whelan et al., 1970; Smith and Epstein, 1970). Many factors including phenotypic and environmental variation may account for these differences. Sorghum reportedly exhibits a range of isotopic discrimination amongst different phenotypes (Henderson et al., 1998; Christin et al., 2009).

2.4.2.4 Isotopic Characterisation of Marri Leachates (150 day)

The same analytical method used for the 100 min leachates – which included separate complimentary analysis with PDMS and PA fibres – was subsequently applied to the 150 day leachates. Table 2.11 shows the δ^{13}C values for the compounds determined in the 150 day Marri leachates.

Figure 2.5 shows the δ^{13}C values of the major products which span a range of biochemical classes (lipids, lignin, and carbohydrate). With the exception of 4-*iso*propyl-2,5-dimethoxybenzylacetate (**56**), all the products were ^{13}C depleted relative to the bulk value which as previously discussed is typical of lipid (Jux et al., 2001) or lignin (Benner et al., 1987, Goni & Eglinton, 1996, Spiker & Hatcher, 1987, Loader et al., 2003, Preston et al., 2006; Dungait et al., 2008b) sources. Carbohydrate-derived products were ^{13}C enriched (0.5–2.1 ‰) relative to the bulk, which is also consistent with previous reports (Leavitt & Long, 1982; Sternberg et al., 1984; Badeck et al., 2005).

Lipid Derived Compounds

The majority of compounds detected in the 150 day Marri leachate were lipid derived terpenoids with δ^{13}C values ranging from -31.4 ‰ (α-terpineol, **24**) to 34.7 ‰ (2-methyl-5-(1-methylethyl)-phenol; **31**). Most products fell within the range -31.4 ‰ to -33.5 ‰. This is consistent with δ^{13}C values (-24.7 ‰ to 30.2 ‰) determined for natural oils (including 2-

methyl-5-(1-methylethyl)-phenol and thymol) from a range of herbs and savoury plants by Greule et al. (2008). The relatively consistent values for most terpenoids show that they are produced from the same pathways.

Table 2.11 $\delta^{13}C$ of products determined from separate SPME-CSIA analysis of 150 min Marri leachate with PA and PDMS fibres (fibre specified next to product name - values for PDMS fibre are displayed for products detected by both). Biochemical class also indicated where possible

	Marri 150 Day Leachates		
Peak No.	**ID**	**Group**	**$\delta^{13}C$ ‰**
9	*p*-cymene (PDMS)	Terpenoids (lipid)	$-32.0 \pm 0.17^{(2)}$
16	pinocarvone (PDMS)		$-31.6 \pm 0.87^{(2)*}$
20	*iso*-borneol (PA)		$-33.5 \pm 0.84^{(2)*}$
21	terpinen-4-ol (PDMS)		$-32.7 \pm 0.70^{(2)*}$
24	α – terpineol (PDMS)		$-34.7 \pm 0.08^{(2)}$
29	thymol (PA)		$-31.6 \pm 0.87^{(2)*}$
29	thymol (PDMS)		$-33.9 \pm 0.90^{(2)*}$
30	vitispirane (PDMS)		$-32.8 \pm 0.25^{(2)}$
31	2-methyl-5-(1-methylethyl)-phenol (PA)		$-34.7 \pm 0.07^{(2)}$
59	veridiflorol (PA)		$-33.2 \pm 0.59^{(2)}$
63	cadenene (PDMS)		$-33.9 \pm 0.30^{(2)}$
34	hydrocinnamic acid (PA)	Flavonoid (lipid)	$-32.0 \pm 0.30^{(2)}$
65	3,5,5,-trimethyl-4-(3-oxobutyl) 2-cyclohexen-1-one (PDMS)	Ketone (lipid)	$-36.9 \pm 0.35^{(2)}$
58	2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid, isobutyl ester (PDMS)	Ester (lipid)	$-40.0 \pm 0.09^{(2)}$
43	1,6 dimethyl naphthalene (PDMS)	Aromatic	$-33.9 \pm 0.14^{(2)}$
54	1,2,3,4 tetrahydro-1,5-dimethyl-napthalene (PA)	Aromatic	$-33.7 \pm 0.05^{(2)}$
62	6-methoxy-1-phenyl-3,4-dihydronapthalene (PA)	Aromatic	$-32.2 \pm 0.11^{(2)}$
56	4-*iso*propyl-2,5-dimethoxybenzylacetate (PDMS)	Aromatic (Carbohydrate)	$-28.4 \pm 0.67^{(2)}$

$^{(n)}$ = number of analyses.
*= Standard deviation high due to the low peak height, an inherent limitation of this method and the nature of the technique with DOC.

A trend of increasing ^{13}C depletion of the terpenoid products with their decreasing volatility implied from increasing MWs (Table 2.5) was observed (Fig. 2.5). This is particularly evident between products 9 (-32.0 ‰) to 24 (-34.7 ‰). This effect was also seen in the $\delta^{13}C$ values of fatty acids in the 100 min Marri leachate (between products 17 and 35 in Table 2.8 and Fig.2.4). The observation is consistent with trends reported by Ballentine et al. (1998) for $\delta^{13}C$ values of individual FAs determined in lipid isolates of Eucalyptus sp. Collister et al. (1994) suggested that the production of specific members of a homologous series at different growth stages may be responsible the depletion effect.

Short chain lipids are synthesised earlier than long chain members of the same series, and thus, are not subjected to 'isotopic fractionation' associated with chain elongation (as described in DeNiro & Esptein, 1977 and Monson & Hayes, 1980).

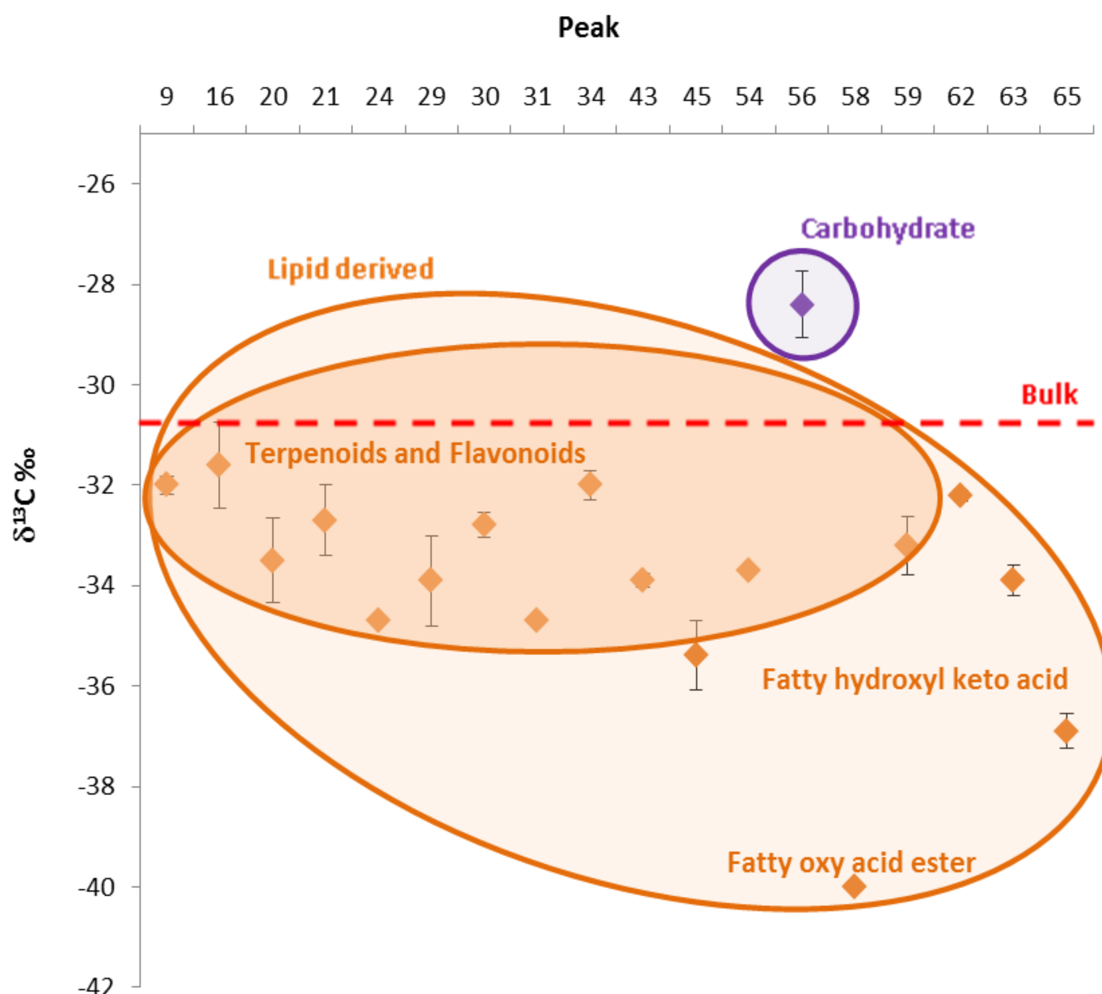


Figure 2.5 $\delta^{13}C$ values of products from SPME (PA or PDMS fibre, values for PDMS fibre are displayed for products detected by both) CSIA of 150 day Marri leachate.
Bulk Marri $\delta^{13}C$ value (-30.7 ‰) indicated by the red dashed line.
Product identity and details given in Table 2.5 and Table 2.11.
Lipid-grouped products circled orange and carbohydrate-grouped products circled purple.
Error bars correspond to standard deviation (refer Table 2.11).

The $\delta^{13}C$ values determined for 2,2,4-trimethyl-3-carboxyisopropyl pentanoic acid, isobutyl ester (**58**; -40.0 ‰) and the substituted hydroxyl ketone, 3,5,5,-Trimethyl-4-(3-oxobutyl) 2-Cyclohexen-1-one (**65**; -36.9 ‰), were the most depleted compounds detected. This corroborates the overall trend of greater ^{13}C depletion with increasing MW of lipid products. The effect could be real, or may be due to isotopic fractionation on the analysis of this particular compound—evident in both present (i.e., established with standards) and

previous work (on C_{2-5} fatty acids; Dias & Freeman, 1997) attributed to mass dependent kinetic shifts upon dissolution of each analyte into the organic SPME fibre coating. It is also possible that the compounds leached from C3 plants simply express a range of $\delta^{13}C$ values of varying degrees ^{13}C depletion, with compound **58** being particularly depleted.

Hydrocinnamic acid (**34**; -32.0 ‰) could derive from one of several possible sources (Michael, 2000). However, its isotopic value of -32.0 ‰ (1.3 ‰ depleted relative to the bulk) is typical of flavonoid sources (e.g., the flavonoid ceratiolin; Tanrisever et al., 1986).

Hydroxy ketones (e.g., **65**) are considered fermentation products (de Revel & Bertrand, 1994; Knapp et al., 2002; Winterhalter & Rouseff, 2002) and may also be produced by cyanobacteria (Hockelmann & Juttner, 2005). Their detection suggests not all microbial activity was quenched by sodium azide—a respiratory inhibitor (Wilson & Chance, 1967) which may not act on other metabolic processes.

Lignin/Lipid Derived Aromatic Products

The aromatic products for which $\delta^{13}C$ values were measured included 1,6 dimethylnapthalene (**43**; -33.9 ‰) and 6-methoxy-1-phenyl-3,4-dihydronapthalene (**62**; -32.2 ‰). These products may be lignin or lipid derived. Stable isotopic composition can help distinguish these sources. If the naphthalenes (**43 & 62**) were derived from the same lipid-precursor as TDN (**38**, ~-32.7 ‰,) their $\delta^{13}C$ values would be expected to be similar, which is the case (shown in Fig. 2.5).

Some research has reported relatively ^{13}C-enriched (up to 5 ‰) values of lignin-derived components relative to bulk $\delta^{13}C$ values (e.g., lignin pyrolysates of peat; Kracht & Gleixner, 2000). More typically however, lignin moieties isolated from plant products range from 2 ‰ to 7 ‰ depleted in ^{13}C compared with the bulk $\delta^{13}C$ values of whole biomass (Boutton, 1991; Schweizer et al., 1999; Yeh & Wang, 2001; Dungait et al., 2007). Due to the overlap of $\delta^{13}C$ value signatures, a lignin source for the naphthalene products cannot be ruled out entirely. However, the proximity of the determined ^{13}C values to that of the *nor*-terpenoids in these leachates strongly suggests a common biochemical precursor.

Carbohydrate Derived Compounds

The ester, *iso*propyl-2,5-dimethoxy benzyl acetate (**56**; -28.6 ‰ average), was ^{13}C enriched relative to the bulk $\delta^{13}C$ value. This relatively ^{13}C enriched isotopic value reflects a carbohydrate origin (Leavitt & Long, 1982; Gleixner et al., 1993; Badeck et al., 2005;

Dungait et al., 2009), consistent with the source suggested by molecular profiling (Table 2.5). Carbohydrate derived material (i.e., Primary metabolism product, refer Section 1.3.3.3) is typically ^{13}C enriched relative to bulk biomass.

Comparison of $\delta^{13}C$ Values of Primary and Secondary Plant Products

The distinction between products of Primary and Secondary plant metabolism and the isotopic differences between the two is discussed in Section 1.3.3.4. The majority of products of Secondary plant metabolism detected (i.e., lipids, terpenoids, flavonoids, naphthalenes) had $\delta^{13}C$ values which ranged from -31.6 ‰ to -34.7 ‰. In contrast, carbohydrate derived (i.e., Primary metabolic) products reflected heavier values (e.g., **56**, -28.4 ‰, Table 2.11).

Several previous isotopic studies of plant biochemical fractions (Galimov, 1981; Schmidt et al., 1995; Hobbie & Werner, 2004 for a review) have similarly reported a 3–6 ‰ difference in the $\delta^{13}C$ of lipid-sourced compared to carbohydrate-sourced (e.g., cellulose) fractions. Although these studies were not compound-specific, but focused on biochemical fraction isolates, the findings are consistent (with the exception of the highly depleted fatty-acid derivative products, **58** & **65**) with the findings of all the lipid-derived products (Table 2.11) which ranged from 3.0–6.1 ‰ ($\delta^{13}C$ depleted compared to carbohydrate derived **56,** -28.4 ‰).

2.4.2.5 Isotopic Characterisation of C4 Plant Leachates (150 day)

The compounds detected by SPME-CSIA of the three C4 plant leachates are shown in Table 2.12. Several abundant products detected via SPME-GCMS (e.g., phenyl propanedioic acid, 3-methyl butanoic acid and 1,6-dimethyl naphthalene) were detected via GCirMS with insufficient reproducibility to allow accurate $\delta^{13}C$ determination. This combined with GC resolution inefficiencies and matrix effects (a visible surface frothing was seen in Maize leachates—attributed to natural detergents, surfactants and waxes) restricted reliable $\delta^{13}C$ determination of only one product (**70**).

Maize leachate vials were not homogeneous (even with vigorous magnetic stirring). The extraction of analytes from a non-homogeneous matrix combined with the manual adsorption and desorption (introducing the possibility of operator error in absolute timings) of four sequential SPME fibres is likely to have contributed to the lack of reproducible $\delta^{13}C$ values for many Maize leachate compounds which were detected adequately by SPME GCMS. However, the $\delta^{13}C$ value of 4-methyl-2,5-

dimethoxybenzaldehyde (**70**; -12.5 ‰) was significantly heavier than any of the Sorghum or Spinifex measurements, and was ^{13}C enriched relative to the bulk (1.7 ‰), indicative of a sugar source.

Table 2.12 $\delta^{13}C$ of products from SPME CSIA of 150 Day C4 plant (Maize, Sorghum and Spinifex) leachates. Each value reported was based on at least 2 replicates. $^{(n)}$ = number of analyses. $\Delta = {}^{13}C_{bulk} - {}^{13}C_{product}$ ‰. nd = not detected.

Peak	Product ID	Sorghum		Maize		Spinifex	
		$\delta^{13}C$ ‰	Δ	$\delta^{13}C$ ‰	Δ	$\delta^{13}C$ ‰	Δ
9	*p*-cymene (PDMS)	-20.7 ±0.73$^{(2)}$	-3.2	nd	nd	-15.0 ±0.33$^{(2)}$	-0.7
16	pinocarvone (PDMS)	-22.2 ±0.89$^{(2)}$	-4.7	nd	nd	nd	nd
36	4-hydroxy benzaldehyde (PA)	-18.7 ±0.63$^{(2)}$	-1.2	nd	nd	nd	nd
41	1,1 diethylpropyl benzene (PA)	-18.4 ±0.20$^{(2)}$	-0.9	nd	nd	nd	nd
50	pentamethyl 2,3-dihydrobenzofuran (PA)	nd	nd	nd	nd	-17.3 ±0.14$^{(2)}$	-3.0
54	1,2,3,4 tetrahydro-1,5-dimethyl-napthalene (PA)	-17.6 ±0.57$^{(2)}$	-0.1	nd	nd	-16.7 ±0.23$^{(2)}$	-2.4
57	tetrahydro-trimethyl-benzofuranone (PA)	nd	nd	nd	nd	-18.5 ±0.21$^{(2)}$	-4.2
70	4-methyl-2,5-dimethoxy benzaldehyde (PDMS)	nd	nd	-12.5 ±0.07$^{(2)}$	1.7	nd	nd

The $\delta^{13}C$ values of products grouped according to lipid, lignin or carbohydrate origin are shown in Fig. 2.6. The same general isotope-precursor trend of lipid < lignin < carbohydrate observed for the C3 Marri leachate (Fig. 2.5) was again evident for the $\delta^{13}C$ data of the C4 plant leachates. Lipid derived terpenoids detected in Sorghum (cymene, **9**; and pinocarvone, **16**) were overall the most ^{13}C depleted products of the C4 plant leachates and these were also the most depleted (-3.2 ‰ and -4.7 ‰ for **9** and **16**, respectively) products relative to the bulk $\delta^{13}C$ value of the parent plant. The Marri leachate products showed a similar level of ^{13}C depletion relative to bulk Marri. The monoterpene cymene (**9**) detected in the Spinifex leachate was only slightly (0.7 ‰) ^{13}C depleted relative to the bulk.

Sorghum leachate showed an intermediate level of ^{13}C depletion of 4-hydroxybenzaldehyde (**36**) and 1,1-diethylpropyl benzene (**41**) relative to the terpenoids and bulk Sorghum which suggests a probable lignin source. 4-Hydroxybenzaldehyde (**36**) is a reported lignin

oxidation product (Baker et al., 1968; Meyer & Norris 1974; Obst., 1983). An alternative cyanogenic glucoside origin (which is known for compound **36** in Sorghum; Hosel et al., 1987) would have been expected to be more ^{13}C enriched (due to the influence of the 'heavy' sugar group). Whilst 1,1-diethylpropyl benzene (**41**) is not a specifically characteristic product of lignin origin, C_1 to C_{10} alkylated benzenes are commonly reported products both from chemical (e.g., Gluckstein et al., 2010; Lapierre, 2010) and thermal (e.g., Saiz-Jimenez & De Leeuw 1987) degradation studies of lignin, lignin is therefore a likely source.

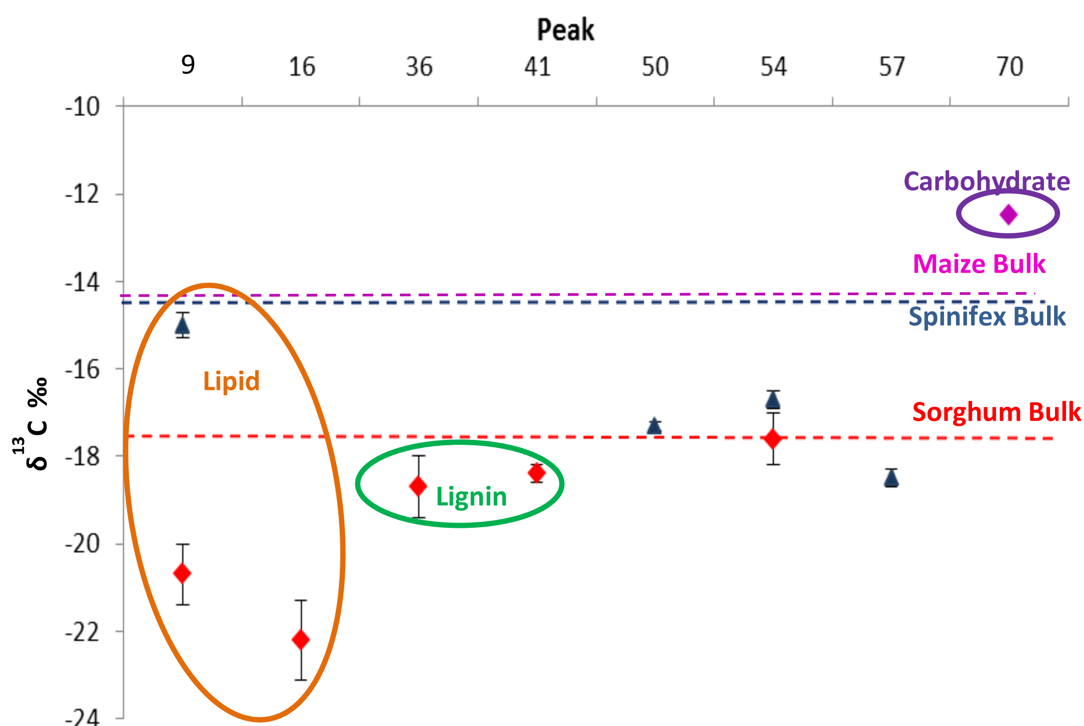


Figure 2.6 $\delta^{13}C$ values of products from SPME CSIA of three C4 plant leachates (150 min) with PA or PDMS fibre. Lipid-derived products shown in orange oval; possible lignin/ligno-cellulose derived products shown in green oval; and carbohydrate products are in purple oval. Bulk Sorghum $\delta^{13}C$ value indicated by red dashed line, bulk Maize $\delta^{13}C$ value indicated by pink dashed line and bulk Spinifex $\delta^{13}C$ value indicated by dark blue dashed line (Table 2.12).

1,2,3,4 Tetrahydro-1,5-dimethyl-napthalene (**54**) was slightly heavier than **36** and **41**, with a $\delta^{13}C$ value of -16.7 ‰ compound **54** was the second most isotopically heavy Spinifex product (**9** being the heaviest) and was ~3 **‰** heavier than bulk Spinifex, indicating a carbohydrate source. Similarly, compound **54** was isotopically the heaviest of Sorghum products, but had almost the same $\delta^{13}C$ value as bulk Sorghum. The full significance of these isotopic values is unclear, but discussed in greater depth below.

The $\delta^{13}C$ signatures of pentamethyl 2,3-dihydrobenzofuran (**50**) and tetrahydro-trimethyl-benzofuranone (**57**) were highly ^{13}C depleted relative to bulk Spinifex (-3.0 ‰ to -4.2 ‰). This isotopic range is consistent with a lignin source, but may also derive from a mixture of sources. This makes deciphering the origins of some of the compounds detected difficult without a more complete data-set.

Isotopic Differences between Sorghum & Spinifex Products

Sorghum products were generally more ^{13}C depleted than Spinifex products (Fig 2.6). Cymene (**9**) was depleted by 3.2 ‰ relative to the bulk $\delta^{13}C$ value in Sorghum leachate and 0.7 ‰ in the bulk Spinifex leachate. These differences could be explained by variation between species—DeNiro and Epstein (1977) reported up to 3 ‰ species-specific differences in the magnitude of the $\delta^{13}C$ value of lipids. It was suggested this variance may be due to different kinetic isotope effects during the pyruvate dehydrogenase reaction, and also from different rates of flow of pyruvate to other metabolic intermediates (DeNiro & Epstein, 1977). Collister et al. (1994) and Chikaraishi et al. (2004) reported similar variation in degree of ^{13}C-depletion in lipid between species.

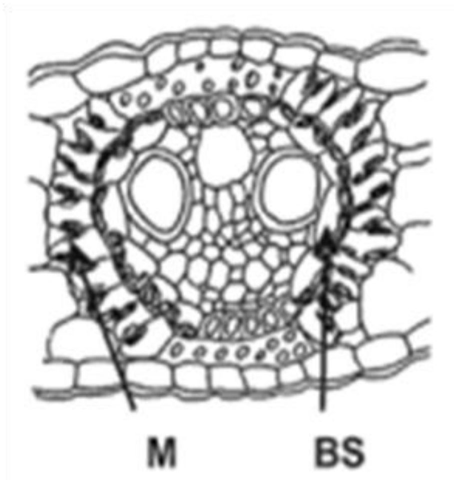
A more specific explanation for the isotopic differences evident in the products of the C4 plants Sorghum and Spinifex, relates to their different sub-pathways of CO_2 fixation. Sorghum uses the classical Nicotinamide adenine dinucleotide phosphate-Malic Enzyme (NADP-ME) pathway (Sage, 2004) and Spinifex uses the Nicotinamide adenine dinucleotide-Malic Enzyme (NAD-ME) pathway and also possesses a unique extreme variant from classical NAD-ME plants (Sage, 2004) referred to as a 'draped form' 'triodioid' type-Kranz anatomy (Hattersley & Watson, 1992: Edwards & Voznesenskaya, 2011).

In the unconventional triodioid anatomy, 'sheaths' drape over adjacent bundles, resulting in an arrangement where the photosynthetic carbon reduction (PCR) cells are distant from the vascular tissue (Hattersley & Watson, 1992). This difference is shown in Figure 2.7.

C4 plants concentrate CO_2 around 1,5-bisphosphate carboxylase oxygenase (RuBisCo) using phosphoenolpyruvate carboxylase (PEPCase) and other enzymes (Hatch, 1987). Sorghum uses NADP-ME whereas Spinifex uses NAD-ME for decarboxylation. When NADP-ME is used (i.e., in the case of Sorghum) PEP is carboxylated forming oxaloacetic acid, which is then converted to malate (this occurs in the cytosol of the photosynthetic carbon assimilation (PCA) tissue. Malate then diffuses into the PCR tissue (mestome sheath cell,

Fig. 2.7) where decarboxylation occurs, resulting in the formation of pyruvate which diffuses back out to the PCA tissue and reforms PEP. However, in NAD-ME plants (i.e., Spinifex) malate is first trans-aminated to asparatate which diffuses into the PCR (bundle sheath cell, Fig.2.7) and diffuses out as alanine following the decarboxylation and transamination reaction.

a) Classical NADP-ME



b) Triodioid Variation

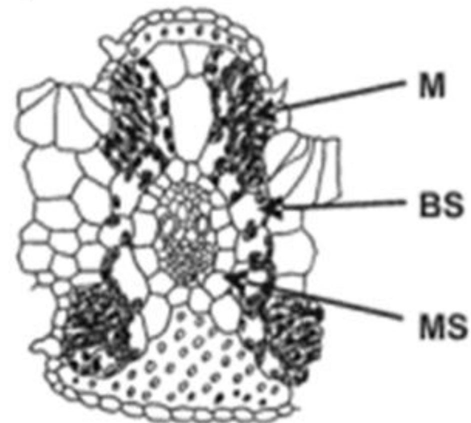


Figure 2.7 Illustrations of different Krantz anatomy, (classical NADP-ME (a) example is of Maize, which is similar to Sorghum). Triodioid variation (Spinifex) shown in (b). Sketches after Edwards and Voznesenskaya (2011). M = Mesophyll, BS = Bundle sheath, MS = Mestome sheath.

C4 plants concentrate CO_2 around 1,5-bisphosphate carboxylase oxygenase (RuBisCo) using phosphoenolpyruvate carboxylase (PEPCase) and other enzymes (Hatch, 1987). Sorghum uses NADP-ME whereas Spinifex uses NAD-ME for decarboxylation. When NADP-ME is used (i.e., in the case of Sorghum) PEP is carboxylated forming oxaloacetic acid, which is then converted to malate (this occurs in the cytosol of the photosynthetic carbon assimilation (PCA) tissue. Malate then diffuses into the PCR tissue (mestome sheath cell, Fig. 2.7) where decarboxylation occurs, resulting in the formation of pyruvate which diffuses back out to the PCA tissue and reforms PEP. However, in NAD-ME plants (i.e., Spinifex) malate is first trans-aminated to asparatate which diffuses into the PCR (bundle sheath cell, Fig.2.7) and diffuses out as alanine following the decarboxylation and transamination reaction.

Bundle sheath 'leakiness' arises when CO_2 from decarboxylation in the bundle sheath 'leaks' back into mesophyll cells (Hatch et al., 1995). Details and consequences of the

phenomenon are provided in Hattersley (1976) and Farquhar (1983). Structural variation in C4 plants have been shown to influence bundle sheath leakiness (Farquhar, 1983). 'Leakage' is a 'branch' from the main path of carbon and allows isotopic discrimination to occur (Farquhar, 1983). Rubisco (which strongly discriminates against $^{13}CO_2$; von Caemmerer *et al*. 1997) becomes relatively more dominant in plants which have a greater degree of CO_2 leakiness, which results in greater discrimination against $^{13}CO_2$ (Kubasek et al., 2007).

Although no studies have specifically addressed the isotopic fractionation effects associated with the 'triodioid' variation of NAD-ME pathway, different degrees of leakiness have been identified in a variety of C4 grasses (including NADP-ME and NAD-ME subtypes). Kubasek *et al*. (2007) determined that NADP-ME subtypes (Maize and Sorghum) discriminated $^{13}CO_2$ to a lesser degree (~1 ‰) than the NAD-ME species (although these were *Amaranthacaeae*, not *Poaceae* like Spinifex). More specifically, Yeoh et al., (1981) investigated the Michaelis constant (i.e., affinity of the enzyme for substrate based on the rate constants within the reaction, K_m) of RuBisCo in different plant species. Yeoh et al., (1981) showed that K_m(RuBP) is greater for Spinifex (57 μmol) than it is for Sorghum (21 μmol). These differences are likely to affect isotopic discrimination although the details of how this occurs are not entirely understood at present.

Leakiness can also be influenced by temperature and irradiance (Fravolini et al., 2002; Kubasek et al., 2007). The Sorghum was cultivated in a controlled laboratory environment, whereas the Spinifex was collected in the field. These effects must also be considered. Background information of the isotopic fractionation effect of variations in CO_2 leakiness on terpene biosynthesis is scarce. It is possible that a novel effect is observed here in the isotopic fractionation of a monoterpene extracted from a triodioid NAD-ME C4 plant (Spinifex) that is different from a classical NADP-ME plant (Sorghum). In contrast to the monoterpene, naphthalene (**54**) shows the opposite trend. This is of less significance as naphthalene is of ambiguous biological source (carotenoid or from a lignin precursor). The monoterpene, on the other hand is source diagnostic, which makes the finding worthy of further research.

2.5 Results Summary

Table 2.13 summarises the main insights gained from SPME-CSIA ($\delta^{13}C$) of plant leachates.

Distinction of Photosynthetic Pathways

The $\delta^{13}C$ values of the SPME products were generally reflective of the photosynthetic pathway of the parent plant. For example, molecular constituents of the Marri (C3) leachate showed quite light $\delta^{13}C$ values typical of C3 plants (i.e., -22 to -35‰; O'Leary, 1988; Ehleringer et al., 1991; Ehleringer & Monson, 1993). Some compounds had exceptionally ^{13}C depleted signatures (e.g., -40 ‰ of 2,2,2,4-trimethyl-3-carboxy*iso*propyl pentanoic acid isobutyl ester, **58**), which may be due to isotopic fractionation on the analysis of this particular compound—evident in both present (i.e., established with standards) and previous work (Dias & Freeman, 1997) or simply the expression of a range of $\delta^{13}C$ values of varying degrees ^{13}C depletion, with compound **58** being particularly depleted.

The $\delta^{13}C$ value of products of the C4 plant leachates were much heavier with Spinifex and Maize products, both showing $\delta^{13}C$ values in the range -14.1 ‰ to -14.4 ‰. Sorghum products were slightly more ^{13}C depleted with $\delta^{13}C$ values of ~17.5 ‰, still quite typical of the $\delta^{13}C$ range of C4 plants (O'Leary, 1981; Hattersley, 1982; Krull et al., 2003). As would be expected, $\delta^{13}C$ values of individual SV and VOC components reflected relatively more ^{13}C depleted values than bulk biomass.

Insights gained from C3 Leaching Investigation

Isotopic data were obtained for approximately twice as many compounds in 150 day leachates than 100 min leachates. Surprisingly, the compounds determined were not the same at each time point. This variation may simply be due to biochemical changes occurring during leaching (e.g., hydrolysis) or the loss of some volatiles. Further investigation, e.g., sampling at more frequent time intervals would help investigate this issue further. An integrated analytical approach, such as the combined use of SPME, LLE, GCMS or direct LCMS would also be useful to determine whether the differences reflect those of the entire leachate or represent more specific differences in the subset of SVs and VOCs. Nevertheless, the isotopic values and associated trends evident for different biochemical classes (e.g., lipid < lignin < carbohydrate) are similar, suggesting there was no significant 'isotopic fractionation' over the 150 day leaching period. Both samples show increasing ^{13}C depletion with decreasing volatility.

Table 2.13 Summary of major findings of SPME-GCMS of plant leachates.

Sample	Plant	DOC (mgL^{-1})	No. GCMS Peaks	Major product	Dominant product class	Bulk (Raw Biomass) ‰	No. CSIA Peaks	Range $\delta^{13}C$ values ‰	Key trends
C3 100 min	Marri	760	31	8-phenyloctanoic acid & pentadecanoic acid	Carboxylic Acids	-30.7	10	-32.9 to -38.9	$\delta^{13}C$ Bulk>lignin>lipids In lipids, slight ↑ MW = ↓^{13}C
C3 150 day	Marri	1400	35	vitispirane	Terpenoids	-30.7	17	-28.4 to -40.0	$\delta^{13}C$ carb>bulk>lignin>lipids In lipids, slight ↑ MW = ↓ ^{13}C Biochemical transformation of lignin products
C4 150 day	Maize	1510	10	4-methyl-2,5-dimethoxy benzaldehyde	Terpenoids	-14.2	1	-12.5	n/a
C4 150 day	Sorghum	1195	11	cymene	Aromatics	-17.5	5	-17.6 to -20.7	Cymene ^{13}C depleted by 3.2‰ relative to bulk $\delta^{13}C$
C4 150 day	Spinifex	145	24	2-methoxy-3-(2-propenyl) phenol	Phenolics	-14.3	4	-15.0 to -18.5	Cymene only 0.7‰ ^{13}C depleted relative bulk $\delta^{13}C$

Source Assignment

SPME-CSIA can help improve the source assignment of leached VOCs and SVs. Many DOC compounds are simple primary substances such as carbohydrates (e.g., furans, furanones, pyrans and some esters) and lignins (e.g., hydroxyl and methoxy phenols) which reflect a close $\delta^{13}C$ relationship to the parent biomass (i.e., bulk $\delta^{13}C$ values). Differentials in the $\delta^{13}C$ value of some products reflect specific biosynthetic mechanisms. For example, 4-*iso*propyl-2,5-dimethoxybenzylacetate, a common product of the Marri leachate, was consistently enriched in ^{13}C relative to the bulk by 2 to 2.5‰ which identifies it as having a carbohydrate source.

The majority of the VOCs/SVs of the leachates derive from Secondary metabolism. Fatty acids and terpenoids showed the largest ^{13}C depletion, attributed to the kinetic isotope effect of the pyruvate dehydrogenase reaction (Melzer & Schmidt, 1987; Gleixner et al., 1993). Lignin, aromatic compounds and flavonoids were also generally ^{13}C depleted (see Table 2.11) due to the kinetic isotope effect of the shikimic acid pathway (Gleixner et al., 1993; Schmidt et al., 1995).

The degree of depletion depends on the metabolic distance of the product from key branching points along this pathway, as described in Section 1.3.3.2. Such pathways are complex and clear trends can be obscured by simultaneous metabolism and catabolism, of biochemical fractions (e.g. lipids) spanning a range of $\delta^{13}C$ values, as well as overlap of isotopic values (e.g. lignin and lipid products) making interpretation difficult. Further work on less complicated model systems (e.g., algal cultures, bacterial isolates) using SPME-CSIA would help elucidate specific pathways.

2.6 Conclusions

- SPME GCMS has been widely used to characterise the VOC and SV composition of various liquid samples, although analysis is typically limited to a semi-quantitative level due to quite variable recovery of some analytes (Kopinke et al., 1999; Zander et al., 2007).

- Minor modification of a previously developed SPME-GCMS method used to analyse the VOCs and SVs of freshwater leachates of terrestrial plants (Zander et al., 2005 & 2007) was successfully used to measure the $\delta^{13}C$ value of these compounds from several plant leachates.

- SPME-CSIA of several chemical standards (representative of NOM compounds) and plant leachates using a multi-fibre adsorption/injection strategy to increase the analytical sensitivity showed only negligible isotopic fractionation. This was consistent with lack of obvious isotope fractionation (<1.5 ‰) recognised in previous studies using SPME and GC-irMS to analyse toluene, methylcyclohexane, hexanol and C_{2-5} fatty acids (Dias & Freeman, 1997), chlorinated methanes, ethanes and ethenes (Hunkeler & Aravena, 2000)and MTBE (Gray et al., 2002) in aqueous samples and confirms the isotopic integrity of the SPME sample introduction approach.

- The sensitivity (even with four fibres) of SPME-CSIA allowed for the $\delta^{13}C$ analysis of only a limited number of VOCs and SVs, although GC resolution of these products – often a limitation in the CSIA of environmentally complex organic samples – was very high. $\delta^{13}C$ data was obtained for 18 VOCs/SVs in Marri (C3 plant) leachate, and a total of 8 VOCs/SVs in the Maize, Sorghum and Spinifex (C4 plant) leachates. It was established from these measurements that these products, in a very general sense, maintained the $\delta^{13}C$ integrity of the primary carbon source (i.e., $\delta^{13}C$ of C3 sourced products < $\delta^{13}C$ of C4 sourced products). In this respect, SPME-CSIA was demonstrated to offer some potential to enhance DOC characterisation based solely on molecular analysis.

- Useful information from the isotopic data which was obtained by SPME-CSIA included:

    1. Distinction in δ13C values of Secondary and Primary metabolites. An example is 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid (81, a component of lignocellulose) in 100 min Marri leachate, which was 10 ‰ lighter than the bulk biomass, a value consistent with a lignin source.

Another example is the source apportion of 1,6 dimethylnapthalene (43; -33.9 ‰), 6-methoxy-1-phenyl-3,4-dihydronapthalene (62; -32.2 ‰) and TDN (38, ~-32.7 ‰) to the same lipid-precursor in 150 day Marri leachate. Similarly, isopropyl-2,5-dimethoxy benzyl acetate (56), which was 13C enriched relative to the bulk Marri δ13C value, reflected a carbohydrate origin. These findings are consistent with more traditionally obtained δ13C data (e.g., Hayes, 2001; Grice & Brocks, 2011).

2. A noticeable ^{13}C depletion with increasing MW in C3 Marri leachate, consistent with trends reported previously for Eucalyptus leaf lipid extract (Ballentine et al., 1998) and probably indicative of a biosynthetic effect (Collister et al., 1994).

3. Elucidation of divergent δ^{13}C values of the monoterpene cymene (9) from Spinifex (an extreme triodioid variant NAD-ME plant, which had comparable δ^{13}C values of cymene and bulk material) and Sorghum (a classical NADP-ME plant cymene was ^{13}C depleted by 3.2 ‰ Cf. bulk)—ascribed to differing isotopic discrimination. NADP-ME subtypes (Sorghum) discriminate $13CO_2$ to a lesser degree (~1 ‰) than the NAD-ME species, (Kubasek et al., 2007) however the Michaelis constant of RuBisCo is greater for Spinifex (57 μmol) than it is for Sorghum (21 μmol; Yeoh et al., 1981). The differing affinity of the enzyme could result in the greater 13C depletion seen in Sorghum. Although the details of how this occurs are not entirely understood at present, such data may extend our understanding of the isotopic fractionation affects associated with the 'triodioid' variation of NAD-ME pathway as reflected in a monoterpene of non- ambiguous biological source.

Outcomes such as these suggest that SPME-CSIA could be a beneficial analytical tool if future developments can overcome current sensitivity issues. However, considering the relatively small proportion of the >>100 products VOCs/SVs detected by SPME GCMS) for which δ^{13}C measurement could be made the sensitivity of SPME CSIA needs to be

significantly improved for this technique to become a practically relevant method of DOC characterisation.

Chapter 3

Characterisation of Aquatic Microorganisms by Molecular and Stable Carbon Isotope Analysis of Phospholipid Fatty Acids

3.1 Overview

The analysis of PLFAs is a well-established ecological tool for investigating microbial communities, but it has not been widely applied to the biota of potable water systems. A few PLFA based studies have investigated the nature of pipeline biofilms (Keinanen et al., 2002; Keinanen et al., 2003a&b) and related changes in PLFA distributions such as carbon chain lengths, degrees of and cyclopropyl PLFA groups to adaptive changes in community structure. PLFA $\delta^{13}C$ signature can also reveal information about substrate utilisation and cycling (Cifuentes & Salata, 2001; Burke et al., 2003). Here, molecular and stable carbon isotopic analysis of the PLFAs from microbial-rich plant leachates and biofilms from distribution pipes throughout Australia have been studied, with particular interest in assessing the added value of the isotope analysis.

3.2 Background

3.2.1 Aquatic Microbiology

The NOM of aquatic ecosystems both influences and is influenced by microbial communities (Boon et al., 1996). The metabolites from microbial biodegradation of organic substrates can cause changes to the chemical and physical environment. Some, such as geosmin and MIB can also significantly impact the taste and odour aesthetics of drinking waters (Chen et al., 1997). Organisms that inhabit aquatic environments are involved in important biogeochemical cycling processes as illustrated in Fig. 3.1. Most of the aquatic biota is intimately linked to a host substrate, typically occur as a consortium of different species and often in the form of biofilms. Biofilms and other aquatic biota represent a quantitatively significant component of aquatic NOM and can entrain endogenous inorganic and organic compounds and other extracellular products.

Monitoring of microbial dynamics can help define the organic matter and nutrient fluxes of the water bodies (Donlan, 2002). Studying such organisms is important to gain an understanding of the carbon dynamics of aquatic ecosystems and to help maintain healthy drinking water systems. Notwithstanding the present knowledge of freshwater microbiology, there is still much to learn about species identity and function.

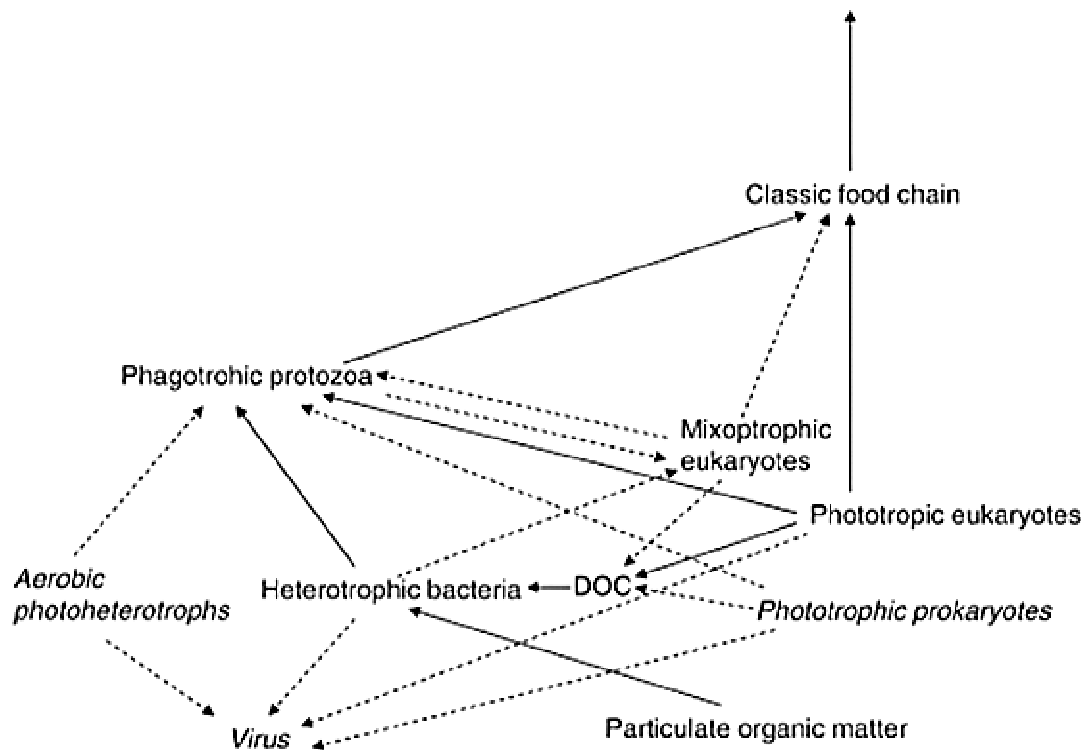


Figure 3.1 A schematic model of the microbial loop (from Azam et al., 1983).

Biological characterisation of aquatic environments presents significant analytical challenges due to the heterogeneity of environmental conditions and typically low biomass concentrations (Köster et al., 2003). Bacterial abundances determined by direct cell counts are in the order of ca 10^7 cells per ml of freshwater (David & Paul, 1989), which is equivalent to 2.2 ngC μm^{-3} using a bacterial conversion factor of 2.2 x 10^{-13} gC μm^{-3} and assuming a mean cell volume of 0.1 μm^{-3} (Bratbak & Dundas, 1984).

The ubiquitous occurrence of microbial organisms means they can impact water catchments, reservoirs, treatment processes and distribution systems. Their impact on drinking water systems can include outbreaks of disease, although a number of coinciding

circumstances are necessary for outbreaks of waterborne disease (Craun, 1991; Addiss et al., 1995; Hrudey & Hrudey, 2004; Wu et al., 2009b). From a public health perspective, the microorganisms of most concern are viruses (minute infectious agents), bacteria (e.g., *Shigella, Salmonella, Campylobacter, E. coli, Vibrio, Yersinia, Aeromonas, Streptobacillus, Mycobacterium, Legionella;* Thornsberry et al., 1984*;* Bagley, 1985*;* duMoulin & Stottmeier, 1986*;* Galbraith et al., 1987) and protozoa (*Giardia, Entamoeba, Cryptosporidium, N.fowleri*; Geldreich, 1991; Trolio et al., 2008). The biological monitoring and effective management of the drinking water system is therefore essential in order to remove, or at least minimise, the concentration of contaminating microorganisms.

A multiple barrier approach is often applied to ensure the provision of safe drinking water as summarised in Table 3.1 (O'Connor, 2002; LeChevallier & Au, 2004). Microbiology is a fundamental component of each of the barriers, separately relating to source water protection, treatment, distribution, monitoring and contingency. Source protection and treatment require detailed characterisation and understanding of the associated microbiology. Effective control of microbiological structure and function is important for the optimisation of treatment efficiency, and thus the reduction of health issues to consumers. In distribution systems 'chlorine residual' is maintained to limit biofilm growth.

Table 3.1 Multiple barrier approach to ensuring safe and reliable drinking water (O'Connor, 2002).

Barrier	Focus
Source Protection	Keeping raw water as clean as possible. Catchment area risk planning. Examine vulnerability of surface and groundwater sources. Determine major contaminant pathways.
Treatment	Removing or inactivating contaminants.
Distribution System	Protecting treated water from intrusion of contaminants. Ensuring appropriate free chlorine residuals throughout the system.
Monitoring program	Detecting concentrations of contaminants beyond acceptable limits. Returning systems to normal operation through automatic control devices.
Contingency responses	Responding to adverse conditions with well-rehearsed plans.

3.2.2 Microbial Ecology of Surface Waters

'Source water protection' involves the development and implementation of policies, plans and activities to prevent or minimise chemical pollution and high biologically supportive

nutrient loads on surface or groundwater sources of drinking water (Krewski et al., 2004; Ivey et al., 2006). Initial plans for new source waters for potable use include a risk assessment of potential contaminant sources, a catchment management plan and the development of appropriate monitoring methodologies (Ivey et al., 2006). Monitoring of groundwater resources, for example, would typically involve an investigation of private wells, recharge areas and whole aquifers (NRC, 2000).

Readily biodegraded organics can promote biological activity and potential eutrophy, increasing the chances for development of opportunistic viruses and other harmful microbes. Faecal or industrial chemical contamination and the development of eutrophic waters are major microbial concerns for source waters. Faecal wastes, from domestic pets, livestock, wildlife and humans, are a constant threat to many water catchments. Pristine water bodies are rare (Medema et al., 2003) and even remote surface water catchments where human activities are minimal are subject to some impacts, particularly in areas where seasonal wildlife colonisation occurs (Geldreich, 1972).

Potential for faecal or pathogen outbreaks has also increased with urbanisation and the intensification of domestic animal husbandry. The risk of pathogens increases near areas of extensive agriculture and below sewage outfalls of some cities (Kreisel, 1991). Rainfall can cause extensive runoff into rivers and water bodies above and below ground and has been directly correlated with increased occurrences of water borne outbreaks (Thomas et al., 2006). Several major outbreaks in industrialised countries over the last two decades have highlighted the importance of robust microbiological maintenance of drinking water catchments (Hrudey & Hrudey, 2004). For example, the 1993 Milwaukee Cryptosporidium outbreak, which caused illness in approximately 400 000 people (Mackenzie et al., 1994), was the result of drinking water contaminated with sewage effluent from a municipal waste water treatment plant.

Other biological issues frequently challenging the quality of Australian surface waters include thermophilic protozoa and cyanobacterial blooms. Thermophillic protozoa such as *N. fowleri* can inhabit drinking water supplies at consistently high temperatures (i.e., >40°C) and have caused fatal incidences of Primary Amoebic Meningoencephalitis (Trolio et al., 2008). Cyanobacterial blooms occur globally in eutrophic lakes (Hunter et al., 2009; Vareli et al., 2009; Zeng et al., 2009) and occasionally, in drinking water reservoirs (von Sperling et

al., 2008; Dzialowski et al., 2009). Cyanobacteria may produce toxic compounds, such as neurotoxic alkaloids or the more common hepatotoxic peptides thought to cause liver damage, gastroenteritis and hepatitis. Approximately half of Finnish lakes (Sivonen et al., 1990) and British water bodies (Codd & Bell, 1996) were found to be affected by blooms at toxic levels. In Western Australia, blooms occur periodically in the Peel Harvey Estuary, Harding Dam Reservoir and the Vasse Wonnerup and Torbay-Lake Powell systems (WAEPA, 2007; WQRA, 2009).

3.2.3 Microbial Ecology of Ground Waters

Ground water systems are typically oligotrophic, containing low DOC concentrations with slow turn-over rates. As such, microbial concentrations are generally low. Most subsurface bacteria occur as biofilms attached to soil particles (Durham et al., 1993; Kilham, 1994).

Contamination of ground water sources can occur when hydrological flows, which increase with rainfall, mobilise harmful organic substances (e.g., faecal matter). Septic tanks and other local wastewater storage and treatment facilities represent highly concentrated point sources of viruses, bacteria and parasites (Ritter, 2002). In the USA, septic tanks are the most common source of groundwater contamination (Yates, 1985). An infamous tragedy attributed to groundwater contaminated with *E.coli* (from livestock waste) occurred in the town of Walkerton in Canada in 2000, where seven people died and at least 2300 people became severely ill (O'Connor, 2002).

3.2.4 Biological Treatment Processes

Biological treatment of drinking and waste water such as primary biological treatment of sludge, biologically activated carbon and aquifer recharge are common treatment strategies to help remove NOM. Biologically activated carbon filtration harnesses microbes that are capable of removing the biologically available portion of DOC (Zhang et al., 1991) and is typically used following conventional treatment (Simpson, 2008), and often with other pre-treatment technologies e.g., ozone or UV irradiation (Buchanan et al., 2008). The nature and function of microorganisms in biological filters has attracted recent attention in efforts to optimise the degradation of algal metabolites and other organic substituents (Ho et al., 2006). However, the microbial ecology of some processes such as artificial groundwater recharge has only recently been studied at length (Kolehmainen et al., 2009).

As climate change intensifies and high quality source water becomes increasingly scarce, there is growing interest in the recycling of waste water for direct potable use as well as well as increasing use of grey water to help preserve primary source waters. Therefore, microbiological processes (e.g., aerated lagoons, activated sludge and slow sand filters) are a particularly important aspect of wastewater treatment and there has been rapid growth in such water treatment technologies (Lalbahadur et al., 2005; Le-Clech et al., 2006; Tuncsiper, 2009). Wastewater treatment processes are continually evaluated and effluents monitored for the occurrence of pathogens, pharmaceuticals, endocrine-disruptive products and other priority pollutants.

Whilst useful when applied in a controlled manner, biological activity can also impede some treatment strategies including membrane separation and bioreactors. Bacterial fouling is a serious problem for membrane filtrations (Le-Clech et al., 2006). This issue is discussed in greater detail in Chapter 4.

3.2.5 Biological Growth in Distribution Systems

3.2.5.1 Bacteria

Biofilms comprise heterogeneous and diverse microbial communities, but consist mostly of bacteria (Zacheus et al., 2001) including pathogens (Pavlov et al., 2004; Bauman et al., 2009). The interaction of bacterial pathogens such as *L. pneumophila*, *E. coli* and *S. typhimurium* can contribute to the development of biofilms (Hood & Zottola, 1997; Camper et al., 1998; Murga et al., 2001; Bauman et al., 2009).

Occurrence of pathogens in distribution pipelines is inversely related to chlorine residual and can deteriorate the quality of drinking water prior to it reaching consumers (Robertson et al, 2003). The concentration of free bacterial cells in the water of distribution networks increases with erosion, sloughing and abrasion of biofilm (van der Wende et al., 1989). This occurs commonly as a consequence of changes in flow velocity (Donlan, 2002).

Coliforms (Camper et al., 1991), *Legionella* (Rogers et al., 1994), *H. pylori* (Mackay et al., 1998), *E. coli* (Camper et al., 1996; Banning et al., 2003), *P. aeruginosa* (Banning et al., 2003) and *Mycobacterium* (September et al., 2004) have all been found in distribution systems (Bauman et al., 2009).

The majority of microorganisms that colonise the pipes in drinking water distribution are generally thought to be closely related to those in the system's source water (Camper et al., 1996), although some bacteria may be local to the pipes (Pepper et al., 2004; Storey & Kaucner 2009). Exogenous microorganisms can also enter the distribution system via cracks in pipelines or during routine maintenance of distribution systems (Bauman et al., 2009).

3.2.5.2 Fungi

There has been recent interest in occurrences of fungi in water systems, with positive identification in source waters, wastewater treatment plants, treated waters in distribution systems and biofilms (Hinzelin & Block, 1985; Rosenzweig et al., 1986; Doggett, 2000; Hageskal et al., 2009). Concentrations, determined by culture and cell counting of water sampled from 14 different water supply networks around Norway, were determined to be three times higher in surface-sourced water compared with groundwater (Hageskal et al., 2007). Species abundance and diversity are highly dependent on source water, temperature, treatment conditions and maintenance regimes of distribution systems (Geldreich, 1996).

3.2.6 Overview of Aquatic Microbial Analysis

3.2.6.1 Traditional Analysis

Several methods have been used to characterise aquatic biota and standard methods have been developed to test for the major indicator organisms of interest to water quality (Weiss & Hunter, 1939; Köster et al., 2003; van Vliet et al., 2009). Concentrations of high risk bacterial species, such as total coliforms and *E. coli*, are continually monitored to ensure the safe quality of drinking water (NHMRC, 2011). Naturally occurring coliform bacteria do not themselves cause disease, but are used to indicate whether other potentially harmful bacteria may also be present in the water. Faecal coliforms and *E. coli* are exclusively sourced from human and animal faecal waste sources (Rompre et al., 2002). Bacteriophages have also been proposed to be appropriate indicators of the biological quality of water (Havelaar, 1991; Nasser & Oman, 1999; Storey & Ashbolt, 2003a; Storey & Ashbolt, 2003b; Langmark et al., 2005).

Traditional cultivation techniques include plating and direct counting by microscopy (Skinner et al., 1952), but these are selective and in general only reveal a quantitatively small bacterial component of the microbial consortium (Ward et al., 1990; Ranjard et al.,

2000). Generally less than 0.5 % of the microbes inhabiting source waters are culturable (Miettinen et al., 1997), yet cultivation based techniques still remain an integral part of routine drinking water monitoring regimes (Fewtrell & Bartram, 2001).

Modified approaches of cell culture target protozoa and cultivation in host cells target viruses (Slifko et al., 1997; Köster et al., 2003). Chromogenic enzyme-based detection methods commonly referred to as 'defined substrate methods' represent another improvement on the traditional methods and are suitable for the identification of target bacteria. The Colilert-18® method, for example, is now used as a test for coliforms and *E.coli* in potable water (Fricker et al., 1997; Edberg et al., 1988; Chao et al., 2004). Because of the severe limitations of cultivation methods (Ward et al., 1990; Amann et al., 1995) they are now being increasingly complemented with culture independent methods.

3.2.6.2 Molecular Analysis

More sophisticated molecular approaches have also been used to explore microbial structure, and to investigate biological incidents or issues in greater detail. Such techniques typically target the detection and identification of nucleic acids by complimentary hybridisation, restriction, amplification and cloning methods (Köster et al., 2003). Cloning or amplification of genes is often used to sequence individual fragments or a family of fragments all ending with a given type of nucleotide. Sequencing can be complemented with database correlations, however, the Deoxyribonucleic Acid (DNA) of living and dead bacteria cannot be distinguished (Wilson, 1997). To address this, emerging methods e.g., reverse transcriptase Polymerase Chain Reaction (PCR), utilise messenger Ribonucleic Acid (mRNA) which has a half-life of a few minutes, thereby facilitating the distinction between living and dead bacteria (Köster et al., 2003).

Other contemporary techniques for microbial community structural analysis include:

- Community level physiological profiling (CLPP; Mayr et al., 1999).
- PCR amplification-based techniques such as Denaturing Gradient Gel Electrophoresis (DGGE; Ramsey et al., 2006).
- Phospholipid fatty acid (PLFA) analysis (e.g., White, 1998).

Some cellular lipids can be diagnostic of specific microbial species. Taxonomically valuable biomarkers such as PLFAs can be extracted directly from biological and environmental

samples to provide a lipid fingerprint of the microbial community and often also information about function, physiology and abundance of community members. PLFA profiling was reported to be more useful in assessing the effects of treatment regimes on the microbial communities of soils than CLPP or PCR based molecular methods (Ramsey et al., 2006). In some instances, highly sensitive PLFA profiling has been able to resolve treatment effects on microbial dynamics where CLPP could not (Baath et al., 1998; Bossio et al., 2005). In many cases, multi-method studies have provided little information above that from PLFA profiling alone (Ellis et al., 1995; Ritchie et al., 2000; Widmer et al., 2001; Ritchie & Perdue, 2008). PLFA profiling has the additional advantage of being a relatively inexpensive and straightforward technique, particularly compared with molecular approaches such as DGGE-PCR (Desnues et al., 2007; Ivnitsky et al., 2007).

PLFA characterisation provides a lipid profile of entire microbial communities, and the most commonly detected PLFAs have multiple microbial sources. Hence, care needs to be taken to avoid over interpretations of product-precursor relationships. Furthermore, most PLFA databases have largely been established through analysis of cultivated microbial isolates, thus suffering the same limitations as other culture-dependent methods. As such, the resolution of sub-community dynamics provided by PLFA profiles can be crude, and statistical treatment of PLFA data is often necessary for meaningful interpretations. Multivariate statistical analyses, including modified Principal Component Analysis (PCA), have been widely applied to PLFA studies (Moll & Summers, 1999; Ritchie et al., 2000; Canuel, 2001; Keinanen et al., 2003b; Drenovsky et al., 2004; McKinley et al., 2005) and have successfully correlated the data to many different variables.

3.2.7 Phospholipid Fatty Acid Analysis

3.2.7.1 Background to PLFAs

Phospholipids are major biochemical components of the cytoplasmic cell membrane of microorganisms. They are asymmetric lipid molecules comprising a phosphate head and a fatty acid tail of generally less than 20 carbon atoms (Fig. 3.2) that form a bi-layer, with the hydrophilic head projecting towards the outer surface of the membrane and the hydrophobic tail buried in the interior—enabling the cell membrane to function as a diffusion barrier with specific permeability for different substances. PLFAs are present in all bacterial cells in roughly equivalent amounts and rapidly degrade after cell death (Zelles &

Bai, 1993). Their analyses therefore provides an accurate indication of living cells at the time of sampling (White et al., 1979; Pinkart et al., 2002; Petsch et al., 2003), and their compositional dynamics may be reflective of biological or abiotic environmental processes (Zelles et al., 1995; Frostegård et al., 1996; Bossio & Scow, 1998).

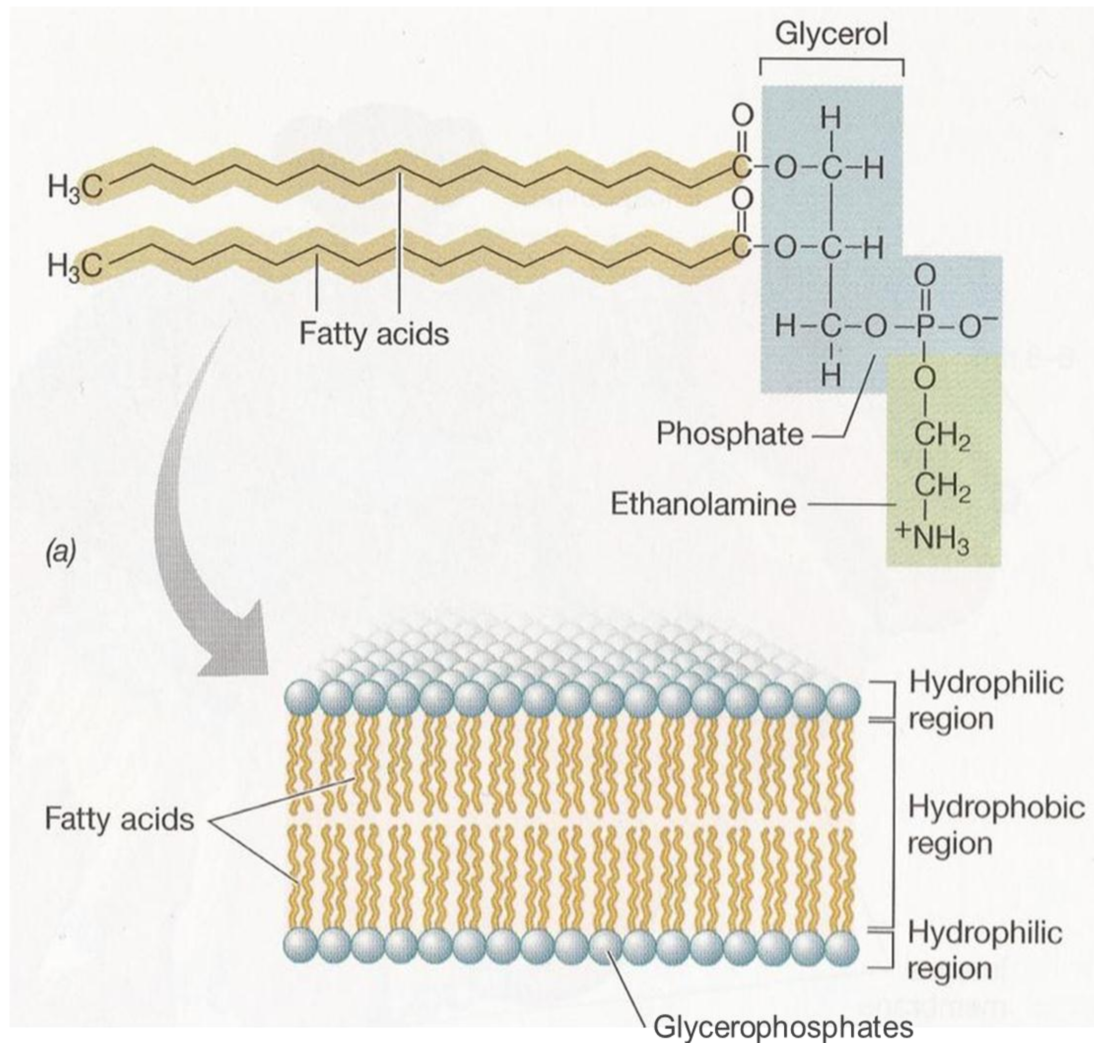


Figure 3.2 Molecular model of phospholipid bilayer of the cell membrane (from Madigan et al., 2011).

Most PLFA compounds are rarely exclusive to any one group of organism (Zelles, 1999a). Nevertheless, several individual or small classes of PLFAs have been assigned to particular types or groups of bacteria (White et al., 1979; LeChevallier, 1989; White, 1998; Green & Scow, 2000). PLFA differences between Gram-positive and Gram-negative bacteria arise from distinctions in cell wall structure. Gram-negative bacteria have an outer membrane while Gram-positive bacteria typically, do not.

Gram-positive bacteria make up the phylum *Firmicutes* and also include the genera *Clostiridium* and *Bacillus*. Most bacteria which are pathogenic in humans are Gram-negative including *E.coli, Salmonella, Shigella, Legionella, Pseudomonas, Heliobacter* and also *Cyanobacteria* (Madigan et al., 2011). Gram-negative bacteria have been observed to adapt to low nutrient conditions so can still threaten drinking water distribution systems of limited nutrient supply (Christine et al., 1992).

PLFA classes, defined by extent and positions of double-bonds and branching, can distinguish different types of microbial species (Ratledge & Wilkinson, 1988). Straight-chain fatty acids are common to most microbial species, but those > C_{20}, which are not so prevelant, are mainly attributed to eukaryotes. Branched fatty acids are characteristic of Gram-positive bacteria (Haack et al., 1994). Monounsaturated PLFAs are widely regarded as indicative of Gram-negative bacteria (e.g., White et al., 1996; Peacock et al., 2001), more specifically when unsaturation occurs at the 7th or 9th carbon from the aliphatic end can be used as actual diagnostic biomarkers for Gram-negative bacteria (Zelles, 1999a).

Cyclopropyl and hydroxyl substituted PLFAs are also generally restricted to Gram-negative bacteria (O'Leary & Wilkinson, 1988). Table 3.2 illustrates the major PLFAs used as indicators for particular microorganisms as reported by Zelles (1999a) and others (Zelles et al., 1992; Frostegård et al., 1993; Frostegård & Baath, 1996; Frostegård et al., 1996; Zak et al., 1996; Zelles, 1999b).

Table 3.2 Fatty acids commonly used as indicators of taxonomic groups (Zelles et al., 1992; Frostegard et al., 1993; Frostegard & Baath, 1996; Frostegard et al., 1996; Zak et al., 1996; Zelles., 1999a).

Microbial Group	PLFA Biomarker
Gram +ve bacteria	*i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, *a*17:0, *i*20:0 & *a*20:0
Gram -ve bacteria	cy17:0 & cy19:0, Monounsaturated PLFAs
Fungi	18:2(9,12), 18:1ω9*t* & 16:1ω5
Actinomycetes	10Me18:0
Sulfate reducing bacteria	*i*17:1ω7c& 10me16:0
Methanogens	16:1ω8 (Type I) & 18:1ω8 (Type 2)

The following convention is used for fatty acid nomenclature: *px:y(z)*

Where (*x)* designates the total number of carbon atoms (e.g., 16:0 in the case of hexadecanoic acid). The number after the colon (*y*) indicates the degree of unsaturation e.g., 16:1 for mono-unsaturated hexadecenoic acid. The (*z*) that follows indicates the position of double bonds, relative to the carboxyl end. Alternatively, ω is used to indicate the member (*z*) relative to the aliphatic terminal end. A prefix (*p)* is often used to describe *anteiso (a), iso (i)* or cyclopropyl (cy) terminal branching and non-specific branching (Br), respectively.

3.2.7.2 Application of PLFAs to the Study of Aquatic Systems

PLFA analysis has been used for quantitative and qualitative analysis of community dynamics and composition, in a wide range of ecosystems, including;

- Soil microflora (McKinley et al., 2005; Ramsey et al., 2006; Greenwood et al., 2009).
- Aquatic biota (Guckert et al., 1992; Scholz & Boon, 1993; Banowetz et al., 2006; Jin & Kelley, 2007).
- Flooded agricultural ecosystems (Bai et al., 2000; Shimizu et al., 2002).
- Characterisation of groundwater communities (Glucksman et al., 2000).
- Biological treatment of wastewaters (Liu et al., 2000; Quezada et al., 2007).
- Potable water treatment and distribution systems (Moll et al., 1998; Smith et al., 2000; Keinanen et al., 2002; Kontro et al., 2006; Yu et al., 2009).
- DOC quality (Bossio et al., 2006).

The insightful outcomes of these studies suggest PLFA analysis offers considerable potential to understanding the biological parameters of drinking water systems.

3.2.7.3 Application of PLFAs to the Study of Drinking Water Systems

PLFA analysis of water systems has been mostly concerned with filtration and biofilm issues. For example, PLFA analysis has been used to provide an early indication of the occurrence of pathogenic bacteria in biofilms (White et al., 2003) and the extent and consequences of chlorine residual in distribution systems (Smith et al., 2000).

Keinanen et al. (2002) using PLFAs reported that phosphate amendments increased the proportion of Gram-negative bacteria (i.e., 16:1ω7c and 18:1ω7c) in distribution biofilms

and also that microbial community structure changes with water temperature and treatment.

PCR treatment of the PLFA data from biologically active drinking water filters by Moll and Summers (1999) provided baseline information about microbial biomass, community structure profiles and their dynamics. The outcomes of these studies helped refine source water management and treatment strategies (e.g., ozonation, contact time, backwashing with disinfected water) to minimise NOM levels and DBP precursors. Moll et al. (1999) also reported an increasing gradient in Gram-negative bacteria and microeukaryotes, at the expense of Gram-positive bacteria and sulfate-reducing bacteria, with decreasing biofilter operation temperature.

Yu et al. (2009) recently used PLFA profiles and PCA analysis to study the impact of organic matter concentrations on the microbial community of drinking water biofilters. By examining the diversity of bacterial biomarkers on drinking water the biological composition of the biofilters were observed to differ when exposed to glucose (greater response on biota) or acetate substrates, attributed to organic loading and media depth (Yu et al., 2009). PCA analysis of the PLFA identified carbon source as the principal factor determining total PLFA variance.

3.2.8 Stable Carbon Isotope Analysis of PLFAs

As described in Chapter 1, stable carbon isotope analysis of biomarkers such as PLFAs may also provide a useful way of tracing substrate utilisation and carbon dynamics. The $\delta^{13}C$ measurement of individual lipid biomarkers is a mature technology widely applied in a variety of fields. The stable isotopic composition of PLFAs is frequently used to help characterise extant microbial populations, and offers wide utility to microbial ecology research (Cifuentes & Salata, 2001; Boschker & Middelburg, 2002; Zhang et al., 2003; Hallmann et al., 2008; Cowie et al., 2009). These analyses can improve our understanding of complex microbial communities and biogeochemical processes. CSIA of PLFAs by GCirMS can be conducted after a single, simple derivatisation step (using alkaline methanolysis). This methylation process adds an extra carbon atom to the original PLFA compound, but the associated $\delta^{13}C$ fractionation is systematic and easily corrected (Coffin et al., 1989; Abrajano et al., 1994; Abraham et al., 1998).

Many factors can influence the $\delta^{13}C$ values of PLFAs, some of the main influences including carbon substrate (Hayes, 1993; Abraham et al., 1998; Boschker et al., 1998; Hanson et al., 1999; Kramer & Gleixner, 2006), biological assimilation and lipid biosynthesis (Blair et al., 1985; Boschker & Middelburg, 2002; Cowie et al., 2009;). Their measurement can therefore, complement molecular analysis with potential to yield further insight into the study of microbiological substrate and system dynamics (Abraham et al., 1998; Cowie et al., 2009).

3.2.8.1 Carbon Substrate

The close similarity of the $\delta^{13}C$ composition of bacterial biomass and its primary carbon substrate has been well documented (Blair et al., 1985; Coffin et al., 1989; Demmelmair et al., 1997; Coffin et al., 2001). Many microbes show metabolic preference to easily assimilable organic compounds (Evershed et al., 2006; Kramer & Gleixner, 2006; Kramer & Gleixner, 2008; Nottingham et al., 2009). The substrate preference of soil microflora (i.e., Gram-positive and Gram-negative bacteria) has been well researched.

Kramer & Gleixner (2006) observed that PLFAs (16:1, 17:1, 18:1, *i*15:0, *a*15:0, 16:0, 17:0cy, Br-Me-17:0, 18:0 and 19:0cy) of soil microbes exposed to C4 vegetation became more enriched in ^{13}C (by ~10 ‰) over a timeframe of 39 years. Furthermore, Gram-negative bacteria, known for their opportunism towards easily assimilated C sources (Paul & Clark, 1996), showed a greater preference for the new plant substrate than Gram-positive bacteria which have a capacity to utilise more recalcitrant carbon materials (Burke et al., 2003).

The use of ^{13}C-labelled substrates has helped identify the microbial species responsible for some mineralisation processes, and expanded the CSIA application of PLFAs to ecological studies (Créach et al., 1999; Evershed et al., 2006; Dornbush, 2007; Stemmer et al., 2007; Kramer & Gleixner, 2008; Williams & Xia, 2009). However, ^{13}C-labelled substrates need to be used with caution as they can contaminate irMS instruments (which are typically used for natural abundance measurement) such that prolonged 'memory' effects distort subsequent analysis. Few studies have addressed the natural abundance measurement of $\delta^{13}C$ fractionation of PLFAs specifically, with respect to their carbon substrates (Abraham et al., 1998; Schouten et al., 1998; Cifuentes & Salata, 2001; Bouillon & Boschker, 2006) somewhat limiting their interpretive value.

3.2.8.2 Assimilation and Metabolism

Stable carbon isotopic fractionation of bulk organic matter and PLFAs with respect to source is largely due to metabolic influences (Boschker & Middleburg, 2002). $\delta^{13}C$ of bacterial lipids are typically depleted by 5 to 13 ‰ relative to $\delta^{13}C$ of the parent biomass and its organic substrate (Monson & Hayes, 1982; Blair et al., 1985; Boschker et al., 1999). The $\delta^{13}C$ of PLFAs of heterotrophic organisms are typically less depleted (by 3 ‰) relative to bulk cellular biomass (Blair et al., 1985; Teece et al., 1999; Hayes, 2001). Greater isotopic fractionation is evident in autotrophic organisms, largely due to fixation of inorganic carbon (Hayes, 2001; Londry et al., 2004) and also influenced by cell size, geometry and environmental factors such as seasonality (Goericke et al., 1994; Bidigare et al., 1997; Grice & Brocks, 2011).

The $\delta^{13}C$ value of PLFAs can help identify metabolic processes (Abraham et al., 1998). By investigating the $\delta^{13}C$ gradient of PLFAs of major bacterial and fungal species cultivated with four isotopically distinct carbon sources (glucose -10.10 ‰; mannose -23.74 ‰; glycerol -28.57 ‰; lactose -27.35 ‰), Abraham et al. (1998) helped identify related assimilation effects. For example, one of the glucose treatments exhibited a relatively ^{13}C depleted 16:0 and the mannose treatment showed consistently more ^{13}C enriched PLFAs.

Molecular and $\delta^{13}C$ analysis of PLFAs was used by Augspurger et al. (2008) to investigate stream microbial biofilm food webs. The carbon isotope signature of sucrose derived from C3 (-24.01 ‰) or C4 (-11.36 ‰) plants was traced in different trophic levels via PLFAs representative of bacteria (terminally branched PLFAs), algae (di- and poly- unsaturated C_{16} PLFAs) or protozoans (highly polyunsaturated PLFAs $>C_{18}$) present in 2 and 6 week old biofilms. The $\delta^{13}C$ of PLFAs of bacteria and algae were combined with more traditional analysis – e.g., microscopic enumeration methods; chlorophyll *a* and sucrose consumption determinations; and statistical analysis, to provide insight into the grazing and competition controls on the microbial community structure the biofilms (Augspurger et al., 2008). A limitation however, was the lack of less common PLFAs specifically diagnostic of many microbial sub groupings, particularly protozoans.

3.2.8.3 Biosynthetic Effects

Isotopic fractionation occurs at key carbon branch points of lipids during biosynthesis (Monson & Hayes 1980; Fang et al., 1993; Abrajano et al., 1994; Schouten et al., 1998; Grice et al., 2008; Zhou et al., 2010; Grice & Brocks, 2011).

These isotopic effects have been used to establish biosynthetic pathways, investigate enzymatic catalytic mechanisms and estimate fluxes at various metabolic branching points (e.g., Monson and Hayes, 1980). For instance, *anteiso*-alkanes (3 ‰ to 4 ‰) and *iso*-alkanes (0 to 2 ‰) extracted from leaves of tobacco (*Nicotiana tabacum*) showed differing ^{13}C enrichments compared to *n*-alkanes, reflecting different biochemical sources (Grice et al., 2008; Zhou et al., 2010). Precursors for odd- and even- carbon-numbered *iso*-alkanes are *iso*-butyryl-CoA (a C_4 unit derived from valine) and α-methylbutyryl-CoA (a C_5 unit derived from *iso*-*l*eucine), respectively, with high MW extension of each series by similar by C_2 elongation and decarboxylation (Fig. 3.3).

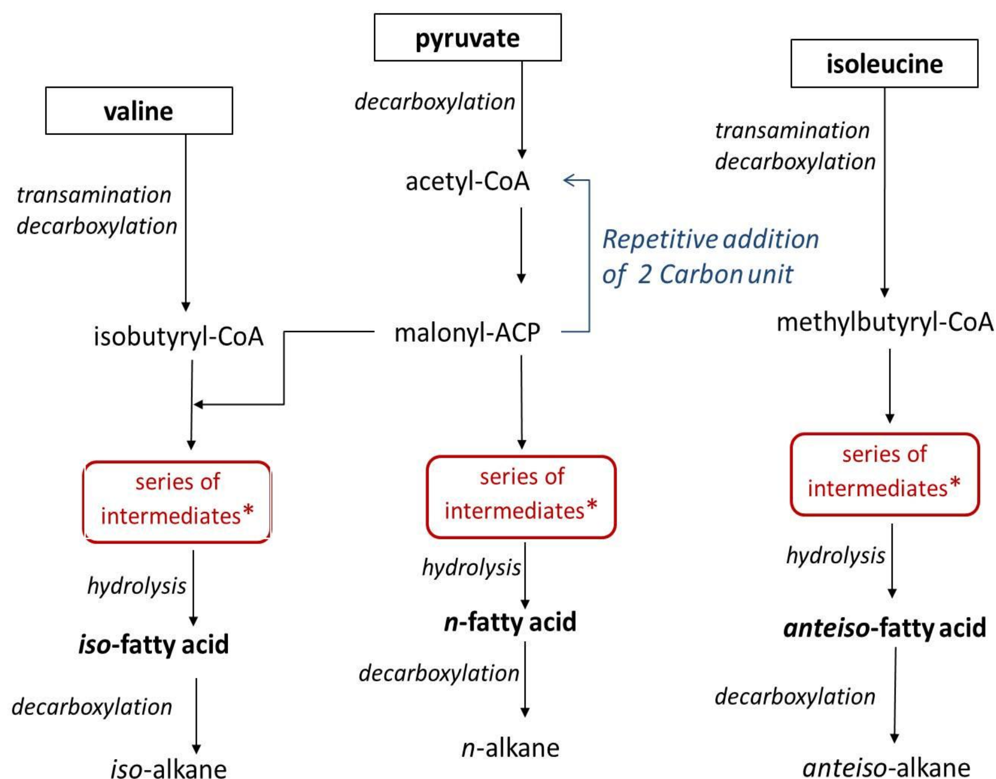


Figure 3.3 Biosynthesis of straight chain, anteiso-branched and iso-branched fatty acids. For further information refer to Grice et al. (2008) and Zhou et al. (2010).

$\delta^{13}C$ analysis of PLFAs has been able to differentiate the isotopically distinct carbon sources of different heterotrophic communities (Boschker et al., 2001; Cifuentes & Salata, 2001), including resolution of heterotrophic from autotrophic processes (Pancost & Sinninghe Damsté, 2003). ^{13}C-labelled compounds have also been cleverly used to identify PLFA biomarkers associated with microbes responsible for the utilisation of particular substrates. For example, Boschker et al. (2001) demonstrated preferential uptake of ^{13}C-labelled acetate by PLFAs with even numbers of carbon atoms (16:1ω7*c*, 16:0, 18:1ω7*c*), and of ^{13}C-labelled propionate by PLFAs with odd numbers of carbon atoms (*a*15:0, 15:0, 17:1ω6, 17:0).

3.2.9 Aims of Research

The present research aims to further investigate the feasibility of measuring the ^{13}C values of individual PLFAs in drinking water systems, particularly with respect to source waters and distribution systems, and assess the microbial characterisation value this provides. Microbial communities associated with laboratory-leached vegetation (i.e., leachates from Chapter 2) and a suite of biofilms collected from potable water distributions from a range of Australian regions were separately studied. The isotopic measurements of individual PLFA compounds from microbial communities of distribution system biofilms have not previously been analysed.

3.3 Experimental

3.3.1 Samples

Two different sets of samples were analysed. The first set was the vegetation leachates described in Chapter 2. The second were biofilms from potable and recycled water distribution systems from around Australia, made available from a previous CRC-WQT study (detailed in Section 3.3.1.2).

3.3.1.1 Microbiota from Vegetation Leachates

Marri, Maize, Sorghum and Spinifex leachates were prepared as described in Sections 2.3.2 and 2.3.4. Briefly, the leaching process was performed over a 150 day duration, after which samples were filtered through a binder-free 90mm-diameter GF/F borosilicate filter (nominal pore size 0.7 mm).

3.3.1.2 Biofilms from Water Distribution Systems

A suite of 35 biofilm samples used previously in CRC WQ&T research project 2.0.2.5.0.7 *'Understanding the Growth of Opportunistic Pathogens within Distribution Mains'* and outlined in Storey et al. (2008) and Storey & Kaucner (2009) was made available for the present study. General details about the types of distribution systems from which biofilms were collected and sampling times are shown in Table 3.3. They were obtained from distribution systems of five adjacent drinking and recycled waters (Sites A, B, C, D and E) from different Australian localities reflecting different climatic regions. Site E was only sampled in summer, and Site A only in winter whereas both summer and winter samples were collected from the other three sites.

Table 3.3 General details of biofilm sample suite from potable and recycled systems. **=not chlorinated.

Site	Biofilm sampling date/ number of sampling instances		Treatment at site	Pipeline material details
Potable A	June 2005 (Winter only)		n/a	CLDI
Potable B	June 2005	Feb 2006	n/a	CLDI
Potable C	July 2005	March 2006	n/a	Copper/PVC
Potable D	March 2006 (Summer only)		n/a	PVC/CLDI
Potable E	June 2005 (Winter only)		n/a	AC
Recycled A	June 2005 (Winter only)		Tertiary	uPVC
Recycled B	June 2005	Feb 2006	Tertiary	CLDI
Recycled C	July 2005	Feb 2006	Secondary	PVC
Recycled D	July 2005	March 2006	Secondary **	PVC
Recycled E	March 2006 (Summer only)		Secondary	CLDI/cast iron

Samples were stored and transported frozen until lyophilisation (conducted upon receipt at Curtin University), which was performed immediately preceding lipid extraction. Nutrient and water quality characteristics of distributions systems from which the samples were taken are presented in Table 3.4. Photographs of two separate recycled water distributions systems are presented in Fig. 3.4. The visual difference in biofilm growth is immediately apparent.

Figure 3.4 Photos of biofilms from left, Site A tertiary treated recycled water (Biofilm 12) and right, Site C secondary treated recycled water (Biofilm 150). Photos from Storey & Kaucner (2009).

3.3.2 Sample Extraction and Derivatisation

3.3.2.1 Lipid Extraction of Biomass

The filtered residue of the leachates was ultrasonically extracted using a modified Bligh-Dyer extraction method (Bligh and Dyer, 1959). Biofilm material (12 to 54 mg) was lyophilised in a Dynavac Freeze-dryer equipped with a FDA/3RH refrigerator. These leachate and biofilm materials were then prepared using the same experimental procedures. Samples (~2 mg) were sonicated in a single-phase mixture of chloroform:methanol:phosphate buffer solution (1:2:0.8 v/v/v; 30 mL). This mixture was then centrifuged (3500 r.p.m., 5 min, centrifuge 5810, Eppendorf) and the organic supernatant removed by addition of water and separation of the aqueous phase. The total extract was reduced to incipient dryness under a gentle stream of nitrogen gas. All glassware used for lipid analysis was heated at 500°C for at least 6 h prior to use. Analytical grade solvents and chemicals were used and stored at ≤4°C.

3.3.2.2 Fractionation of Lipid Extracts

Extracted lipids were separated into neutral-, free- and phospho-lipids using solid phase extraction (SPE) cartridges packed with 0.5 g silica (Supelclean LC-Si, Supelco). The SPE cartridges were preconditioned with methanol (2 mL) and then chloroform (2 mL). Neutral lipids were first eluted with chloroform (2 mL), free lipids with acetone (2 mL), and lastly

phospholipids with methanol (2 mL). Procedural blanks were run alongside each daily batch of samples to monitor for any contamination.

3.3.2.3 Methylation of PLFAs

The phospholipids were reconstituted in methanol-toluene (1:1, v/v, 0.2 mL) and methyl esterified by addition of a potassium hydroxide-methanol mixture (0.2M, 0.5 ml) at 75°C. On cooling to room temperature, fractions were neutralised with acetic acid (0.2M, 0.5 mL). Chloroform (1 mL) and deionised water (1 mL) were added and the two phases separated. The remaining aqueous phase was re-extracted, and the two chloroform phases combined. Nonadecanoic acid (19:0) methyl ester (200 µl; 15 ng μL^{-1}) was added as an internal standard for quantitative GCMS purposes.

3.3.3 Molecular Analysis of PLFAs by GCMS

The PLFA fractions were analysed by GCMS using a HP 5973 MSD interfaced to a HP 6890 GC. The GC was fitted with a 60 m × 0.25 mm i.d. fused silica column coated with 0.25 µm 5 % phenyl-methylpolysiloxane (DB5, Agilent J&W). The GC oven was programmed from 70 to 310°C at 3°C min^{-1} with initial and final hold times of 1 and 15 min, respectively. Samples were injected (split/splitless injector) via a HP 6890 series autosampler used in pulsed-splitless mode. UHP He was used as carrier gas at a constant flow rate of 1.0 mL min^{-1}. PLFA samples were analysed simultaneously in both full scan (*m/z* 50–550 at approximately 2 scan s^{-1}) and selected ion (*m/z* 74, 199, 250, 268, 264, 278, 294, 298, 312, 326; 20 ms dwell time) monitoring modes with standard MS conditions including an electron energy of 70 eV, filament current of 200 µA and a source temperature of 250°C.

The GCMS was operated with Chemstation software. Identification of compounds was achieved by GC retention time and MS correlation to library (NIST 05, Wiley 275) and published data (Keinanen et al., 2003b; Kontro et al., 2006) and also compared to retention times and spectra of a 26 component Bacterial Acid Methyl Ester (BAME).

Table 3.4 Nutrient concentrations and water quality of distribution systems from which biofilms were sampled. Water quality values are average from 8 weeks of water sampling prior to sample collection.

Site#	ID	Biofilm ID	Temp °C	pH	Free Cl ~ ($mg\ L^{-1}$)	Cond $\mu S\ cm^{-1}$	Total N ($mg\ L^{-1}$) ** = TKN * = Organic N	Total P ($mg\ L^{-1}$) ^ = Sol. Reactive P	TOC ($mg\ L^{-1}$) * = DOC
A w	Potable	7, 9	15.7	8.00	0.05	19.46	0.50	0.0 ^	2.74 *
	Recycled	10, 11, 12	16.3	7.83	0.30	86.13	6.15	0.11^	6.06 *
B w	Potable	25, 26	13.6	8.21	0.59	100.00	0.10	0.00	2.00
	Recycled	28, 29, 30	10.8	7.75	0.05	595.00	19.56	3.33	7.30
B s	Potable	128	21.0	8.36	0.65	98.71	0.60	0.09	1.57
	Recycled	130, 131, 132	20.3	8.12	0.22	201.93	2.45	0.34	3.14
C w	Potable	43, 45	14.6	8.16	0.02	78.50	0.20	0.03	2.35
	Recycled	47, 48	16.2	7.64	0.07	980.00	27.56	5.30	21.94
C s	Potable	145, 147	20.9	8.08	0.02	No data	1.92	0.39	1.81
	Recycled	150	22. 9	7.71	0.02	No data	21.50	6.48	23.06
D w	Potable	62, 63	27.7	6.82	0.26	No data	0.27 **	0.01	2.31
	Recycled	64, 65, 66	27.6	7.35	0.08	536.33	14.04*	3.09 ^	40.72
D s	Potable	181, 182, 183	31.4	6.83	0.71	No data	0.31 **	0.01	2.76
	Recycled	185	32.0	6.89	n/d	No data	4.0 **	2.93	13.40
E s	Potable	163, 164, 165	22.3	No data	0.39	639.85	0.26 **	No data	No data
	Recycled	166, 168	23.6	7.27	0.92	1730.00	12.08	2.54	9.68

~ Free chlorine is calculated as the total sum of hypochlorous acid (HOCl) and hypochlorite ion (OCl-). # w = winter, s = summer.

(commercial standard, Cat no: 47080-U, Supelco) and a 37 component Fatty Acid Methyl Ester (FAME) (commercial standard, Cat no: 4788-5U, Supelco, PA, USA). Relative amounts of PLFA products were determined by area integration of total ion chromatograms (TIC) peaks of the consistently more abundant PLFA products.

The concentration of each fatty acid was determined relative to the standard and was calculated as:

µg individual fatty acid per sample = $(P_{FAME} \times \mu g_{STD}) / (P_{STD})$ .... **Eqn. 3.1**

Where P_{FAME} and P_{STD} are the peak area of individual PLFA and internal standard peaks respectively; μg_{STD} is the concentration of the internal standard (200 µL, 15 ng μL^{-1} solvent).

3.3.4 Statistical Analysis of PLFA Data

Multivariate statistical analysis was performed using PRIMER-e software (Plymouth Marine Laboratory). The complete data set is shown in the Appendix A. ANOVA analysis was performed using IBM® SPSS® Statistics 21 statistical software, the analyses and discussion are shown in Appendix B.

3.3.5 Isotope Analysis of PLFAs by CSIA

A general description of the CSIA method can be found in Section 2.3.5.4. Methylation adds one carbon to each PLFA which contributes to the stable carbon isotopic value of the methyl ester product, thus the measured $\delta^{13}C$ value needs appropriate correction. The $\delta^{13}C$ of the added methyl group is assumed to be same as the methanol used in the derivatisation reaction (Rieley, 1994). Hexadecanoic fatty acid (16:0) was derivatised batch wise and run by CSIA before and after methylating, thus enabling correction using the following equation after Abrajano (1994).

$\delta^{13}C_{FAME} = (x)\delta^{13}C_{FA} + (1\text{-}x)\delta^{13}C_{CH3OH}$ .... **Eqn. 3.2**

Where:

$\delta^{13}C_{FA}$ is the isotope ratio of the original fatty acid

$\delta^{13}C_{FAME}$ is the measured isotope ratio of the free fatty acid

$\delta^{13}{}_{CH3OH}$ is the measured isotope ratio of methanol

x is the fractional carbon contribution of the free fatty acid to the ester (i.e., 16/17 for hexadecanoic acid).

Any isotopic fractionation from the extra carbon added during derivatisation introduced can thus be accounted for (Rieley, 1994). It is assumed that the $\delta^{13}C$ of the added methyl group is the same as that for the methanol used in the derivatisation reaction.

Uncompromised $\delta^{13}C$ measurement of individual products requires baseline GC resolution. This is because the *m/z* 45 peak elutes slightly earlier than the *m/z* 44 peak (refer Chapter 1 for more detailed discussion). Where partial co-elution occurs, only the average value of the combined peaks was determined.

3.4 Results and Discussion

3.4.1 Molecular Analysis of PLFAs from Biota of Plant Leachates

3.4.1.1 Analytical Results

A partial TIC from the GCMS analysis of Marri PLFAs is shown in Fig. 3.5. All plant leachates showed a wide distribution of C_{13} to C_{20} PLFAs, including straight-chain, mid chain and terminally branched saturated and monounsaturated PLFAs (Table 3.5). The concentrations of the PLFA products from each of the plant leachates are listed in Table 3.6.

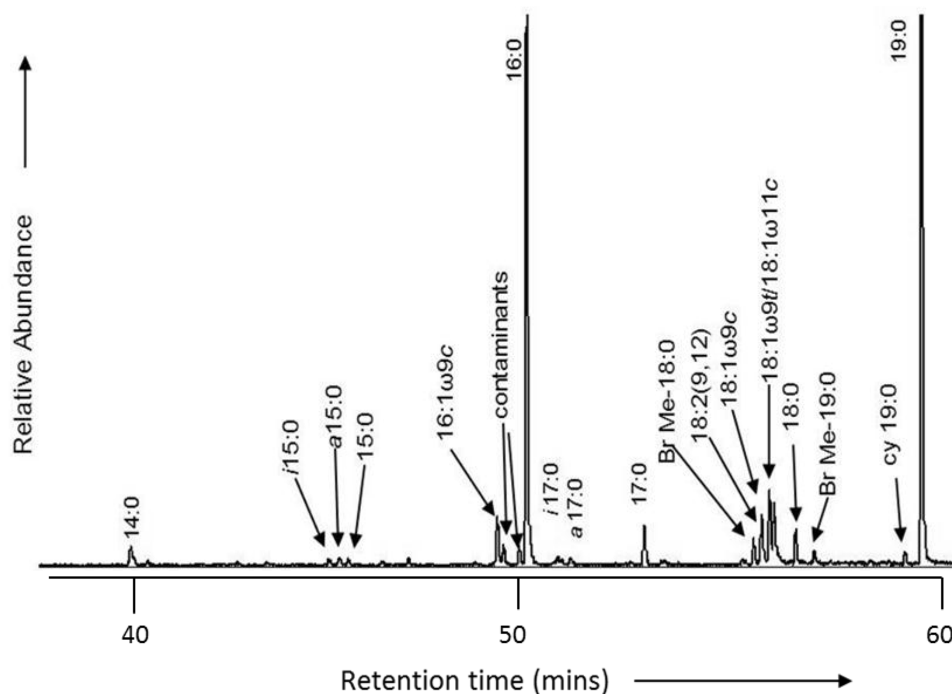


Figure 3.5 Partial TIC from GCMS analysis of the PLFA fraction of Marri leachate (154.4 ng mL^{-1}). 19:0 = internal standard.

Straight chain PLFAs (*n*-PLFAs), particularly 14:0, 16:0 and 18:0, were typically the most abundant products, with lesser amounts of hydroxyl, cyclopropane and polyunsaturated PLFAs. Trace amounts of the generic PLFAs 16:0 and 18:0 were also observed in the control

analysis of abiotic samples and procedural blanks. This indicates sterile conditions were not completely attained, however, background PLFA levels remained very low (16–28 ng mL^{-1}). Twenty eight PLFAs were common to all plant leachates. Additional PLFAs were detected from some of the samples, but typically at relatively low concentrations (<1 ng mL^{-1}) and these were not included in data correlations.

Table 3.5 Major PLFA groupings based on structure.

PLFA Grouping	Abbreviation
Straight chain saturated	Sat
Hydroxyl-branched	OH
Terminally branched	TB
Mid-chain branched	MCB
Mono-unsaturated	MU
Polyunsaturated	PU
Cyclopropyl group	Cyclo

Total PLFA concentrations (i.e., of the common 28) were used as a proxy measure of total biomass (following Zelles, 1999b). The Marri leachate produced the highest concentrations of these products (154.4 ng mL^{-1}) followed by Maize (131.7 ng mL^{-1}), Spinifex (5.4 ng mL^{-1}) and then Sorghum (76.0 ng mL^{-1}) which shows a direct correlation with the DOC value of the leachates (Section 2.4.2.1). The PLFA 16:0 was the most abundant of the Marri leachates, with branched (Br Me-18:0), mono (18:1ω9c, 18:1ω9*t*/18:1ω11*c*) and poly-unsaturated (18:2(9,12)) C_{18} PLFAs also abundant.

Figure 3.6 shows the PLFA group distribution for each of the leachates. Marri followed by Spinifex showed the greatest PLFA group/microbial diversity, with Sorghum and Maize both significantly dominated by the universally abundant 16:0 and 18:0 *n*-PLFAs. Conversely, the Maize leachate showed relatively low concentrations of mono and poly unsaturated, particularly compared to Marri (Fig. 3.6). Terminally branched (TB) PLFAs were common to all samples, but were most abundant in the Sorghum leachate. MCB PLFAs were the group with the largest number of isomers (Table 3.6) and were present in all leachates except Maize where they were not detected. Cyclo PLFAs (cy17:0 and cy19:0), also common to all leachates, were of notably high concentration in the Spinifex leachate. Trace amounts of OH-PLFAs were detected in all samples except the Sorghum leachate. The varied PLFA group distribution reflects the promotion of different microbial communities by the four distinct organic substrates.

Table 3.6 The concentration (ng mL-1) and structural grouping of the major PLFAs detected in the plant leachates. PLFAs in bold verified by authenticated FAME and/or BAME standard. * indicates co-elution - see experimental Section (3.3.3) for detailed discussion. Nd = Not detected.

PLFA	Grouping	Marri	Spinifex	Maize	Sorghum
13:0	Sat	Nd	Nd	Nd	Nd
12:0 (3OH)	OH	Nd	Nd	1.20	Nd
***i*14:0**	TB	Nd	Nd	Nd	Nd
14:0	Sat	7.46	2.70	9.12	13.01
***i*15:0**	TB	2.80	2.95	Nd	0.86
***a*15:0**	TB	1.76	1.07	4.92	2.85
15:0	Sat	Nd	21.72	1.27	1.62
14:0 (3OH)	OH	14.06	8.30	0.41	Nd
***i*16:0**	TB	6.93	Nd	Nd	4.42
Br Me -16:0	MCB	Nd	Nd	Nd	2.46
Br Me -16:0	MCB	Nd	Nd	Nd	1.76
16:1ω9c	MU	4.82	5.45	1.20	Nd
16:1ω7c	MU	Nd	Nd	3.75	Nd
16:0	Sat	15.21	18.21	79.63	29.85
Br Me -17:0	MCB	Nd	1.12	Nd	Nd
Br Me - 17:0	MCB	Nd	2.33	Nd	Nd
***i*17:0**	TB	Nd	Nd	Nd	Nd
*a*17:0	TB	12.92	Nd	Nd	Nd
cy 17:0	Cyclo	2.85	Nd	Nd	Nd
17:0	Sat	Nd	Nd	Nd	2.02
Br Me -18:0	MCB	7.45	Nd	Nd	Nd
18:2(9,12)	PU	17.62	5.46	1.76	2.83
18:1ω9c	MU	20.45	1.38	Nd	Nd
18:1ω9t/	MU	22.97	2.77	1.95	4.28
18:0	Sat	10.58	15.64	19.78	6.30
Br Me -19:0	MCB	3.08	6.04	Nd	2.43
cy 19:0	Cyclo	3.49	19.52	4.10	1.32
20:0	Sat	Nd	10.78	2.65	Nd
Total PLFA	-	154.4	125.4	131.7	76.0

3.4.1.2 Microbial Subgroup Assignment

Many PLFAs (such as straight chain compounds) are ubiquitous and non-specific (Zelles, 1997). It has been suggested that fungi generally contain higher concentrations of straight chain PLFAs than bacteria (Zelles, 1997), although 15:0 has been ascribed a specific bacterial source (Drenovsky et al., 2004). Similarly, MCB fatty acids, such as the C_{15}–C_{18} products detected in high concentrations in all leachates, can derive from a variety of microbial sources (e.g., detrital plankton, bacteria and zooplankton, Wakeham, 1995). Given the limited diagnostic value of MCB PLFAS they are not discussed in detail here.

Gram-Negative Bacteria

The monounsaturated (MU) PLFAs prominent in Marri (Fig 3.6) are reliable biomarkers for Gram-negative bacteria (Ratledge & Wilkinson, 1988; Zelles, 1997), although some (e.g., ω10; Zelles, 1997) can also come from Gram-positive bacteria (Zelles, 1999a; Green & Scow, 2000). Marri contained both the greatest DOC concentrations (Section 2.4.2.1; Table 2.9) and total PLFA concentrations (Table 3.6). Gram-negative bacteria grow rapidly and are able to out-compete Gram-positive bacteria in nutrient-rich environments (Madigan et al., 2011).

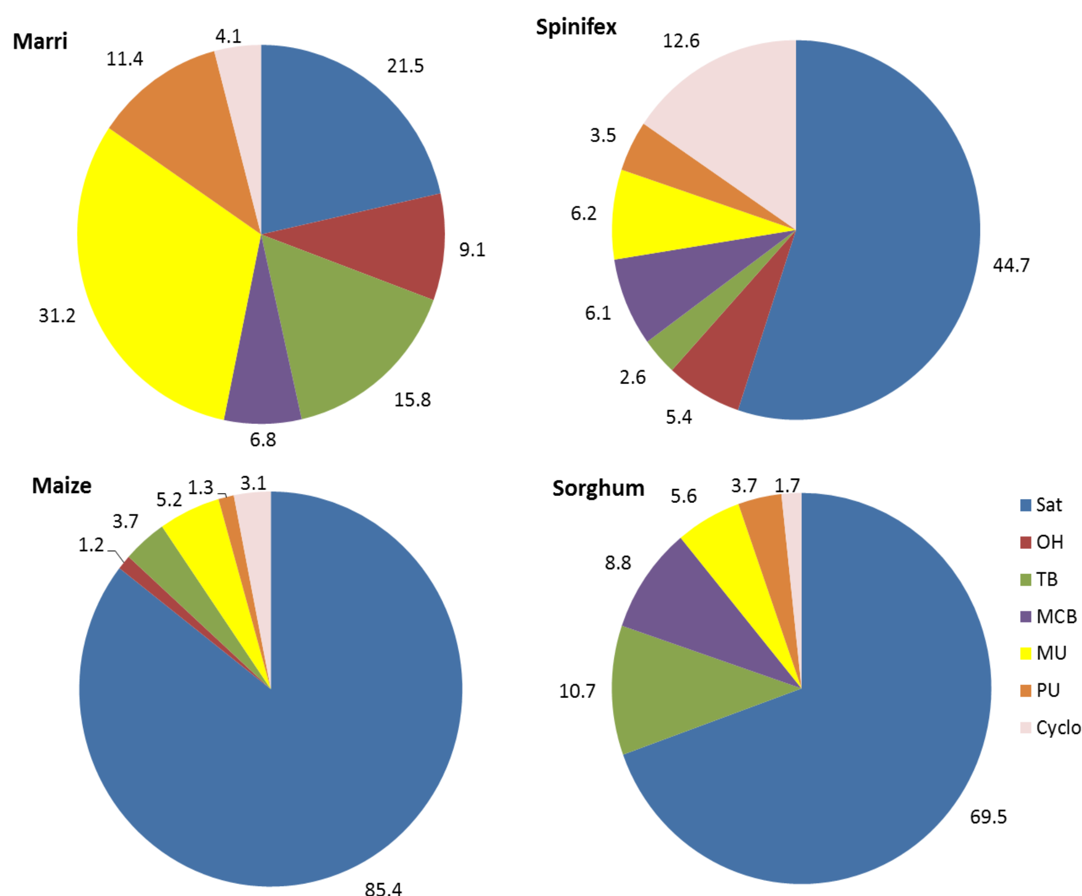


Figure 3.6 PLFA group distributions of plant leachates. Sat = saturates, OH = hydroxyl branched, TB = terminally branched, MCB = mid-chain branched, MU = monounsaturated, PU = polyunsaturated, Cyclo = contains a cyclopropyl group.

Cyclopropyl (Cyclo) and hydroxyl (OH) PLFAs are also generally indicative of Gram-negative bacteria (Ratledge & Wilkinson, 1988; Cavigelli et al., 1995; Zelles, 1997). Both of these PLFA groups were most prominent in the Spinifex leachate, which also showed the next highest proportion of MU PLFA after Maize. Spinifex leachate had the lowest DOC of all the leachates (145 mg L^{-1}, Section 2.4.2.1, Table 2.9).

Gram-Positive Bacteria

Terminally branched (TB) fatty acids are generally indicative of Gram-positive bacteria (Zelles, 1997) and were measured to be in highest proportions in Sorghum (10.8 %) and were also significant in Marri (8.4 %) where they were also higher in abundance than MCB PLFAs (Fig. 3.5). Gram-positive bacteria have been reported to degrade less easily assimilable organic substrates (Paul & Clark, 1996; Olapade & Leff, 2006).

Fungi

Polyunsaturated (PU) PLFAs such as 18:2 (9,12) and 18:1ω9*c* (Zak et al., 1996) are established fungal markers (Frostegard & Baath, 1996; Zak et al., 1996; Zelles, 1997; Kelly et al., 2007). The PLFA 18:2 (9:12) was detected in all samples, and highest proportions of PU PLFAs were detected in Marri, indicating a relatively high fungal contribution to the Marri leachate. The fungi were likely to have been living on the plant prior to leaching. Fungi have the potential to thrive in DOC and nutrient-rich aquatic environments. Fungi, particularly of the class *Hyphomycetes* and also of the Phyla *Chytridiomycota*, have been previously detected in biofilms in natural aquatic systems (Hax & Golladay, 1993; Pozo et al., 1998) as well as biofilms in water distribution systems (Doggett 2000; Hendrickx et al., 2002; Keinanen et al., 2002; Hageskal et al., 2009) and in wastewater systems (Quezada et al., 2007).

Bioavailability and Bio-assimilation of Leaf Leachates

Many interrelated factors influence microbial utilisation of leached DOC including, but not limited to; microbial consortia present, nutrient availability, particle size and age of vegetation detritus (Baldwin, 1999; O'Connell et al., 2000). Plant taxa and specific compounds leached from leaves can also impact the kinds of bacterial communities that might thrive (Wu et al., 2009a). A summary of previously reported PLFA-microbe product-source relationships suggested in the present study is summarised in Table 3.7.

From the PLFA profiles, the Marri leachate showed the largest and most diverse microbial community. Fungi and possibly Gram-positive bacteria may have exploited the more recalcitrant DOC material (Paul & Clark, 1996; McNamara & Leff, 2004 a&b; Olapade & Leff, 2006), whilst opportunistic Gram-negative species utilised the more ‘labile’ portions of the DOC.

DOC leached from the Sorghum plant supported a higher proportion of Gram-positive bacteria. Conversely, Maize which has a relatively low polyphenol content (Mutabaruka

et al., 2007) had a greater representation of Gram-negative bacteria. Maize is known to contain a large proportion of 'labile' sugars (Spielbauer et al., 2006) easily assimilated by opportunistic Gram-negative bacteria.

Table 3.7 Summary of main PLFA features and their likely supporting substrate.

	Diagnostic PLFAs	Microbial Source	Source Reference	Preferred Substrate	Substrate Reference
Marri	18:1ω11*c*	Gram -ve	Zelles, 1997	Assimilable 'labile' material	Zander et al., 2005
	*a*15:0 *i*15:0 *i*16:0 *a*17:0	Gram +ve	Zelles, 1997	Refractory substrates	Olapade & Leff, 2006 Paul & Clark, 1996 McNamara & Leff 2004a&b
	18:2 (9,12) 18:1 ω9*t* 18:1 ω9*c*	Fungi	Frostegard & Baath 1996 Zelles, 1997 Zak et al., 1996 Mckinley et al., 2005	Refractory substrates	Mutabaruka et al., 2007
Spinifex	*a*15:0 *i*15:0	Gram +ve	Zelles, 1997	Refractory substrates	Webster & Benfield 1986
	cy19:0	Gram -ve low Oxygen & nutrient stress	Petersen & Klug 1994	Assimilable 'labile' sugars	Cookson et al., 2006
Maize	few specific indicators, mainly MU	Gram -ve	Zelles, 1997	Assimilable 'labile' sugars	Speilbauer et al., 2006
Sorghum	*a*15:0 *i*15:0 *i*16:0	Gram +ve	Zelles, 1997	refractory substrates	Fishman & Neucere 1980

Spinifex is considered relatively nutrient-poor (Grigg et al., 2008) but can yield DOC with a high 'labile' content that is readily bioavailable (Cookson et al., 2006). This leachate supported an appreciably more diverse PLFA distribution/microbial population than Sorghum and Maize (Fig. 3.6). The thick waxy cuticle of Spinifex blades are high in polyphenols (Webster & Benfield, 1986) which are preferred by Gram-positive bacteria and fungi (Mutabaruka et al., 2007). The Spinifex leachate did show substantial input of cyclo-PLFAs, which have been reported to be high under low nutrient (or low oxygen) levels

(Petersen & Klug, 1994). Cyclo-PLFAs are generally indicative of Gram-negative bacteria (Zelles, 1999a). These results demonstrate that the different plants or DOC substrates of the four leachates do give rise to different microbial communities which can be resolved to a certain degree by the PLFA distributions.

3.4.2 Isotope Analysis of PLFAs from Biota of Plant Leachates

3.4.2.1 General Observations

Measurement of the stable carbon isotope value of individual PLFAs by CSIA may provide further insight into substrate utilisation and/or source (Jones et al., 2003). Accurate CSIA measurements require an analyte signal 50-300 ng and baseline resolution between analytes. This detection sensitivity precluded $\delta^{13}C$ analysis of all but the most abundant PLFAs, even after concentration of samples down to the minimum volume. Incomplete GC resolution also prevented $\delta^{13}C$ measurement of several components which did pass the sensitivity threshold—e.g., *i*15:0 and *a*15:0; 16:1ω9*c* and 16:1ω7*c*. A combined value was instead used for these particular co-elutions where structural similarity was indicative of a common microbial source. As such, data was only obtainable for a small subset of PLFAs. Similar problems were reported by Cifuentes et al. (1996), Jones et al. (2003) and Burke et al. (2003)—where PLFA $\delta^{13}C$ measurements were consistently limited to no more than five PLFAs. Table 3.8 shows the $\delta^{13}C$ values obtained for the most abundant PLFAs of the plant leachates together with the corresponding bulk $\delta^{13}C$ values of the biomass residue of the leachates.

Table 3.8 $\delta^{13}C$ values of bulk leachate biomass and the PLFAs (corrected) extracted from them. * = not baseline resolved so measured as combined value. (n) = corresponds to number of analyses. (±) = Standard deviations, based on uncorrected data.

PLFAs	Source	$\delta^{13}C$ of PLFAs in Vegetation Biomass (‰)			
		Marri	Spinifex	Maize	Sorghum
i&*a*15:0*	G+ve	-30.1 ±0.37$^{(3)}$	-19.0 ±0.54$^{(3)}$	-	-19.9 ±0.46$^{(3)}$
15:0	Bact.	-	-28.0 ±0.66$^{(2)}$	-22.4 ±0.98$^{(3)}$	-
16:1ω9*c*&7*c**	G-ve	-38.8 ±0.84$^{(3)}$	-	-20.4 ±0.86$^{(2)}$	-
16:0	Multi	-42.0 ±0.68$^{(3)}$	-20.8 ±0.20$^{(3)}$	-23.3 ±0.42$^{(3)}$	-20.2 ±0.28$^{(2)}$
18:2 (9,12)	Fungi	-	-18.1 ± 0.31$^{(3)}$	-	-
18:0	Multi	-30.8 ±0.30$^{(3)}$	-17.9 ± 0.86$^{(2)}$	-17.4 ±0.18$^{(2)}$	-27.9 ±0.25$^{(3)}$
cy19:0	G-ve	-	-18.9 ±0.62$^{(3)}$	-	-
20:0	Multi	-	-17.8 ±0.39$^{(3)}$	-	-
Bulk $\delta^{13}C$ of Raw Biomass		-30.7	-14.3	-14.2	-17.5

All PLFAs were ^{13}C depleted by 1–12 ‰ relative to the biomass residue with the exception of *i*&*a*15:0 in the Marri (+0.6 ‰) and 15:0 in the Spinifex (-14.5 ‰) leachates. This is similar

to the 3 ‰ to 11 ‰ range reported from several previous studies (De Niro & Epstein, 1977; Monson & Hayes, 1982; Blair et al., 1985; Hayes, 1993; Abraham et al., 1998; Boschker et al., 1999; Jones et al., 2003) and reflects the response of different microbial populations to different substrates. PLFAs extracted from heterotrophic organisms are typically 3 ‰ depleted in ^{13}C relative to bulk cellular biomass (Blair et al., 1985; Teece et al., 1999; Hayes, 2001). The biomass contains a large array of additional biochemical components (e.g., sugars and proteins) which are relatively enriched in ^{13}C compared to the lipid material (van Dongen et al., 2002).

The $\delta^{13}C$ values of bulk biomass and individual PLFAs (Table 3.8) both reflect the photosynthetic pathway of the respective vegetation. The -30 ‰ to -42 ‰ range of the Marri PLFA data is consistent with a C3 photosynthetic pathway (Kramer & Gleixner, 2006), whereas Maize, Sorghum and Spinifex leachates show generally more ^{13}C enriched values consistent with a C4 photosynthetic pathway (Kramer & Gleixner, 2006).

3.4.2.2 Marri (C3)

The Gram-positive *i*&*a*15:0 (-30.1 ‰, Table 3.8) was the only isotopically heavy PLFA of the four measured compared to the bulk $\delta^{13}C$ value. Similar results were reported for previous studies carried out on several soil types (Burke et al., 2003), sedimentary bacteria and seagrasses (Jones et al., 2003). Burke et al. (2003) attributed this to Gram-positive bacteria being more efficient at utilising recalcitrant SOM, whilst Jones et al. (2003) attributed it to being reflective of bacterial sub-group consuming and efficiently preserving the $\delta^{13}C$ signature of the plant biomass. PLFA 18:0 (30.8 %) was only slightly more depleted in ^{13}C than *i*&*a*15:0, suggesting it also may be indicative of Gram-positive bacteria utilising the leached Marri biomass as a primary carbon source.

The $\delta^{13}C$ of 16:0 (-42.0 ‰) was the most ^{13}C depleted of the PLFAs, but the potential multiple sources of this common PLFA limits its diagnostic value. The Gram-negative 16:1ω9c&16:1ω7*c* (-38.8 ‰) was also significantly lighter than the bulk biomass (8.1 ‰; Table 3.8). Burke et al. (2003) reported that 16:1ω7*c* was more depleted in ^{13}C (0.4–4.0 ‰) and even more so was the Gram-negative Cy19:0, (4–7 ‰) relative to the Gram-positive *i*15:0 in the soils they studied. Gram-negative bacteria tend to utilise easily assimilable organic substrates (Madigan et al., 2011), which in the present leachates may be ^{13}C depleted material of lipid origin. SPME-GCMS (Chapter 2) determined that isoprenoids were the most abundant constituents of the Marri leachates. The isoprenoids gave $\delta^{13}C$

values ranging from -31.4 ‰ to -34.7 ‰ (Table 2.11), much more depleted in ^{13}C than several lignin derived products (-28.4 ‰ to -33.9 ‰; Table 2.11). Gram-positive bacteria are able to degrade complex substrates like lignin and humic acid (Paul & Clark, 1996) which does contribute to ^{13}C- enriched signatures relative to lipid-derived material (Preston et al., 2006; Dungait et al., 2008a). Separate substrate utilisation by different bacterial species has previously been identified in soils (Burke et al., 2003; Waldrop & Firestone, 2004; Kramer & Gleixner, 2006) and aquatic environments (Cifuentes & Salata, 2001; Jones et al., 2003; Augspurger et al., 2008).

3.4.2.3 Maize, Sorghum and Spinifex (C4)

Bulk and individual PLFAs from the leachates of the C4 plants showed consistently more ^{13}C enriched values than the corresponding Marri data. The Spinifex and Maize residues had similar bulk $\delta^{13}C$ values of -14.2 ‰ and -14.3 ‰, respectively (Table 3.8), whilst Sorghum was slightly more ^{13}C depleted with a value of -17.5 ‰. The PLFAs from these leachates displayed a slightly broader range of values, likely reflecting the complexity and heterogeneity of microbial communities utilising different carbon substrates. Many factors can influence the $\delta^{13}C$ of lipid membranes, including, growth rate, competition, type and bioavailability of substrate, light and temperature effects (Jahnke et al., 1999; Wick et al., 2003; Londry et al., 2004; Zhang et al., 2005; Staal et al., 2007).

Most of the $\delta^{13}C$ values of PLFAs from these leachates were similarly more ^{13}C enriched than the corresponding Marri PLFAs. But similar to the Marri data, the $\delta^{13}C$ value of most of the PLFAs from the C4 plant leachates showed a 2–9 ‰ depletion compared to the bulk $\delta^{13}C$ value of the raw biomass (Table 3.8). Values of 15:0 (-28.0 ‰) from Spinifex and 18:0 (-27.9 ‰) from Sorghum were the most depleted of the C4 plant derived PLFAs. Metabolic processes such as methanogenesis may contribute to these depleted values (Summons et al., 1998). Additionally, trophic levels within the microbial community may also have had an influence (Breteler et al., 2002).

The Sorghum 16:0 was only a few ‰ more ^{13}C depleted than the raw biomass bulk $\delta^{13}C$ value. A similar $\delta^{13}C$ trend was seen in the Gram positive *i*&*a* 15:0. Conversely, Sorghum 18:0 was highly depleted in ^{13}C (10.4 ‰, Table 3.8) *Cf.* the raw biomass. Given the ubiquity and multiple sources of these PLFAs, these isotopic trends are difficult to interpret, however the 16:0 signature (-20.2 ‰, Table 3.8) suggests its main microbial source has no strong preference of organic substrate.

Relatively high concentrations of the Spinifex sourced PLFAs allowed δ^{13}C measurement of seven PLFA peaks. Apart from the more ^{13}C depleted 15:0 and less so 16:0, the other PLFAs—which included markers of Gram-positive (i&a15:0,), Gram-negative (cy19:0) and fungal (18:2 (9,12)) microbial communities—showed quite similar δ^{13}C values (-18 ‰ to -19 ‰). This suggests that the microbes these reflect share a similar part of the available substrate. It is also possible that these values reflect a similar average of the complex substrate utilisation of complex sub-communities (Cifuentes & Salata, 2001; Burke et al., 2003).

3.4.2.4 Summary Vegetation Leachate Isotope Analysis

Insights gained from isotope analysis of the leachate PLFAs are presented in Table 3.9.

Table 3.9 Summary of isotopic findings of vegetation leachates. * Burke et al., 2003. ~ Jones et al., 2003. ^ Mutabaruka et al., 2007.

	PLFA	Represents	Δ bulk ‰	Δ 16:0 ‰	possible substrate
Marri	*i*&*a*15:0	Gram +ve bact.	+0.6	+11.9	Marri biomass * relatively recalcitrant portion ~
	16:1ω7c&ω9c	Gram -ve bact.	-8.1	+3.2	Lipids Utilisation of the most abundant easily assimilable substrate *
Spinifex	*i*&*a*15:0	Gram +ve bact.	-4.7	+1.8	Mixed
	18:2 (9,12)	Fungi	-3.8	+2.7	Utilisation of the most abundant easily assimilable substrate (Possibly polyphenols^)
	cy19:0	Gram -ve bact.	-4.6	+1.9	Utilisation of the most abundant easily assimilable substrate * Possibly sugars
Maize	15:0	Non-specific bact.	-8.2	+0.9	Mixed
	16:1ω7c&ω9c	Gram -ve bact.	-6.2	+2.9	Utilisation of the most abundant easily assimilable substrate * Possibly sugars
Sorghum	*i*&*a*15:0	Gram +ve bact.	-2.4	+1.8	Mixed Utilisation of tannin-protein complexes ^

Molecular characterisation (i.e., PLFA distributions) showed that PLFA concentrations and diversity varied between leachates. The greatest diversity was observed in Marri, and the least in Maize. Gram-positive *i*&*a*15:0 was detected in all samples, but Maize. The δ^{13}C

discrepancy between *i*&*a*15:0 and the bulk leachate was 0 ‰, 2 ‰ and 5 ‰ in Marri, Sorghum and Spinifex, respectively (Table 3.9). These data suggest that the Marri leachate supported a Gram-positive bacteria community that was able to utilise a greater proportion of recalcitrant ^{13}C enriched material, possibly reflecting adaptation due to a limited supply of readily bioavailable substrates.

Gram-negative 16:1ω7*c*&ω9*c*, detected in all samples but Sorghum, was depleted in ^{13}C by more than 6 ‰ compared to the bulk biomass. This suggests that Gram-negative bacteria, which prefer 'labile' DOC, are utilising ^{13}C enriched sugars rich in both C3 and C4 plant leachates. This pattern of substrate utilisation is also consistent with what is known about the opportunistic growth of Gram-negative bacteria.

3.4.3 Molecular and Isotopic Characterisation of Distribution System Biofilms

3.4.3.1 Analytical Results of Molecular Characterisation

Representative partial TICs for Site C Biofilms (BFs) 47 (winter) and 150 (summer) are shown in Fig 3.7. Both were sampled at the same point of the same the distribution system but at different times to reflect opposing climatic seasons. The summer sample (BF 150) showed highest concentrations and diversity of PLFAs, reflecting an increase in microbial activity with climatic temperature.

The biofilm concentrations of 28 common PLFAs highlighted in the leachate study are listed in Table 3.10. The biofilms showed qualitatively similar PLFA distributions to the leachates. Quantitatively, the biofilm concentrations varied with season and water treatment—PLFA concentrations were greatest for summer recycled systems (Table 10d) and lowest for winter potable systems (Table 10a). The PLFA concentrations obtained for the potable system biofilms (24–97 ng mL^{-1}) were lower than those of the vegetation leachates (76–155 ng mL^{-1}). Generally, the PLFA concentrations obtained for recycled systems were comparable to or higher than the vegetation leachates.

For biofilms of the recycled systems, total PLFA concentrations ranged from 17–6973 ng mL^{-1}, the higher values of the latter reflecting the more biologically rich nature of recycled systems. Substantially higher PLFA concentrations were detected in biofilms from secondary treated (Class C) recycled water—i.e., site C (BFs 47 & 150) and site D

(BF 185). All biofilms were able to be resolved by MDS and cluster multivariate analysis of individual PLFA concentrations (Section 3.3.4. and Appendix).

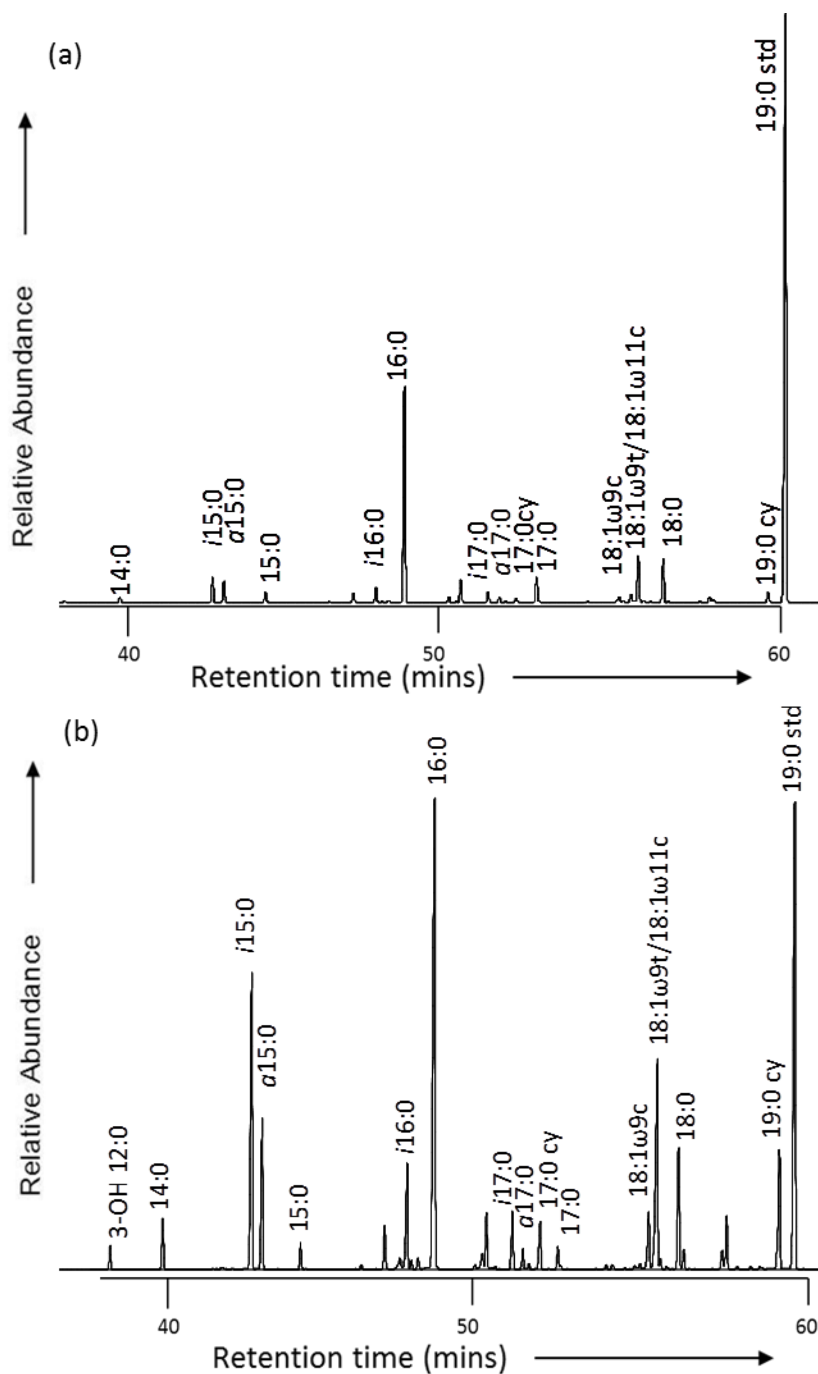


Figure 3.7 Partial TIC of PLFA distribution of (a) BF 47 (winter recycled, Site C); and (b) BF 150 (summer recycled, Site C).

Table 3.10 (a) Concentrations (ng mL^{-1}) of major PLFAs detected in biofilms collected during winter from potable distribution systems. The collective concentration of these PLFAs is expressed as Total PLFA concentration. PLFAs verified by authenticated standard (BAME and/or FAME) indicated in bold. Nd = not detected.

	Site A		Site B		Site C		Site D	
PLFA	**7**	**9**	**25**	**26**	**43**	**45**	**62**	**63**
13:0	0.18	0.18	0.18	0.35	0.18	0.18	0.35	0.18
12:0 (3OH)	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
***i*14:0**	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
14:0	0.18	0.53	0.18	0.35	0.18	0.18	0.18	0.18
***i*15:0**	Nd	0.18	Nd	Nd	Nd	Nd	Nd	Nd
***a*15:0**	Nd	0.18	Nd	0.18	Nd	Nd	0.18	0.18
15:0	0.18	0.88	0.35	0.53	0.18	0.18	0.35	0.35
14:0 (3OH)	Nd	0.18	0.18	0.18	Nd	Nd	0.18	0.18
***i*16:0**	Nd	0.18	Nd	Nd	Nd	Nd	Nd	Nd
Br Me -16:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Br Me -16:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
16:1ω9c	Nd	0.18	0.18	2.11	0	0.35	3.7	0
16:1ω7c	4.93	17.25	6.69	10.74	5.1	3.52	14.08	0.18
16:0	17.42	14.61	18.48	16.54	12.5	15.14	12.85	28.34
Br Me -17:0	0.18	0.18	Nd	Nd	Nd	Nd	Nd	Nd
Br Me - 17:0	Nd	0.18	0.18	0.18	0	0.18	0	0.35
***i*17:0**	Nd	0	Nd	Nd	Nd	Nd	Nd	Nd
*a*17:0	Nd	0.18	Nd	Nd	Nd	Nd	Nd	Nd
cy 17:0	Nd	0	Nd	Nd	Nd	Nd	Nd	Nd
17:0	0.35	0.53	0.35	0.35	0.35	0.35	0.53	0.35
Br Me -18:0	Nd	0.18	0.35	1.94	0	0.35	3.34	0.18
18:2(9,12)	0.18	0.35	0.35	0.18	0.18	0.18	0.35	0.35
18:1ω9c	Nd	0.53	Nd	Nd	0.18	Nd	Nd	Nd
18:1ω9t/18:1ω11c	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
18:0	4.4	9.15	4.22	7.57	5.46	3.17	10.21	13.2
Br Me -19:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
cy 19:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
20:0	0.53	0.53	0.7	2.99	0.35	0.7	4.22	0.53
Total PLFA conc	28.5	46.2	32.4	44.2	24.7	24.5	50.5	44.6

Table 3.10 Continued (b) Major PLFAs detected in biofilms collected during winter from recycled distribution system.

	Site A			Site B			Site C			Site D	
PLFA	**10**	**11**	**12**	**28**	**29**	**30**	**46**	**47**	**48**	**64**	**65**
13:0	0.18	0.18	0.18	0.18	0.35	0.18	0.18	0.53	0.18	0.18	0.18
12:0 (3OH)	Nd	Nd	Nd	Nd	0.18	0.18	Nd	3.17	Nd	0.35	Nd
***i*14:0**	Nd	Nd	Nd	Nd	Nd	Nd	0.18	0.18	Nd	2.11	Nd
14:0	0.18	0.18	0.18	0.18	0.7	0.35	0.53	14.26	0.18	Nd	0.88
***i*15:0**	Nd	Nd	0.18	Nd	1.41	0.18	0.35	64.94	0.18	9.15	2.82
***a*15:0**	Nd	0.18	0.18	Nd	1.23	0.35	0.35	52.98	0.18	5.81	0.88
15:0	0.18	0.35	0.18	0.18	1.41	0.18	0.53	26.58	0.53	4.4	1.23
14:0 (3OH)	0.18	0.18	Nd	0.18	Nd	Nd	0.35	0.7	0.18	Nd	1.23
***i*16:0**	Nd	Nd	0.18	Nd	0.88	Nd	Nd	35.55	Nd	9.15	8.1
Br Me -16:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	2.64	Nd	Nd	1.58
Br Me -16:0	Nd	Nd	Nd	Nd	Nd	Nd	0.35	4.4	Nd	2.29	1.23
16:1ω9c	0.18	Nd	Nd	Nd	2.29	0.35	0.18	1.41	0.18	Nd	Nd
16:1ω7c	9.15	10.4	8.45	6.51	18.7	15.1	18.5	56.5	28.86	19. 9	15.1
16:0	14.6	20.4	15.1	15.1	28.7	23.2	15.1	14.6	15.1	0.53	64.5
Br Me -17:0	Nd	0.18	0.18	Nd	2.99	Nd	0.35	1.76	Nd	10.5	0.18
Br Me - 7:0	Nd	Nd	0.18	Nd	Nd	Nd	0.18	60.02	0.18	0.18	0.35
***i*17:0**	Nd	Nd	Nd	Nd	1.23	Nd	0.35	27.63	Nd	6.86	0.88
*a*17:0	Nd	Nd	0.18	Nd	0.53	Nd	0.35	14.08	0.18	2.82	0.35
cy 17:0	Nd	Nd	Nd	Nd	0.53	Nd	Nd	12.5	0	3.17	0.53
17:0	0.35	0.35	0.18	0.35	3.34	0.53	0.53	69.3	0.7	15.5	1.23
Br Me -18:0	Nd	Nd	0.35	Nd	2.29	0.35	Nd	0.7	0.18	0.35	Nd
18:2(9,12)	0.18	0.35	0.35	0.18	0.7	0.35	0.88	1.94	0.7	7.92	0.35
18:1ω9c	0.35	0.18	0.35	0	5.46	Nd	1.94	129.9	0.18	28.8	8.1
18:1ω9t/ 18:1ω11c	Nd	Nd	Nd	Nd	Nd	Nd	Nd	4.22	Nd	Nd	28.34
18:0	7.39	8.1	5.63	5.46	9.86	12.9	12.1	116.2	27.1	29.0	16.2
Br Me -19:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	3.17	Nd	Nd	0.88
cy 19:0	Nd	Nd	0.18	Nd	0.7	Nd	0.18	27.3	Nd	3.17	2.46
20:0	0.35	0.53	0.35	0.35	2.64	0.7	0.53	8.45	0.88	Nd	0.7
Total PLFA	33.3	41. 6	32.6	28.7	86.1	54.9	54.1	755.6	75.9	162.8	158.8

Table 3.10 Continued (c) Major PLFAs detected in biofilms collected during summer from potable distribution system.

PLFA	Site B 128	Site C 145	Site E 163	164	165	Site D 181	182	183
13:0	Nd	1.23	0.18	1.23	2.29	0.18	0	0.18
12:0 (3OH)	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
***i*14:0**	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
14:0	Nd	0.35	0.18	0.53	1.58	0.88	1.06	0.53
***i*15:0**	Nd	Nd	Nd	Nd	Nd	0.18	0.18	0.18
***a*15:0**	Nd	Nd	0.18	Nd	Nd	0.35	0.18	0.18
15:0	Nd	Nd	0.18	0.18	Nd	0.88	0.88	0.53
14:0 (3OH)	Nd	Nd	0.18	Nd	Nd	0.35	0.18	0.18
***i*16:0**	Nd	Nd	Nd	Nd	Nd	Nd	0.18	Nd
Br Me -16:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
Br Me -16:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
16:1ω9c	0.18	1.76	0.18	2.11	Nd	0.35	2.46	0.53
16:1ω7c	Nd	1.76	3.87	7.92	3.17	17. 8	Nd	22.2
16:0	1.76	22	15.7	16.2	9.68	41.7	37.1	18.3
Br Me -17:0	0.18	Nd	Nd	Nd	Nd	Nd	0.35	Nd
Br Me -17:0	Nd	Nd	Nd	Nd	Nd	0.53	Nd	0.18
***i*17:0**	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.18
*a*17:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
cy 17:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
17:0	0.35	1.76	0.35	0.7	1.06	0.88	0.88	0.7
Br Me -18:0	0.18	1.76	0.18	2.11	2.99	Nd	2.46	0.53
18:2(9,12)	Nd	1.94	0.18	0.53	0.53	0.7	1.23	0.35
18:1ω9c	Nd	1.94	Nd	0.7	Nd	Nd	Nd	0.18
18:1ω9t/ 18:1ω11c	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
18:0	2.99	1.94	2.29	15.1	10.0	31.9	9.0	10.2
Br Me -19:0	Nd	Nd	Nd	Nd	Nd	Nd	Nd	0.35
cy 19:0	Nd	Nd	0.18	Nd	Nd	0.18	Nd	Nd
20:0	0.88	2.11	0.35	3.52	4.93	0.88	4.05	0.88
Total PLFA	38.6	24.1	50.9	36.3	97.7	80.0	60.2	56.4

Table 3.10 Continued (d) Major PLFAs detected in biofilms collected during summer from recycled distribution system. * = unusually low concentration. ** = unusually high concentrations. ^= very high 16:0.

	Site B			Site C	Site D	Site E	
PLFA	**130**	**131**	**132**	**150**	**185**	**166**	**168**
13:0	Nd	0.18	0.18	0.35	Nd	0.18	0.18
12:0 (3OH)	Nd	Nd	Nd	7.04	67.8	Nd	Nd
***i*14:0**	Nd	Nd	Nd	Nd	Nd	Nd	Nd
14:0	0.18	0.7	0.18	23.1	149.1	0.88	0.35
***i*15:0**	Nd	0.18	Nd	282.0	1007.3	0.18	0.35
***a*15:0**	Nd	0.35	Nd	110.4	459.0	0.35	0.35
15:0	0.35	1.23	0.18	22.5	80.4	0.88	0.7
14:0 (3OH)	Nd	0.18	0.18	16.0	55.1	0.35	0.53
***i*16:0**	Nd	Nd	Nd	103.8	389	Nd	Nd
Br Me -16:0	Nd	Nd	Nd	7.22	28.7	Nd	Nd
Br Me -16:0	Nd	Nd	Nd	7.22	33.3	Nd	Nd
16:1ω9c	0.53	0.53	1.94	Nd	55.1	0.35	0.18
16:1ω7c	7.7	19.7	3.17	15.5	56.5	17.8	17.1
16:0	18.3	27.6	0.53	695.6	1950.4^	41.7	18.0
Br Me -17:0	Nd	Nd	Nd	50.7	176.9	Nd	0.18
Br Me - 17:0	Nd	Nd	Nd	Nd	Nd	0.53	0.35
***i*17:0**	Nd	Nd	Nd	85.4	185.7	Nd	0.18
*a*17:0	Nd	Nd	0.18	22.5	65.3	Nd	0.18
cy 17:0	Nd	Nd	Nd	47.3	160.7	Nd	Nd
17:0	0.35	1.23	1.06	25.0	57.6	0.88	0.88
Br Me -18:0	0.7	0.53	1.94	4.93	Nd	Nd	0.18
18:2(9,12)	0.18	0.7	0.18	80.8	223.9	0.7	0.53
18:1ω9c	Nd	Nd	0.18	244.5	Nd	Nd	0.7
18:1ω9t/18:1ω11c	Nd	Nd	Nd	7.57	842.2	Nd	Nd
18:0	6.34	17.6	4.05	152.2	402.2	31.9	18.3
Br Me -19:0	Nd	Nd	Nd	18.0	62.8	Nd	Nd
cy 19:0	0.7	0.18	Nd	138.7	428.9	0.18	Nd
20:0	1.23	1.41	2.99	12.14	35.5	0.88	0.88
Total PLFA	36.6	72.3	17.0*	2180.3**	6973.1**	97.7	60.0

3.4.3.2 Relative Abundances of PLFA Groupings in Biofilms

Fig. 3.8 illustrates the averaged distribution of the major PLFA groups detected in distribution biofilms of potable (summer and winter) and recycled (summer average) waters. The potable-winter BFs had straight chain saturated PLFAs (70 %) in highest abundance, followed by monounsaturated PLFAs (24 %; indicative of Gram-negative bacteria) and mid-chain branched saturated PLFAs (2 %; non-specific PLFAs). Straight chain PLFAs have been previously shown to account for most (55–79%) of the PLFAs of drinking water biofilms (Keinanen et al., 2003a).

The potable-summer biofilms showed a similar distribution of PLFA groups, although polyunsaturated (PU) PLFAs (e.g., 1.4 %; 18:2(9,12)) indicative of fungi were more significant. Other studies have similarly detected fungal PLFAs 18:2(9,12) in the biofilms of non-disinfected drinking water (Keinanen et al., 2002) or provided other evidence for fungi in drinking water distribution systems (Rosenzweig et al., 1986; Zacheus & Martikainen, 1995; Hageskal et al., 2007; Hageskal et al., 2009).

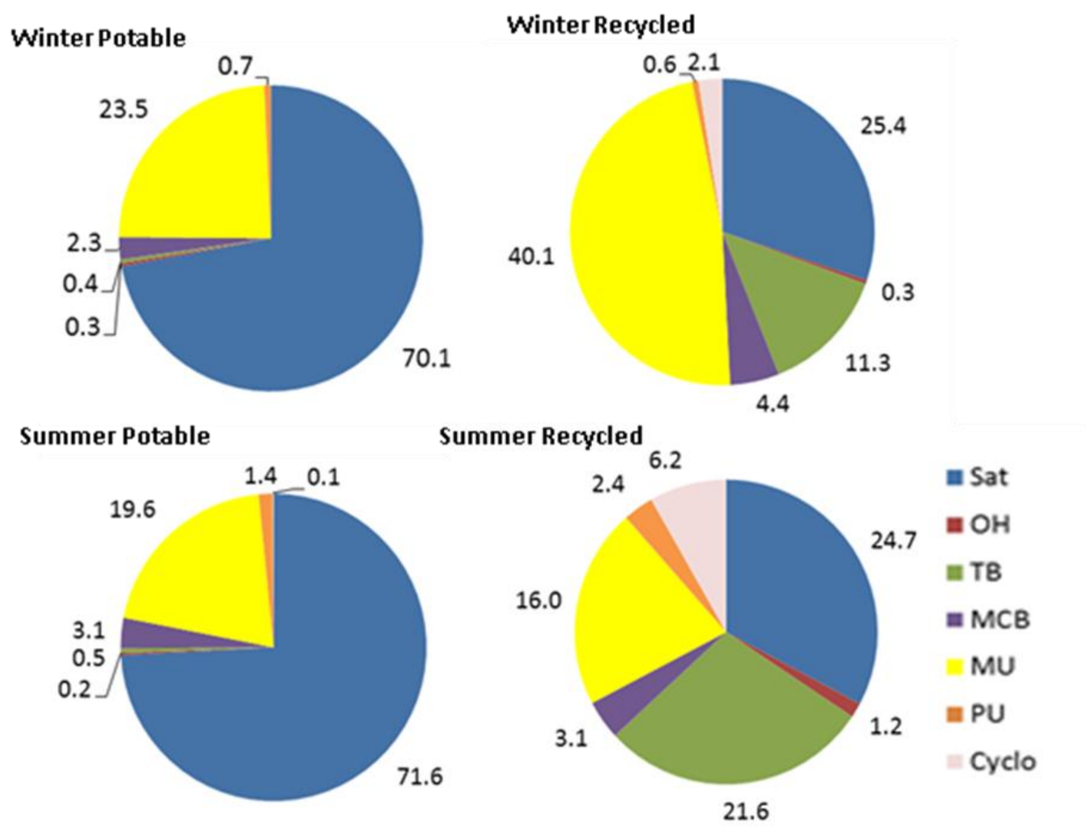


Figure 3.8 Distributions of major PLFA groups detected in biofilms. Sat = saturates, OH = hydroxyl branched, TB = terminally branched, MCB = mid-chain branched, MU = monounsaturated, PU = polyunsaturated, Cyclo = contains a cyclopropyl group.

Biofilms from recycled schemes showed very different PLFA signatures compared to potable systems. The quantitatively most significant PLFA group in the winter recycled biofilm was MU PLFAs (40 %), followed by Sat (25.4 %), TB (11 %), MCB (4.4 %) and Cyclo PLFAs (2.1 %). Negligible amounts of PU and OH-PLFAs were detected.

The summer sampled biofilms of recycled systems showed the greatest diversity and concentrations of PLFAs, suggesting warm recycled water contains high organic substrate and nutrient supply able to support a greater proportion of microbial species. Sat PLFAs were again significant (25 %) and the sum of PLFAs indicative of Gram-negative bacteria (i.e., 12:0(3OH), 14:0(3OH), 16:1ω9*c*, 16:1ω7*c*, cy17:0, 18:1ω9*t*/18:1ω11*c*, cy19:0) made up a comparable amount (26 %). Gram-positive TB PLFAs (22 %) were also high in relative abundance. PU PLFAs (2.4 %) were also more abundant in these biofilms than all others.

3.4.3.3 Microbiological Sub-groupings

Figure 3.9 shows the percentage contribution of PLFAs attributed to specific microbial species class as defined below (based on Parker et al., 1982; Frostegard et al., 1993; Cavigelli et al., 1995; Frostegard & Baath, 1996; Zak et al., 1996; Zelles 1997; Zelles, 1999a; Drenowsky et al., 2004):

- Bacterial PLFAs = ∑ [*i*15:0, *a*15:0, *i*16:0, 16:1ω5*c*, *i*17:0, *a*17:0, 17:0cy, 18:1ω7*c*, 19:0cy].
- Gram +ve = ∑ [*i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, a17:0]
- Gram -ve = ∑ [12:0(3OH), 14:0(3OH), 16:1ω9*c*, 16:1ω7*c*, cy17:0, 18:1ω9*t*/18:1ω11*c*, cy19:0]
- % Fungi (or general Eukaryotes) = ∑ [18:2 (9,12) + 18:1 ω9*c*]

Biofilms of all potable and recycled waters systems, apart from recycled Site D, were dominated by Gram-negative bacteria. Relatively high abundance of Gram-negative bacteria have also been reported in previous studies of biofilms from potable water systems (LeChevallier et al., 1987; Payment et al., 1988; Lahti, 1993; Moll et al., 1998; Percival et al., 1998; Moll & Summers, 1999; Moll et al., 1999; Smith et al., 2000; Keinanen et al., 2002). Gram-negative bacteria are opportunistic and typically faster growing (Paul & Clark, 1996). Biofilms associated with the recycled distributions of Sites C and D showed much greater proportions of Gram-positive bacteria, particularly those passing secondary treated waters, and fungi. Similar abundances of fungal and Gram-negative PLFAs were

measured from the Site B summer biofilm. Potential controls on these unusual PLFA distributions are discussed in detail in Section 3.4.4.

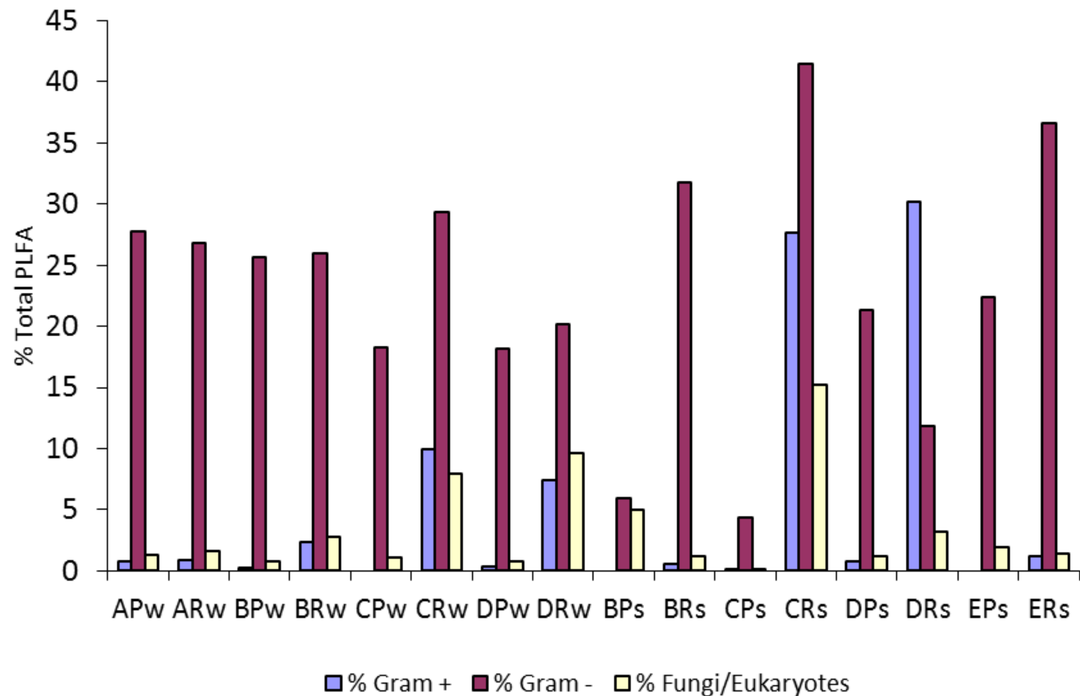


Figure 3.9 PLFA determined microbial group distribution of winter and summer sampled biofilm samples for potable (P) and recycled (R) distribution systems.
w = winter, s = summer.

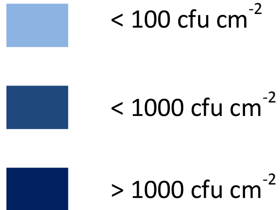
Samples from Sites C and D also contained high concentration of 18:1ω11*c* (Table 3.13) indicative of Gram-negative *proteobacteria* (Smith et al., 2000), assuming 18:1ω11*c* is the main component of the co-eluting 18:1ω11*c*/18:1ω9*t* peak - 18:1ω9*t* is a fungal marker and likely to be less significant. *Proteobacteria* have been shown to be a dominant microbe of some wastewater treatment plants (Werker & Hall, 1998; Werker, 2003; Werker, 2006) and distribution systems (Kalmbach et al., 2000; Santo Domingo et al., 2003; Schmeisser et al., 2003; Martiny et al., 2005; Hoefel et al., 2005).

Microbiological Insights

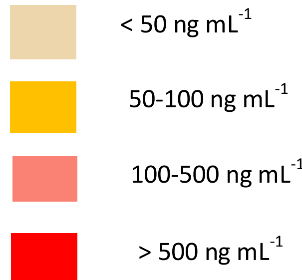
The present PLFA data was generally consistent with the previous characterisation of the biofilm suite via conventional cultivation techniques (Heterotrophic Plate Counts, HPC; Storey & Kaucner, 2009). A brief summary of outcomes from the Storey & Kaucner (2009) study together with PLFA concentrations from the present study are presented in Fig. 3.10.

	A		B				C				D				E	
	Winter		Winter		Summer		Winter		Summer		Winter		Summer		Summer	
	Pot	Rec	Pot	Rec	Pot	Rec	Pot	Rec	Pot	Rec	Pot	Rec	Pot	Rec	Pot	Rec
Coliforms																
E.coli																
Enterococci																
Aeromonads																
Pseudomonads																
Legionaellae																
Clostridia																
Mean PLFA																

HPC Results (Storey & Kaucner 2009)



PLFA Results (Present study Tables 3.10-3.13)



Pot = Potable, Rec = Recycled

Figure 3.10 Total PLFA concentrations (shown shaded in tan/gold/pink/red) and HPCs (shaded in blue; Storey & Kaucner, 2009) of recycled distribution system biofilms.

Pathogenic bacteria were found to be most prevalent in biofilms sampled from Sites C and D (Fig. 3.10; Storey & Kaucner 2009), which also showed highest PLFA diversity. The relatively high total PLFA concentrations of Site C and D BFs (Table 3.11) and the dominant Gram-negative bacteria (e.g., *Coliforms* (except *proteus*), *E. coli*, *Aeromonads*, *Pseudomonads* and *Legionaellae*) and Gram-positive bacteria (e.g., *Enterococci* and *Clostridia)* coincide with pathogenic species of interest identified by Storey & Kaucner, (2009). The respective Gram-negative PLFAs (i.e., OH, Cyclo, MU groups) and Gram-positive PLFAs (i.e., TB group) might relate to these bacteria. The HPC data compliments the PLFA data, by providing species specific diagnosis.

3.4.4 Insight into Environmental Effects on Biofilm Communities

Biofilm communities react to specialised environmental conditions at the macro and micro-level. Influences which can affect biofilm growth include nutrient availability, pipeline material, temperature, flow rates and presence of a pre-established microbial community. To investigate the main controls on the microbial community composition of the biofilms, the PLFA data was correlated with other known environmental and water quality information (Table 3.4).

3.4.4.1 Water Temperature

Aquatic bacterial growth typically increases with temperature (White et al., 1991) from approximately 20–40°C but may be impeded by lower or higher temperatures (Melo & Pinheiro, 1992; Smith et al. 2000; Ollos et al., 2003; Rao*,* 2010), so low temperatures are typically maintained in distribution systems to minimise biofilm development (Moll et al., 1999; Smith et al., 2000). The Australian Drinking Water Guidelines (NHMRC, 2011) recommend maintaining water below 20°C in order to minimise bacterial regrowth.

The water temperatures of the present sample suite differ with climatic season and geographical location (Table 3.4). Fig. 3.11 shows the relationship between total PLFA concentration (Table 3.10) and water temperature (Table 3.4) divided into seasonal and distribution system groups. Biofilm data shows a general increase in concentration with temperature— consistent with the previous findings

However, there were a few exceptions to this general trend. The influence of climatic temperature on biota had different effects on some biofilms. The summer sample of the

biofilm (BF 150) of the recycled water at Site C (22.9°C, 2180.3 ng mL^{-1}) showed an almost 20 times greater PLFA concentration than the corresponding winter sample (BF 47, 16.2°C, 755.6 ng mL^{-1}), illustrating the importance of the 20°C threshold described in the Australian Drinking Water Guidelines (ADWG; NHMRC, 2011).

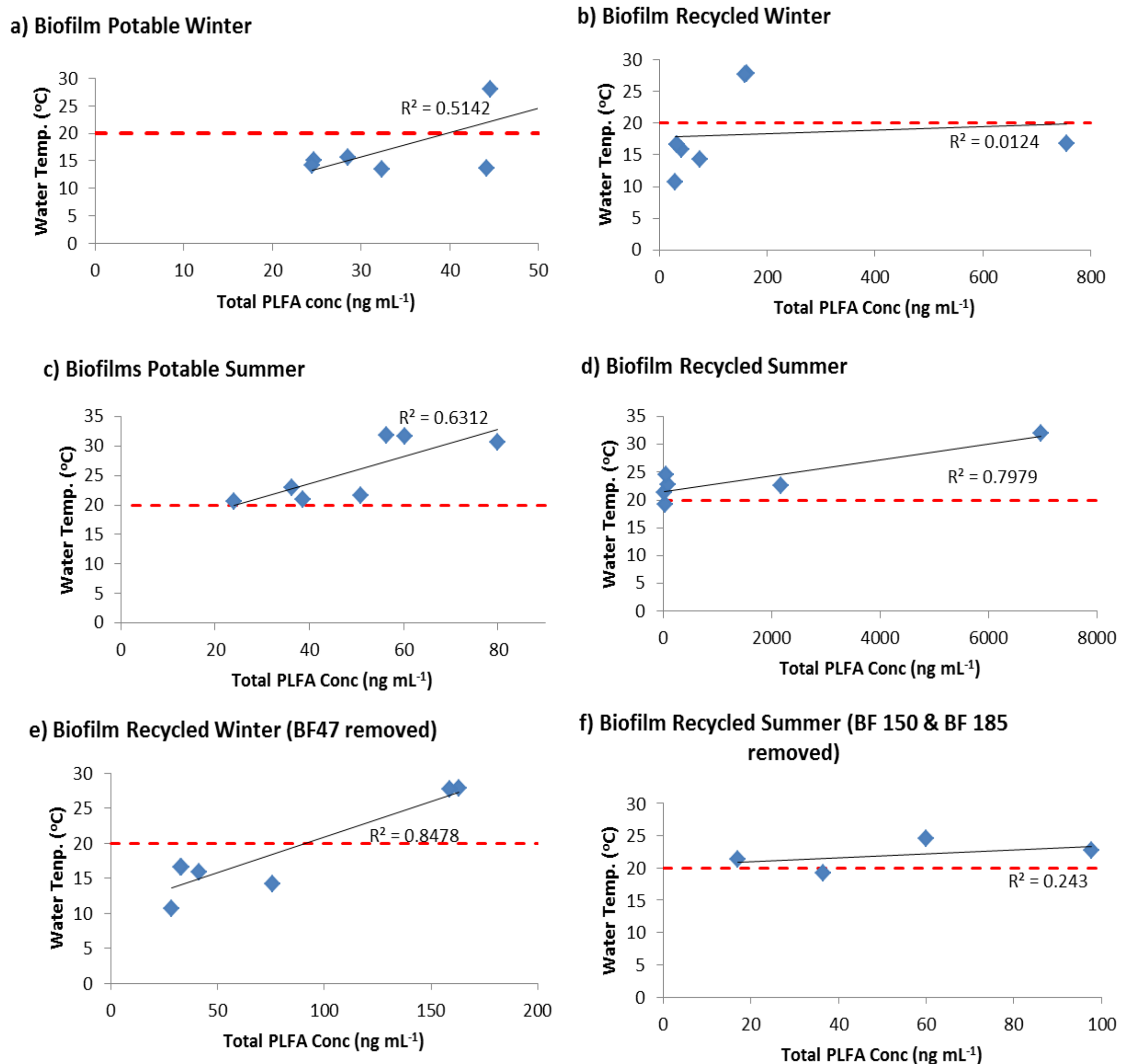


Figure 3.11 Linear regression showing water temperature against PLFA concentration for each sample grouping, graph (e) shows the stronger correlation between temperature and PLFA concentration (*Nb*. BF 47 - (with an unusually high PLFA concentration - was not included in this plot. Red dash line indicates 20°C AWDG (NHMRC, 2011) recommendation.

Whereas the summer recycled water BF 131 at site B (20.3°C, 72.3 ng mL^{-1}) was only slightly greater than the corresponding winter sample BF 29 (13.6°C, 54.9 ng mL^{-1}) despite the fact that the temperature difference in both cases were identical (6.7°C). On this basis, the temperature differential between seasonal samples is less important than the maximum

temperature when above 20 ^{o}C. Removing the BFs with very high PLFA concentrations from the summer recycled plot (Graph f, Fig. 3.11) results in a lower correlation (compared to Graph d, Fig. 3.11) showing that temperature has a strong influence. Nevertheless, the existence of very high PLFA concentration from some biofilms during both winter and summer (as illustrated by BF 47 in Graph b, Fig. 3.11) indicates other factors besides high water temperature can promote microbial growth (discussed in 3.4.4.2 & 3.4.4.3).

No obvious changes in the distribution of PLFA groups with climatic season were evident from the present analysis (Fig. 3.10). Previous studies have reported altered proportions of some PLFA groups in response to water temperature fluctuations, for example Moll et al. (1999) showed a decrease in monounsaturated fatty acids with lowered temperature. Altered production of *iso*-PLFAs (especially *i*15 and *i*17) and increase in cyclopropyl fatty acids in response to increasing temperature have also been reported (Petersen & Klug, 1994).

3.4.4.2 Nutrient Availability

Biofilms of recycled waters showed higher PLFA concentrations than those of potable waters mirroring their higher concentrations of TOC, P and N. Higher nutrient loads will support biofilm growth, with P often the most biologically limiting nutrient of drinking water distribution systems. Water treatment aims to reduce nutrients to very low levels. Gram-negative bacteria are often the most abundant microbial group in low nutrient substrates such as potable water (Christine et al., 1992; Smith et al., 2000; Lehtola et al., 2004a). Most pathogenic bacteria of concern in potable water systems belong to the Gram-negative grouping including coliform bacteria such as *Citrobacter, Enterobacter, Escherichia, Klebsiella* as well as *Salmonella, Shigella, Legionella, Pseudomonas, Heliobacter* and *Aeromonas* (Madigan et al., 2011).

3.4.4.2.1. Influence of Source Water Treatment

The source of the water and the type and degree of treatment are the main controls on the nutrient load of distribution systems. Secondary biological treatment (Sites C, D and E) produces Environmental Protection Agency EPA class C recycled water (EPHC, 2006). Tertiary treatment (Sites A and B), via methods such as sand, micro or dissolved air floatation filtration, or UV irradiation, reduces pathogens and produces higher quality class A recycled water (EPHC, 2006).

Generally higher PLFA abundances were measured in recycled samples. For confidentiality reasons, limited detail of the treatment plants was provided with the samples. It can be noted (Table 3.3) however, that water at Recycled Site D had not been disinfected prior to distribution. Nevertheless, its Gram-negative bacteria dominant PLFA distribution was generally similar to the other biofilms. Significant differences in PLFA/microbial community composition were evident between potable and recycled water at Sites C and D, but less so at Site E (illustrated by Fig. 3.9). Storey & Kaucner (2009) reported that water treatment practiced at Sites C and E were almost identical, so differences observed in community composition must be due to other parameters such as temperature, nutrient availability and hydraulic flow (discussed in this Section and 3.4.4.3).

Like most of the waters studied, potable samples from Sites C and D were dominated by Gram-negative bacteria. A greater PLFA contribution from fungi and Gram-positive bacteria was evident in the recycled samples. Similar Gram-negative bacteria dominant PLFA distributions (Fig. 3.8) were measured from the potable and recycled Site A and B biofilms. The similarity of the recycled water to the potable water (in general) reflects the high degree of treatment to which the recycled water has been subjected. Although the Site B potable summer BF was seen to have a larger proportion of PLFA derived from Gram-positive bacteria and fungi, the sample was still overwhelmingly dominated by Gram-negative bacteria. In general therefore, Class A recycled water (Sites A and B) is closer to potable water quality than Class C (Sites C and D) recycled water. Gram-positive bacteria were the major microbial group of the Site D recycled water, the only sample in which Gram-negative bacteria was not the dominant group.

3.4.4.2.2 Influence of Disinfection

Disinfection and maintaining chlorine residual in the distribution systems aim to limit the development of microbial community structure and function. Extracellular exudates, pipeline materials, as well as attached and free-living bacteria (which can be deposited unevenly along a distribution system) all exert a chlorine demand (Momba et al., 1998; Lu et al., 1999; Chandy & Angles, 2001).

However, as shown by Figure 3.12, there was only a weak correlation between total PLFA concentration and free chlorine residual, particularly during winter when the lower temperatures impede microbial growth.

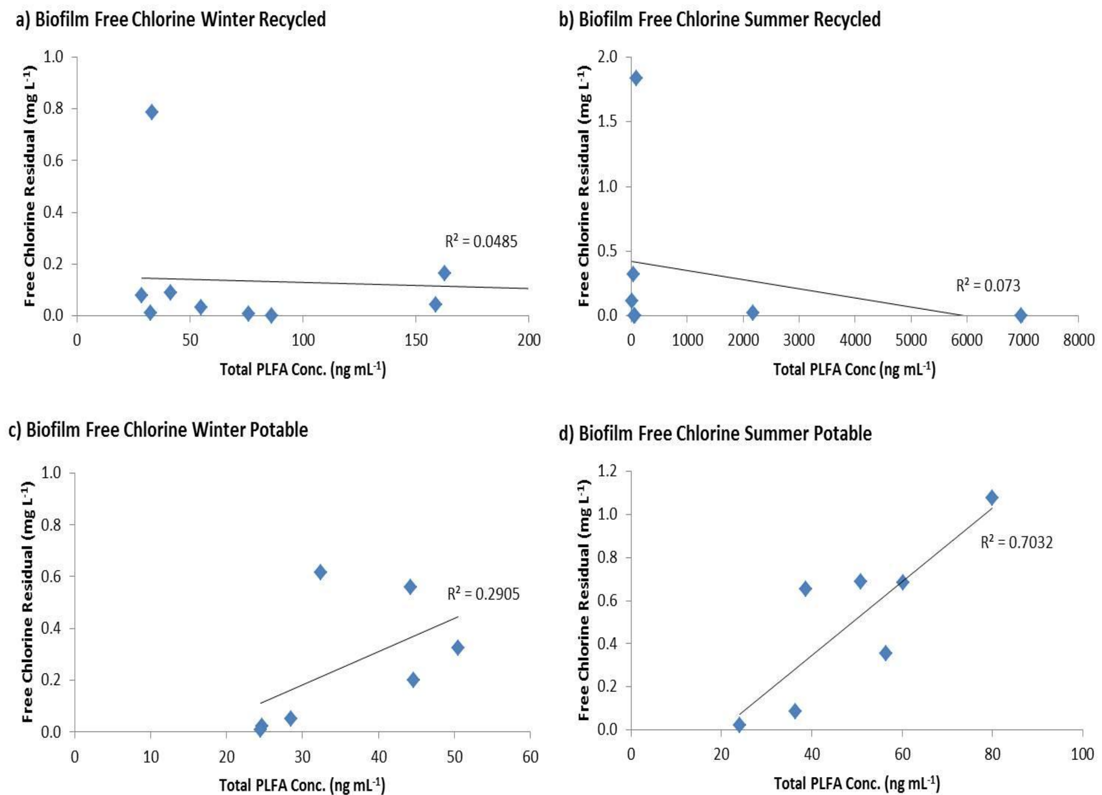


Figure 3.12 Linear regressions showing free chlorine residual against PLFA concentration for each potable and recycled water during winter and summer.

The strongest correlation was seen in summer potable systems (R^2 = 0.7032). The highest free chlorine residual (0.92 mg L^{-1}; Table 3.9) was measured in the recycled water of Site E during summer, and the associated BF showed typical Gram-negative dominant PLFA signature. However, the total PLFA concentration of this BF, and in particular yields of Gram-positive and fungal markers were less than other waters such as summer recycled Sites C and D, which had very low chlorine residual (0.02 mg L^{-1}, Site C; 0 mg L^{-1} Site D; Table 3.9). This suggests Gram-positive bacteria and fungi are more significantly affected by high chlorine residual. The biofilm PLFA concentrations did reflect an increase with distance from the treatment plant, consistent with Storey & Kaucner (2009) finding of an up to four times increase in heterotrophic bacteria along a distribution system.

There are various possible origins of organisms that colonise the pipes in drinking water distribution. Most are generally thought to relate to the source water biota (Camper et al., 1996), but others have reported that bacteria can also be indigenous to the pipes (Pepper et al., 2004; Storey & Kaucner 2009). Another possible source of entry into the system is

via cracks in pipelines or during routine maintenance of distribution systems (Bauman et al., 2009).

3.4.4.2.3 Influence of Organic Carbon Content of Water

Biodegradable organic carbon (BDOC) is thought to be the main control on bacterial growth in drinking water systems (Vanderkooij et al., 1982; Bachmann & Edyvean, 2005). In the absence of BDOC data, the available TOC/DOC data (Table 3.4) was used as a proxy for BDOC, although TOC has not always been found to be a good indicator of BDOC levels (Zacheus and Martikainen, 1995; Volk et al., 1994). The present data set shows a good correlation between PLFA and TOC concentrations for winter recycled systems (Fig 3.13a, R^2 = 0.9759).

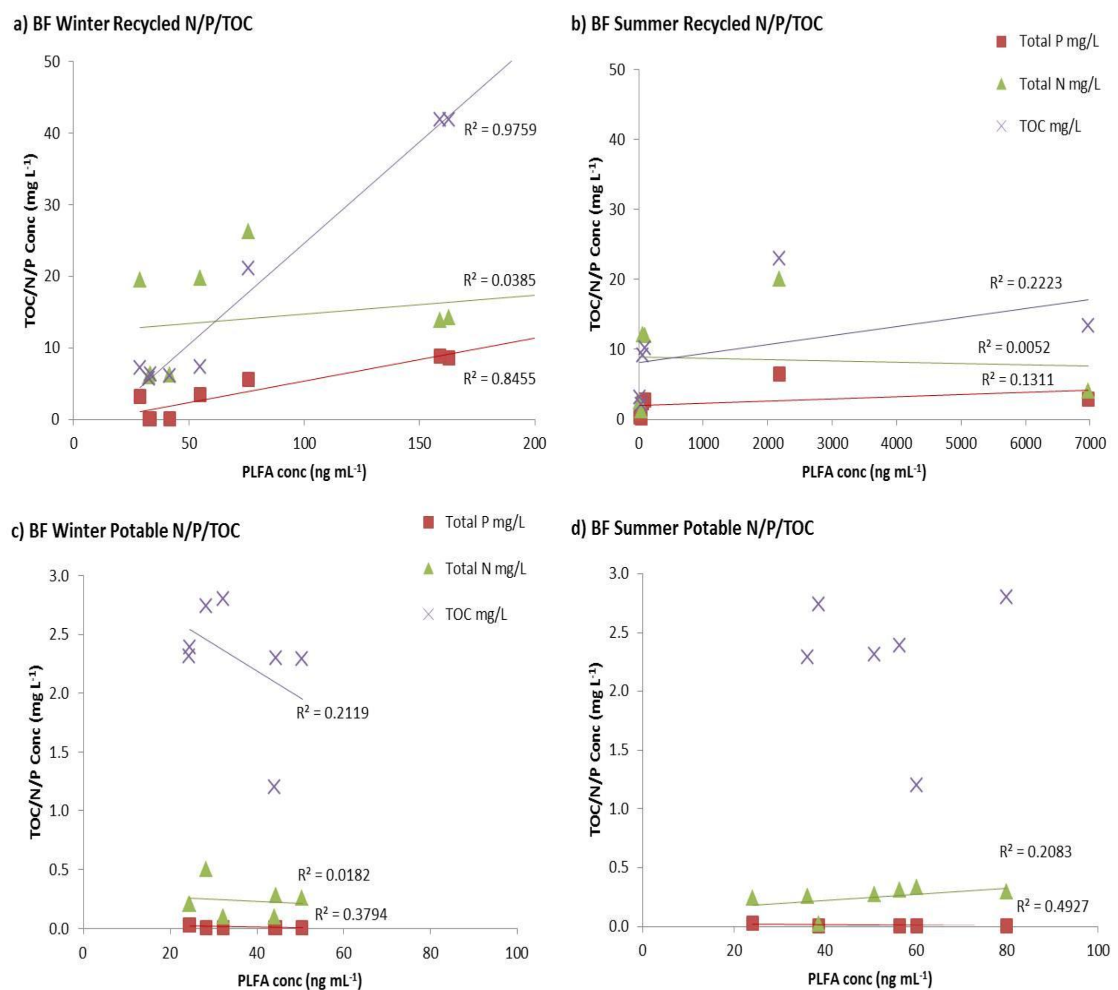


Figure 3.13 Linear regression showing nutrient and TOC concentrations against PLFA concentration for potable and recycled waters during winter and summer.

This relationship contrasts previously reported poor correlation of TOC with total numbers of microbes detected in drinking water (Zacheus & Martikainen, 1995). The highest TOC values were found in recycled winter biofilms from Site D (BF 64, 41.8 mg L^{-1} and BF 66, 41.8 mg L^{-1}), and these BFs also showed high PLFA concentrations (BF64 = 162.8 ng mL^{-1}; BF66 = 158.8 ng mL^{-1}). However, the highest PLFA concentration were detected in BF 47 (755.6 ng mL^{-1} with TOC=21.1 mg L^{-1}) and 150 (2180.3 ng mL^{-1}; TOC= 23.0 mg L^{-1}) despite their significantly lower levels (*Cf*. BF64 and BF66).

3.4.4.2.4 Influence of Nitrogen Content of Water

Five of the highest PLFA concentrations (BFs 150, 47, 64, 65 and 166) were detected in systems which had 'total N' concentrations of over 12 mg L^{-1} (Table 3.4 and Tables 3.10–3.13. *Nb*. 'total N' data was unavailable for BF 185). Beyond this, little correlation between PLFA and N concentration was observed (Fig. 3.13).

3.4.4.2.5 Influence of Phosphorous Content of Water

Freshwater biofilm growth is often limited by naturally low P levels (Miettinen et al., 1997; Sathasivan et al., 1997; Chandy & Angles, 2001; Keinanen et al., 2002; Lehtola et al., 2004a). The winter recycled data (Fig. 3.13a) show a correlation between total PLFA and P concentrations. Increasing P concentration has previously been shown to shift community composition—for example as reflected by concentrations of signature Gram-negative bacterial PLFAs including 16:1ω7*c* (Miettinen et al., 1997, Keinanen et al., 2002). The biofilms with highest concentrations of 16:1ω7*c* (i.e., BFs 28, 29, 20, 47, 48, 185; Table 3.10) were associated with water containing 'total P' concentrations of over 3 mg L^{-1} (Table 3.4).

Fungi has been detected in several potable water biofilms (Doggett 2000; Hendrickx et al. 2002; Hageskal et al., 2006; Hageskal, 2009), and a potential association with high P concentration—nutrient imbalance was suggested by Hendrickx et al. (2002) as a possible explanation. However, the present 123 biofilm suite showed little correlation between P concentrations (Table 3.4, Fig. 3.13) and fungal PLFAs (Fig. 3.9).

3.4.4.3 Significance of Water Age

Continuous flow of water through a pipeline helps to disperse residuals and particulates in the water. However, flows may vary due to pumping action, different pipe diameters, filters, tanks, bends, valves, dead legs and other physical properties of the pipeline. The

biofilms were subject to the prevailing flow conditions at each sampling point (Lehtola et al., 2004a&b).

Recycled Site C water from which biofilms 47 (winter) and 150 (summer) were sampled, represented a 'dead-leg' of the distribution system, (Storey, personal communication, 2007). The increasing water age and decreasing disinfectant residual of dead-legs can promote microbial growth (Fransolet et al., 1985; LeChevallier, 1990), and indeed PLFA concentrations of these biofilms were the highest of the sample suite.

The PLFA concentrations of BF 48 were much lower (Table 3.11). BF 48, whilst further from the chlorination point than BF 47, was located in a free flowing segment of the distribution system with a smaller pipe diameter (BF 48 = 80 mm; BF 47 = 150 mm; Storey & Kaucner 2009), both of which are less favourable to biofilm development (Huck & Gagnon, 2004). Surface roughness is also important to the establishment of biofilm (Percival et al., 1998) whilst the specificity of some biofilms to particular parts of the pipe network—e.g., discrete locations of deposits—has also been noted (LeChevallier et al., 1987). The internal surfaces of the pipes shown in Fig. 3.4 are good examples of differing surface roughness. It is interesting to note that biofilm BF 150, which had very high PLFA concentrations (2180.3 mg L^{-1}) was sampled from the surface of the pipe work at Site C which is shown in Fig 3.4, and the pipe in the photo depicting an obvious biofilm.

3.4.4.4 Summary of Molecular Characterisation

Table 3.11 summarises the main findings of the distribution system biofilms of recycled water. Potable water systems all dominated by Gram-negative PLFAs and their analysis provided little insight. Sites C and D contained the greatest diversity of 'diagnostic' PLFAs. Sites C and D were similarly found to contain high counts of pathogenic bacteria (Storey & Kaucner, 2009). Temperature, substrate availability, chlorine residual and water flow all represent important controls on biomass growth.

Table 3.11 Summary of main molecular findings of PLFA analysis of recycled water system biofilms. * = Similar community composition to potable.

Site	Treatment level	Diagnostic PLFAs		Microbial subgroup	Ref
		winter	summer		
A	Tertiary	n/a	18:1ω11*c**	Gram -ve bacteria*	Zelles, 1997
B	Tertiary	18:1ω11*c* *	18:1ω11*c**	Gram -ve bacteria*	Zelles, 1997
C	Secondary	18:1ω11*c* 18:1 ω9*c* 16:1 ω7*c* *i*15:0	18:2 (9,12) 18:1 ω9*t* 18:1ω11*c* 18:1 ω9*c* 16:1 ω7*c* *i*16:0 *a*15:0 *i*15:0 *a*17:0 *i*17:0	Fungal/Eukaryotic Gram +ve bacteria Gram -ve bacteria	Frostegard & Baath 1996 Zelles, 1997 Zak et al., 1996 Mckinley et al 2005
D	Secondary	18:1ω11*c* 16:1 ω7*c* *i*16:0 *i*15:0	18:2 (9,12) 18:1 ω9*t* 18:1ω11*c* 18:1 ω9*c* *a*15:0 *i*15:0 *a*17:0 *i*17:0	Fungal/Eukaryotic Gram +ve bacteria Gram -ve bacteria	Petersen & Klug 1994
E	Secondary	18:1ω11*c* 16:1 ω7*c*	n/a	Gram -ve bacteria	Zelles, 1997

3.4.5 Stable Carbon Isotopic Characterisation of Abundant PLFAs

Isotopic characterisation of PLFAs may provide further information about the dynamics of carbon cycling and how different environmental factors interact, including the substrate utilisation and metabolic processes related to biofilm development.

Stable carbon isotope ratios of several PLFAs from biofilms of the Site C recycled waters in winter (BF 47) and summer (BF 150) and Site D recycled water in summer (BF 185) are presented in Table 3.12. These samples contained the highest PLFA concentration, yet $\delta^{13}C$ measurement was still limited to just several of their most abundant PLFAs, due to

sensitivity challenges discussed previously for $\delta^{13}C$ measurement of the plant leachate PLFAs (Section 3.4.2). The PLFA concentrations of biofilms from Site E recycled water in summer (BF 64 and BF 66) were just a little lower—despite the high TOC of these samples—yet their slightly lower concentrations proved insufficient for reliable and reproducible $\delta^{13}C$ analysis.

Table 3.12 $\delta^{13}C$ values of biofilm PLFAs. These values have been corrected for methyl ester derivatsation. (n) = number of analyses. Standard deviations were determined from uncorrected data.

PLFAs	$\delta^{13}C$ PLFAs in Biofilm Samples (‰) 47 (site C) Winter Rec	150 (site C) Summer Rec	185 (Site E) Summer Rec
*i*15:0	-	-28.8 ±0.78 (2)	-
*a*15:0	-	-25.9 ±0.56 (2)	-
16:1ω9c/ω7c	-	-23.4 ±1.45 (2)	-27.1 ±0.12 (2)
16:0	-24.8 ±0.04 (2)	-26.0 ±1.23 (2)	-27.7 ±0.26 (2)
*i*17:0	-	-	-24.2 ±0.89 (2)
*a*17:0	-	-	-21.2 ±0.54 (2)
17:0	-26.6 ±1.18 (2)	-27.3 ±0.94 (2)	-
18:1ω9c	-	-21.5 ±0.84 (2)	-24.4 ±0.07 (2)
18:1ω9t/18:1ω11c	-	-21.1 ±0.79 (2)	-23.6 ±0.96 (2)
18:0	-26.4 ±0.04 (2)	-26.6 ±0.23 (2)	-23.6 ±0.23 (2)
cy 19:0	-	-26.7 ±0.19 (2)	-

Seasonal influence (BF 47 & BF 150)

The influence of temperature on substrate utilisation and growth rates can affect $\delta^{13}C$ composition (Jahnke et al., 1999; Boschker et al., 2005; Ekblad et al., 2005). $\delta^{13}Cs$ of the non-source specific straight chain PLFAs 16:0, 17:0 and 18:0 were measured in both the winter (BF 47) and the summer sample (BF 150) associated with Site C recycled water.

Their $\delta^{13}C$ values were up to 1 **‰** lighter in summer than winter. This could be related to increased biomass growth rates or the algal blooms which occurred in summer as wastewater of the recycled system at Site C had been transferred to large outdoor ponds before distribution (Storey & Kaucner, 2009). It is possible that algae may have flourished in the warmer summer months. The absence of 18:1ω9*c* in the related winter sample (BF 47) provides further support for a summer algal bloom theory.

Many factors can influence the $\delta^{13}C$ of lipid membranes however, including, competition, type and bioavailability of substrate, light and temperature effects (Jahnke et al., 1999; Wick et al., 2003; Londry et al., 2004; Zhang et al., 2005; Staal et al., 2007). The isotopically heavier values in winter might be the result of less discrimination against ^{13}C fractionation in colder temperatures and substrate requirements (Wiebe et al., 1992; Yumoto et al., 2004).

Gram-positive bacteria

Jones et al. (2003) reported that straight chain PLFA 16:0 can be used as a proxy for overall biomass. In the present study, Biofilm data was referenced relative to 16:0 in absence of the $\delta^{13}C$ of bulk biofilms, as sample quantities were not sufficient to measure this value. In BF 150, the $\delta^{13}C$ of *i*15:0 was depleted by 2.8 ‰ relative to the 16:0 PLFA, the reverse of the more usually observed depletion of 16:0 relative to PLFAs of Gram-positive bacteria (Burke et al., 2003; Kramer & Gleixner, 2006; described in Section 3.4.2), which have a capacity to degrade and utilise relatively more recalcitrant carbon sources (Burke et al., 2003). However, there was negligible difference between the $\delta^{13}C$ of 16:0 and that of *a*15:0 of the same BF sample. This suggests that the Gram-positive bacteria from which the *a*15:0 and the i15:0 PLFAs were derived may have been from different microbial sub-communities, potentially using different carbon sources, perhaps reworked material that in itself was more depleted in ^{13}C or entrained particulate carbon (e.g., Macko et al., 1987; also refer Chapter 4 for detailed discussion). Alternatively a more ^{13}C depleted carbon source may relate to detrital or isotopically light faecal (e.g., from zooplankton, commonly detected at biological wastewater treatment facilities; Guosheng, 2002; Curtis, 2003) substrate utilisation (Breteler et al., 2002).

The $\delta^{13}C$ values of *i*17:0 and *a*17:0 in BF 185 (not measured in BF 150 due to low concentration) were 3.5 **‰** and 6.5 **‰**, respectively, more ^{13}C -depleted relative to 16:0. The 3 **‰** difference between these terminally methylated PLFAs is further suggestive of different Gram-positive bacterial sub-groups. The differences evident in the $\delta^{13}C$ signatures and molecular distribution of the terminally branched PLFAs at site C (BF 150) and site D (BF 185) also reflect the different Gram-positive bacteria communities at these sites.

A similar ~3 ‰ $\delta^{13}C$ differential was evident between the *iso*- and *anteiso*- isomers of 15:0 and 17:0. A 3–4 ‰ enrichment of *anteiso*-branched alkanes (*Cf*., *iso*-alkanes) was previously

reported for tobacco lipids (Grice et al., 2008). This isotopic trend—separately evident in two different recycled water schemes (BF 150 and BF 185) and tobacco plants—shows that the valine and *iso*-leucine amino acid precursors of *iso* and *anteiso*- branched fatty acids, respectively, are utilised by different sub-communities reflected by different biosynthetic isotopic effects.

Gram-Negative Bacteria

The $\delta^{13}C$ value of co-eluting Gram-negative markers 16:1ω9*c*/16:1ω7*c* were more enriched in ^{13}C than 16:0 in BF 150 (2.6 ‰) and BF 185 (0.6 ‰). This differs from the trend evident in the Maize and Marri leachate data (Table 3.9), where the $\delta^{13}C$ value of 16:1ω9*c*/16:1ω7*c* from Marri was depleted in ^{13}C relative to bulk vegetation biomass by 8.4 ‰ and a similar difference (6.3 ‰) was seen in Maize. This leachate data is typical of Gram-negative bacteria which generally have relatively depleted ^{13}C values (Burke et al., 2003; Kramer & Gleixner, 2006) attributed to their opportunistic feeding behaviour for substrates such as recent plant material (Paul & Clark, 1996; Burke et al., 2003).

Fungi

18:1ω9*c,* which is often assigned a fungal/eukaryotic source (Zak et al., 1996) had a $\delta^{13}C$ value of -21.5 ‰ in BF 150 and -24.4 ‰ in BF 185. These values reflect a ^{13}C enrichment by 3.3 to 4.5 ‰ compared to 16:0, but may be compromised by partially co-elution with the Gram-negative 18:1ω9*t*/18:1ω11*c* (-21.1 ‰) present in lower abundance.

A PLFA based study by Phillips et al. (2002) observed that fungi (identified by PLFAs 18:1ω9*c* and 18:2 (9,12)) were more efficient at utilising the disaccharide cellobiose than other soil microbes. Esperschutz et al. (2009) also separately showed fungi (identified by 18:2 (6,9)) had a notable preference for 'labile' plant carbon. So in the case of the present biofilms, fungi may preferentially utilise the sugar fraction of available organics. As sugars are isotopically enriched compared to many other organic fractions (e.g., Brugnoli & Farquhar, 2000) this would account for the heavier values measured for the fungal PLFA 18:1ω9*c* in BF's 150 and 185. The relatively ^{13}C enriched isotopic values observed for the fungal PLFAs 18:1ω9*c* (or potentially others like 18:2 (9,12) also mirror the ^{13}C heavy value of the perylene (*Cf.* -26.5 to -27.5 ‰, Grice et al., 2009) detected in lignin, a highly condensed polycyclic aromatic suspected to be of fungal origin (e.g., Wolkenstein et al., 2006). The occurrence of lignin in domestic wastewater is generally low. However, Site C

received some industrial wastewater input (Kaucner & Storey, 2009), and this may contain lignin at appreciable levels (Mobius & Helble, 2004) which could support fungal growth.

3.5 Summary

Table 3.12 summarises the main findings of the isotopic characterisation of the biofilms from Site C (BF 150) and Site D (BF 185).

Table 3.13 Major findings from the PLFA $\delta^{13}C$ data of BFs 150(Site C) and 185 (Site D). Nm = not measured. EPS = Extracelluar Polymeric Substances (sugars).

			Assimilation		Biosynthesis	
BF	PLFA	Microbial Group	Δ 16:0 ‰	Possible substrate	Δ *iso*-PLFA ‰	Amino acid precursor
150	*i*15:0	Gram +ve	-2.8	mixed, reworked	+2.9	Valine
	*a*15:0		+0.1	same as bulk microbial community		*Iso*leucine
	16:1ω7*c*/ 16:1ω9c	Gram -ve	+2.6	sugars EPS	Nm	n/a
	18: 1ω11*c*		+4.9	EPS	Nm	Nm
	cy19:0		-0.7	same as bulk microbial community	Nm	Nm
	18:1ω9*c*	Fungi	+4.5	EPS, lignin	Nm	Nm
185	*i*17:0	Gram +ve	+3.4	sugars	+3.0	Valine
	*a*17:0		+6.5	sugars		*Iso*-leucine
	16:1ω7*c*/ 16:1ω9*c*	Gram -ve	+0.6	same as bulk microbial community	Nm	Nm
	18:ω11*c*		+4.1	EPS	Nm	Nm
	18:1ω9*c*	Fungi	+3.3	EPS, lignin	Nm	Nm

The pipeline biofilms comprise cellular and inorganic material embedded in a matrix of sugar and protein rich extracellular polysaccharide substances (EPS; Flemming et al., 2000; Sutherland, 2001), thus representing a more complex matrix than the plant leachates presented earlier (Section 3.3.2) in this chapter.

EPS can comprise 50–80 % of the biofilm mass (Flemming et al., 2000). Such sugar-rich substances tend to be ^{13}C enriched compared to other common sources such as soluble plant matter, which may account for the relatively ^{13}C enriched values of several bacterial PLFAs and possibly also fungal PLFAs. Additionally, several studies have reported that rapid mineralisation of sugars by Gram-negative bacteria and fungi can produce metabolites which can then be utilised by the Gram-positive community (Treonis et al., 2004; Elfstrand et al., 2008), complicating the identification of a well-defined carbon substrate.

The $\delta^{13}C$ (and molecular) results show that the microbial structure and function of distribution system biofilms is very different to the aquatic biota supported by soluble plant material - as observed for the PLFA data of the plant leachates. The data of these systems also differs to the PLFA based microbial data of natural estuarine systems (Jones et al., 2003) and soil organic matter (Burke et al., 2003, Kramer & Gleixner, 2006) further reflecting the biological heterogeneities of both aquatic and terrestrial environments.

3.6 Conclusions

- Molecular and stable isotopic measurement of PLFAs were used to characterise the microbial communities of plant leachates and a suite of distribution biofilms, demonstrating the respective value in understanding the biota of different aquatic systems.

- The CSIA of individual PLFAs encountered significant limitations (e.g., MS sensitivity, linearity and GC resolution), nevertheless, where measurable their $\delta^{13}C$ values can provide important information on carbon substrate utilisation and dynamics as summarised below.

- The PLFA $\delta^{13}C$ data of the plant leachates and distribution biofilm samples were compared to other available water quality and organic substrate or nutrient data where possible. Several important relationships were identified. For example, the degree of treatment of recycled water was identified as a major control on distribution biofilm structure, with the higher DOC and nutrient concentration of secondary treated waters supporting larger microbial concentrations, particularly of Gram-positive bacteria and fungi (or algae). Free chlorine residual and temperature were also important influences on bacterial growth and community

composition. Of all the water quality data, concentrations of the main PLFA groups showed the strongest correlation with increasing water temperature over sample range of 11–32 ^{o}C.

- The δ^{13}C data provided new information about preferential assimilation of some organic moieties of the plants by different microbial sub-groups.

- The most diverse microbial communities of the plant leachates were detected from the Marri and Spinifex, attributed to the plentiful availability of 'labile' organic substrates combined with a relatively refractory pool which could be exploited by niche communities.

- The PLFAs of Gram-negative bacteria supported by the leached plants were ^{13}C depleted compared to other PLFAs, consistent with their utilisation of an abundant and isotopically light terpenoid fraction. Gram-positive bacteria were ^{13}C enriched suggesting utilisation of more refractory and isotopically heavy material such as lignin or lignocelluloses. The respective Gram-positive and Gram-negative isotopic trends were consistent with several previous studies (Burke et al., 2003; Jones et al., 2003; Waldrop & Firestone, 2004; Kramer & Gleixner, 2006).

- The δ^{13}C signature of the photosynthetic pathway of the supporting vegetation (C3 or C4) was reflected by PLFA δ^{13}C values indicating that this CSIA technique can help in elucidating sources of microbial species impacting drinking water systems. It also offers a possibility of tracking inputs, and potentially establishing the connectivity and relationships between organic substrates and the biota they support at different stages of the drinking water system.

- A ^{13}C enrichment of *iso*- (but not significantly *anteiso*-) terminally branched PLFAs (Gram-positive bacteria biomarkers) of two separate recycled water schemes mirrored previous isotopic-PLFA trends identified in tobacco plants (Grice et al., 2008; Zhou et al., 2010). This is attributed to the utilisation of different amino acid precursors (valine and *iso*-leucine for *iso* and *anteiso*- branched fatty acids, respectively) consistent with the molecular data reported for bacteria by Kaneda (1991).

- PLFA composition of the distribution system biofilms showed variance with site, season and source water grade. Gram-negative bacteria were generally dominant, consistent with previous research (LeChevallier et al., 1987; Payment et al., 1988; Lahti, 1993; Moll et al., 1998; Percival et al., 1998; Moll & Summers, 1999; Moll et al., 1999; Smith et al., 2000; Keinanen et al., 2002).

- The interpretation presented here for the measured $\delta^{13}C$ data serves to demonstrate the type of microbial community information and insight into carbon cycling and biosynthetic pathways within the high chemical and biological complexity of water distribution systems. It offers the potential to complement the information provided by molecular composition.

- At present sensitivity levels of this analytical technique application would be restricted to materials with very high microbial concentrations, which does limit its practical application to organic elements of potable water systems, but may be presently more applicable to the high biological loads of recycled or waste water systems.

As a closing statement to this study, the application of CSIA of microbial PLFA biomarkers of aquatic (and terrestrial) environments must be considered to be at an early stage of development. Nevertheless, the microbial characterisation of the plant leachates and biofilm samples did reveal the significant potential of this technique to provide unique information about the sources and fate of the biota of freshwaters and therefore warrants further research and development.

Chapter 4

Off-line Pyrolysis for Molecular and Stable Isotope Characterisation of a Water Treatment Plant Membrane Foulant

4.1 Overview

4.1.1 Pyrolysis and Compound Specific Stable Isotope Analysis

Pyrolysis of complex organics from extant and sedimentary organic matter yields complex product distributions which are not usually compatible with the stringent GC resolution requirements of CSIA. As such, off-line pyrolysis methods conducted on large sample amounts are considered the best approach so that the large pyrolysate yield can be subjected to additional preparation procedures to provide less complex fractions facilitating baseline GC separation of individual compounds to allow accurate stable isotope measurements.

Whilst various off-line pyrolysis methods exist, here the recently developed method of Catalytic HyPy is utilised. HyPy is conducted at moderate pyrolysis temperatures (300–550°C) in the presence of a dispersed sulfided molybdenum catalyst under high hydrogen pressure (>10 Mpa) which effectively quenches the reactive radicals generated by pyrolysis, thus helping preserve primary structural information. Berwick (2010) found that the structural integrity of hopane biomarkers of bacterial cell membranes was maintained with HyPy, confirming it as a useful technique to investigate bacterial and other source inputs, to complex macromolecular material. Significantly, Meredith et al. (2010) showed that the stable carbon isotopic signature of three C_{27} steroid standards retained isotopic integrity in the resultant HyPy sterane products.

In this chapter the potential of HyPy and CSIA to investigate the biological origins of aquatic NOM is explored. This requires a large amount (minimum of 30 mg) of sample which was not available from either the leachates or biofilms described in Chapters 2 and 3. However a suite of membrane biofoulants from a drinking water treatment plant provided sufficient sample quantity of organic-rich material, which beneficially, had also been studied by other

characterisation methods. Analysis of the membrane foulants additionally allowed the treatment step of the 'catchment to consumer' process of the drinking water framework (NHMRC, 2011, described in Chapter 1) to be examined.

4.1.2 Membrane Filtration and Fouling

Membrane technology is used extensively in the water supply industry. Over the past few decades there have been rapid developments in the design of membrane based water filtration systems. Modern drinking water treatment processes include Microfiltration (MF), Ultrafiltration (UF), Nanofiltration (NF), Reverse Osmosis (RO) and Electrodialysis Reversal (EDR), all of which depend on membranes for effective filtration. The flow of water through the membrane is essential to enable efficient filtering, but fouling of the membrane can interfere with flow and severely affect the performance of the filtration process and consequently, water quality.

In practice, the degree of fouling is influenced by membrane type, operating conditions of the treatment plant and cleaning strategies, all of which have direct cost implications to the water utility. As every effort should be made to understand and reduce the fouling process, the following questions are fundamental:

- What is the foulant structure?
- What is its source?

This chapter investigates whether the combined application of off-line pyrolysis and CSIA can help resolve these issues.

4.1.3 Aims of Research

The focus of this research is the molecular and isotopic characterisation of drinking water treatment membrane foulants using a combined off-line pyrolysis and CSIA approach. Characterisation studies of membranes (e.g., Croué et al., 2003a; Berwick 2010) have previously investigated the molecular composition of foulants, but no studies have investigated whether CSIA can add value to the characterisation process. Stable isotopic signatures may help identify the bacterial origins of the foulant substrate, whether it is produced in situ or from exogenous sources (e.g., from the feed water). Isotopic measurement may also help characterise the respective contribution of allochthonous and autochthonous NOM inputs to the foulants. Stable isotopic signatures indicative of

biochemical processes active during simulated enzymatic cleaning of membranes, may also help optimise maintenance practices.

4.2 Background

4.2.1 Thermal Degradation in NOM Studies

4.2.1.1 Pyrolysis

Thermal degradation is an effective way to break the macromolecular fraction of NOM into lower MW products amenable to GCMS analyses, and there is an extensive body of research which investigates the structures of NOM and its precursors in this way (e.g. Douglas & Grantham, 1974; Faix et al., 1987; Dungait et al., 2008b).

Stable carbon isotopic analysis has also been used previously in conjunction with thermal degradation methods to help characterise various HMW environmental samples. Py-GCMS has been directly interfaced with stable isotope ratio mass spectrometry (Py-GC-irMS) to determine $\delta^{13}C$ values of various samples (e.g., lignin derivatives; Dungait et al*.,* 2008b), but resolution and concentrations have been identified as major analytical limitations to this technology. Experiments by Goni & Eglington (1994), however, showed that (off-line) flash pyrolysis of lignin rich samples did not produce major molecular rearrangements or isotopic fractionation of lignin biomarkers. Corso and Brenna (1997) also showed negligible difference in the $\delta^{13}C$ of lignin sample and its pyrolysates. Nevertheless, various researchers have identified off-line pyrolysis combined with additional separation procedures as a more appropriate preparation procedure for GC-irMS (Sephton et al., 1998; Poole & van Bergen, 2002; Grice et al., 2003; Beramendi-Orosco et al*.,* 2006a&b; Dungait et al*.,* 2008b; Grice & Brocks, 2011 for a review).

4.2.1.2 Off-line Pyrolysis

Off-line pyrolysis systems can potentially fulfil the optimum chromatographic separation required for GC-irMS analysis. Pyrolysis products can be trapped and then further fractionated, for example by column chromatography, or treated with derivatising agents (which use apolar groups to block functional groups) prior to analysis (e.g., *N,O-bis*(trimethylsilyl)trifluoro acetamide, BSTFA and trimethylchlorosilane, TMCS for trimethylsilylation, Poole & van Bergen, 2002; Poole et al., 2004). It also enables the use of

internal standards, repeat analyses (important for isotopic reproducibility assessments and the use of optimal GC loads (Faix et al., 1987).

The application of off-line pyrolysis and subsequent CSIA of heterogeneous geochemical mixtures was explored by Goni and Eglinton (1994) on a range of kerogens and kerogen precursors. Analytical pyrolysis, CuO oxidation and then CSIA was similarly combined to investigate vegetation changes (Huang et al*.,* 1999), sources and distribution of organic matter in marine sediments (Goni et al., 1997; Gordon & Goni 2003), and genesis of humic substances (Liu & Huang, 2008). The isotopic values of individual aliphatic and phenolic pyrolysis products were able to be related to their biological source and no major isotopic fractionation was observed upon the thermal dissociation of the macromolecules. Off-line pyrolysis and subsequent derivatisation followed by CSIA has also been applied to the study of fossil wood (Poole & van Bergen, 2002) and lignin (Dungait et al*.,* 2008b).

A consideration to the use of derivatising agents is the possible Kinetic Isotope Effect (KIE) which may arise due to the ratio of differing reaction rates (*K*) of isotopes in a chemical reaction (i.e., $^{12}CK/\ ^{13}CK$). A KIE is only associated with some derivatising procedures (e.g., alditol acetate derivatisation of monosaccarides using sodium boride, Fox et. al., 1988). If the KIE is systematic then it can be quantified for each compound (Rieley, 1994) and measured values can be accurately adjusted by the use of 'correction factors' (Rieley, 1994; Macko et al., 1998; Docherty et al., 2001). Other derivatising procedures (e.g., BSTFA/pyridine silylation or methanolic BF_3 esterification) do not cause a KIE, but the contribution of the BSTFA to the overall $\delta^{13}C$ value obtained (measured via a standard of known $\delta^{13}C$) must be corrected for—commonly using a simple mass balance equation (e.g., Abrajano et al., 1994; Grice et al., 1996).

Despite the potential advantages of combining off-line pyrolysis and CSIA, traditional off-line systems can be challenged with incomplete transfer of pyrolysis products (Hatcher et al., 2001) or isotopic fractionation. The emerging technique of HyPy, however, has several attributes which may help meet the challenges of coupling pyrolysis with CSIA.

4.2.1.3 Catalytic HyPy

Catalytic HyPy is an open-system off-line pyrolysis technique (described in Section 4.1.1). It has proven to be an effective analytical tool in the field of petroleum geochemistry

(e.g., Lockhart et al., 2008; Aboglila et al., 2011), yielding high recoveries (>85 %) of liquid hydrocarbon products from kerogens and coal (Snape et al., 1989 & 1994). Kerogen characterisation provided the main impetus for its development, yet HyPy has more recently been extended to a diverse range of applications including the generation of biomarker profiles from asphaltene fractions of biodegraded oils (Russell et al., 2004), lipid extracts from archaeological cooking ceramics (Craig et al., 2004) and functionalised model compounds (steroids and fatty acids) in forensic studies of plasma samples (Sephton et al., 2005; Meredith et al., 2006).

HyPy has also been used to help characterise immature organic materials (Bishop et al., 1998, Bennett et al., 2004; Beramendi-Orosco et al., 2004, Love et al., 2005; Berwick et al., 2010a). Love et al. (1995) showed that the structural integrity of hopane biomarkers of bacterial cell membranes was maintained with HyPy, confirming it as a useful technique to investigate bacterial inputs to complex macromolecular material.

HyPy products are either collected off-line in a cold-trap (Love et al., 1995; Meredith et al., 2004) or more commonly, adsorbed to silica (Meredith et al., 2004 & 2008). This has the advantage of relatively easy isolation for further separation procedures, such as column chromatography or derivatisation, which can help reach the required degree of baseline separation necessary for CSIA.

The pyrolysis in a high H_2 atmosphere produces high yields of covalently bound structures (Meredith et al., 2006) and, the rapid removal of products from the heated zone of the reactor, combined with the use of moderate heating rates, preserves much of the structural and isotopic integrity of the hydrocarbon precursors (Love et al., 1995, 1997 & 2005; Sephton et al., 2005, Meredith et al., 2010). The isotopic signature of HyPy sterane products have also been measured and shown to be the same as steric acid precursors (Love et al., 2008). The present study is the first to use HyPy and CSIA to analyse the biological foulant of a NF membrane in a full-scale drinking water treatment plant.

4.2.2 Foulant Formation

Biofilms form on a surface when microorganisms and extracellular compounds accumulate due to deposition or growth, or a combination of the two (Characklis & Marshall, 1990; Vrowenvelder, 2009). Biofouling refers to biofilm formation on water treatment

membranes which, in sufficient levels, can lead to the deterioration of membrane performance, consequentially increasing operation and maintenance costs (Characklis & Marshall, 1990; Vrowenvelder, 2009).

Membrane foulants include:

- Inorganic fouling (scaling) resulting from deposition of mainly $BaSO_4$, $CaSO_4$ and $CaCO_3$.
- Particle fouling, resulting from the deposition of silt and clay particles.
- Organic fouling, resulting from interaction and deposition on membranes of humic acids, protein, carbohydrate and other NOM constituent, in feed water.
- Biofouling, resulting from the attachment of microbes, their subsequent growth, multiplication and their exudation products. Biofouling frequently overlaps with organic fouling (Vrouwenvelder et al., 1998; Al-Amoudi & Lovitt 2007).

Despite much investigation, the origin and mechanisms of fouling remain difficult to identify and quantify. Fouling is an extremely complex process (Al-Amoudi & Lovitt 2007) and different types of fouling (e.g., scaling, particulate fouling, organic and biofouling) often occur simultaneously (Flemming, 1993). Many factors contribute to the degree and rate of fouling, including hydrodynamics, feed water composition and membrane properties (Bessiere et al., 2009). Nevertheless, the characterisation of foulants is crucial to understanding the processes and the impact of surface water pre-treatment and catchment management strategies on fouling, as well as developing appropriate cleaning procedures.

The efficiency of chemically cleaning membranes has been of practical interest to drinking water utilities and also the subject of extensive research (Cyna et al*.,* 2002; Liikanen et al*.,* 2002; Houari et al*.,* 2010). Understanding the biological and chemical nature of biofoulants will help the design of appropriate treatment strategies. Bases, acids, metal chelating agents, surfactants, and enzymes are all typically used to clean NF membranes (Cyna et al*.,* 2002, Houari et al., 2010). Such an extensive chemical concoction, however, only partially removes exopolysaccharide (EPS) material from the persistent superficial layer of the biofoulant material (Houari et al*.,* 2010). Pre-treatment steps have been shown to limit scaling and particulate fouling. However, adequate treatment has not been developed to adequately control organic and particularly biological fouling which has placed limits on the practical utilisation of membrane technology (Violleau et al., 2005).

4.2.3 Characteristics of Biological Foulant NOM

The contribution of NOM to the fouling of membranes has been well reported (Paul, 1991; Flemming, 1993; Tasaka et al., 1994; Paul, 1996; Ridgway & Flemming, 1996; Baker & Dudley, 1998; Huisman & Feng Kong, 2004; Schneider et al., 2005; Lee et al., 2006; Karime et al., 2008; Vrouwenvelder et al., 2009). However, there have been conflicting views on the components of NOM that are most responsible.

The colloidal fraction of NOM, which can make up to 20–30 % of surface water DOC (Leenheer et al*.,* 2000; Kennedy et al., 2008), contributes significantly to membrane fouling (Makdissy et al., 2003). Inorganic materials (e.g., clays, silica salt, metal oxides), organics and biological products all concentrate in this fraction which tends to be hydrophilic in nature (Al-Amoudi & Lovitt, 2007). Muco-polysaccharides and peptidoglycan, from bacterial cell wall residues, can also make up a substantial portion of organic colloids (Leenheer et al*.,* 2000; Makdissy et al., 2003). Polysaccharides and amino-sugars have been established as major contributors to membrane fouling (Cho & Amy 1999; Cho et al*.,* 2000; Jarusutthirak et al*.,* 2002; Kimura et al*,.* 2004). Humic fractions can also increase fouling (Jucker & Clark, 1994; Nilson & DiGiano, 1996; Aoustin et al., 2001; Violleau et al., 2005).

4.2.4 Characterisation Methods

Vrouwenvelder et al. (1998) reported that analysing membrane foulants by 'autopsy' (i.e., physical dissection) provided the most useful insight into the mechanics of membrane fouling. The morphology of membranes and the influence of pore structure on fouling have also been investigated by Atomic Force Microscopy (Zhu & Elimelech, 1997), whilst mechanical properties such as viscosity and contact angles have also attracted attention (Houari et al*.,* 2010). Chemical analysis by spectroscopic methods such as FTIR, ^{13}C-NMR (e.g., Croué et al., 2003a) and Py-GCMS speciation (Berwick et al., 2010a&b) can help identify the organic structures and species responsible for fouling.

A suite of contemporary techniques including: Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR), Inductive Coupled Plasma-Atomic Emission Spectrometry (ICP-AES), contact angles, adenosine triphosphate (ATP) measurement and rheometry were used to study the foulant from NF membranes of the Mery-sur-Oise plant (Val d'Oise, France; Houari et al., 2010). This characterisation of material from three

different stages of the plant before and after chemical cleaning showed some properties, such as viscosity and ATP content, do decrease with treatment stage, but a permanent biofilm layer was present at all stages both before and after cleaning. Such a layer was thought to contribute towards irreversible fouling, but the exact mechanism for this process has not been fully established.

Vrouwenvelder et al. (2009) conducted autopsies of the NF membranes foulants of full-scale and pilot-scale installations (as well via membrane fouling monitors). The autopsy and additional ATP measurements and Magnetic Resonance Imaging (MRI) led to the conclusion that biofouling was a feed spacer problem, and further efforts should focus on the relationship between fouling and chemical attributes of feed spacers.

Membrane autopsy followed by comprehensive molecular biological analyses, including by the PCR-DGGE profiling and cloning techniques described in Chapter 2, were used by Bereschenko et al. (2008) to find that *Sphingomonas* spp. was the dominant microbe of the biofoulant from spiral-wound RO membranes of a full-scale drinking water purification plant (ca. five years after installation). Using the same techniques plus additional fluorescence *in situ* hybridisation (FISH), Bereschenko et al. (2010) showed that *Sphingomonas* spp. initiated as well as dominated biofilm communities grown on RO membranes and feed spacer surfaces of a laboratory-based pilot-scale RO drinking water treatment plant.

CSIA has not yet been widely applied to the characterisation of membrane fouling, but may help elucidate sources and possibly establish important substrate-microbial community relationships.

4.3 Methodology

4.3.1 Sample Collection, Treatment and Storage

Lyophilised biofoulant samples—obtained via autopsy of sheets of spiral-wound NF membranes were provided by Professor Jean-Philippe Croué (Poitiers University, France). The membrane material was sourced from a full scale drinking water treatment plant, which was fed by extensively pre-treated surface water.

In order to simply the foulant prior to further analyses, the foulant was separated into two components on the basis of solubility. Preparation of the sample for organic analysis was described by Croué et al. (2003a). Briefly, foulant was ultra-sonicated in Milli-Q water, centrifuged and the insoluble precipitate and soluble supernatant separated and lyophilised. The soluble and insoluble fractions were subjected to an enzymatic (protease) cleaning process (Croué et al., unpublished work) which separated them further into 'before cleaning' (BC) and 'after cleaning' (AC) fractions. The four fractions - all investigated in the present study - are thus identified as follows:

Sol_{BC} = soluble fraction before enzymatic cleaning
Sol_{AC} = soluble fraction after enzymatic cleaning
$Insol_{BC}$ = insoluble fraction before enzymatic cleaning
$Insol_{AC}$ = insoluble fraction after enzymatic cleaning

4.3.2 Bulk Stable Carbon Isotope Analysis

Bulk stable carbon isotopic analysis of the four membrane biofoulant fractions was conducted as described in Section 2.3.5.2.

4.3.3 HyPy

4.3.3.1 HyPy Procedure

Details of the HyPy procedure have been described previously (e.g., Meredith et al., 2004). Briefly, the membrane foulants (~30 mg) were mixed with a dispersed sulfided molybdenum catalyst (10 mg $(NH_4)2MoO_2S_2$) dissolved in a minimum of 20% aqueous methanol, dried gently and then transferred into the pyrolysis reactor. The catalyst loaded samples were then transferred to a stainless steel reactor tube, where they were heated from ambient temperature to 250°C at 300°C min^{-1}, then to 500°C at 8°C min^{-1}. A constant hydrogen flow of 5 L min^{-1} was maintained, ensuring that the volatile products were quickly removed from the reactor vessel. The products were adsorbed onto silica via a silica gel-filled trap cooled with dry ice.

4.3.3.2 Fractionation of HyPy Product

Column chromatography with 20 cm x 0.9 cm i.d. columns and a stationary phase of pre-rinsed activated Silica gel 60 (120°C, >8 hours, 0.063–0.200 mm, Merck) was conducted on

the silica-adsorbed HyPy products. The aliphatic hydrocarbon (saturate) fraction was eluted in *n*-pentane (Ultima-R grade, 35 mL), followed by the aromatic fraction in a solution of dichloromethane (DCM):*n*-pentane 30:70 v/v (both Ultima-R grade, 40 mL) and finally, the polar fraction was eluted with dichloromethane: methanol 50:50 v/v (Ultima-R, 40 mL). The saturate and aromatic fractions were then concentrated by evaporation of the solvent to <1 mL prior to GCMS analysis. The polar fractions were derivatised prior to GCMS analysis as described below.

4.3.3.3 Derivatisation of Polar Fraction

The polar fraction was derivatised by adding an excess of *N, bis*(trimethylsilyl)trifluoro acetamide (BSTFA, derivatisation grade, Supelco product number 33024) and pyridine (Sigma-Aldrich product number 437611) at a ratio of 1:1 v/v and heating the reactants to 70°C for 60 min. The derivatised extract was subsequently filtered using a Pasteur pipette packed with silica gel to remove non-chromatographically resolvable compounds. The solvent was evaporated from the filtered extract at 40°C under a gentle stream of N_2 gas, and samples were reconstituted in DCM. Derivatisation was separately performed on a palmitic acid standard (Aldrich product number P0500, 1 mg mL^{-1}) to establish, and thus allow correction for, the effects of fractionation on isotopic composition (refer Section 4.3.3.5).

4.3.3.4 GCMS of HyPy Fractions

GCMS analysis of the saturate, aromatic and derivatised polar fractions of the HyPy product was performed using a HP6890 GC interfaced to a HP5973 MSD (electron energy 70 eV, source temperature 230°C, electron multiplier 1800 V, transfer line 310°C). Samples (~1 µL) were injected via a HP 6890 autosampler in pulsed splitless mode (207 kPa, 0.5 min) using helium as carrier gas (constant flow, 1 mL min^{-1}). Compounds were separated on a Phenomenex ZB-5 fused silica capillary column (60 m x 0.25 mm i.d. x 0.25 µm) in an oven with a temperature programme of 40°C (1 min) to 310°C (30 min) at a rate of 3°C min^{-1}. Data acquisition was performed in full scan mode (*m/z* 50–550 at ~2 scan s^{-1}). MS data were correlated with previously published data and MS libraries (NIST 05 and Wiley 275). Peak identifications were also based on correlation of GC elution position where possible.

4.3.3.5 CSIA of HyPy Fractions

CSIA analysis was conducted by GC-irMS as described in Section 3.3.5. The $\delta^{13}C$ values obtained for tri-methylsilylated compounds were corrected for the addition of the extra three carbons using the mass balance shown in Equation 4.1 (Abrajano et al., 1994). Error was calculated on the raw (uncorrected) $\delta^{13}C$ values. The $\delta^{13}C$ value for BSTFA (-37.4 ‰) was determined by derivatisation of a palmitic acid standard of known $\delta^{13}C$ value (determined by EA-irMS) and back calculated. These values were used to calculate the $\delta^{13}C$ values of un-derivatised compounds by correcting the $\delta^{13}C$ values of the derivatised compounds determined by CSIA for addition of compounds during derivatisation, according to Equation 4.1

$$n_{cd}\,\delta^{13}C_{cd} = n_c\,\delta^{13}C_c + n_d\,\delta^{13}C_d \quad \text{..} \quad \textbf{Eqn. 4.1}$$

Where:

n = number of carbon atoms

c = un-derivatised compound

d = derivatising agent

cd = derivatised compound

4.4 Results and Discussion

4.4.1 Molecular Characterisation by GCMS

4.4.1.1 Aliphatic Compounds

The aliphatic profiles of all four membrane biofoulants were dominated by *n*-alkanes (range n-C_{14}–n-C_{34}) with other significant contributions from isoprenoids, other methyl-branched alkanes and unsaturated aliphatics. The 50 most abundant saturate compounds (S_x) are listed together with their 4 major fragment ions, MW and compound type in Table 4.2. A representative TIC (from $Insol_{BC}$) is shown in Fig.4.1. The n-C_{14} to n-C_{34} *n*-alkane profile (Fig.4.1) was bimodal, with peaks at C_{20} and C_{26} and displays a strong even-over-odd predominance. The Carbon Preference Index (CPI) of the foulants ranged from 0.33 to 0.42.

Previous analyses with other pyrolytic methods have shown varying CPI trends. Flash-Py of biofoulant samples from this filtration plant for example, revealed only trace levels of *n*-alkanes and their precursor fatty acids, with no CPI pattern of note (Croué et al., 2003b). In

the same study a smooth Gaussian FAME profile from *n*-C_{22}–*n*-C_{36} (*n*-C_{30} maximum) was observed by thermochemolysis, again with no notable carbon preference (Croué et al., 2003b).

The *n*-alkane distribution from previous (Berwick, 2010) MSSV analysis (which, unlike the relatively soft thermal release of HyPy, may cause cleavage of acid group) of the biofoulants showed two different CPI trends, an odd-over-even-carbon preference in the shorter carbon chain lengths (< *n*-C_{16}) and an even-over-odd-carbon preference in the longer *n*-alkanes (> *n*-C_{19}). The different extent of complete reduction reactions of acid groups of HyPy used in the present study *Cf*. MSSV and Flash-Py may explain the differences observed.

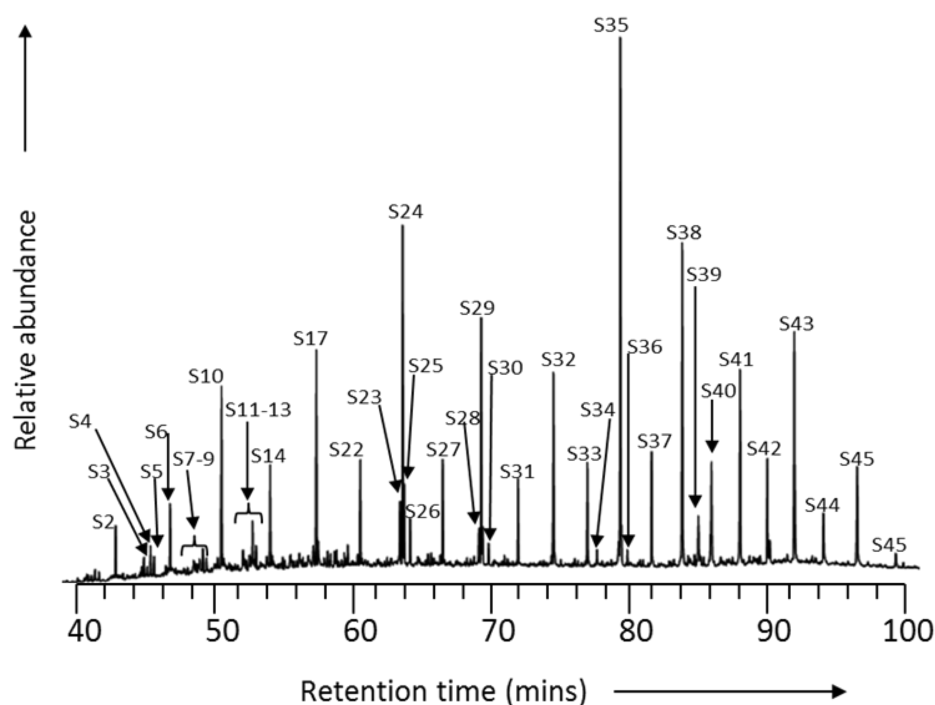


Figure 4.1 Partial TIC of aliphatic fraction of Insol$_{BC}$ sample. Peak assignments correspond to products listed in Table 4.1.

Love et al. (2005) established that rather than decarboxylation, HyPy promoted the complete reduction of fatty acids to *n*-alkanes. The *n*-alkane carbon number shown here therefore directly reflects the chain-length of free or bound carboxylic acids and alcohol precursors (and not one less as is typical of other pyrolysis methods due to cleavage of acid group). The higher MW *n*-alkanes are thought to be indicative of long chain fatty acid precursors, the origin of which were proposed to be microbial (or algal) precursors (representing a biosynthetic source; Grice et al., 2003; Berwick, 2010).

Table 4.1 Major saturated (Sx) products detected by HyPy GCMS analysis of the aliphatic fraction from HyPy of the membrane biofoulant samples. *m/z* of four main fragment ions, molecular weight (MW) and product class details are provided. A = *n*-alkane, MMA = Mono-methyl alkane, U= unsaturated, Ste = Steroid, Hop = Hopanoid.

Peak No.	RT (min)	Compound ID	*m/z*	MW	Class
S1	34.40	tridecane	57, 71, 85, 184	184	A
S2	38.88	tetradecane	57, 71, 85, 99	198	A
S3	41.08	5-methyl tetradecane	57, 85, 71, 169	212	MMA
S4	41.58	2-methyl tetradecane	57, 71, 85, 169	212	MMA
S5	41.88	3-methyl tetradecane	57, 71, 85, 183	212	MMA
S6	43.10	pentadecane	57, 71, 85, 212	212	A
S7	44.95	7-methyl pentadecane	57, 71, 85, 99	226	MMA
S8	45.68	2-methylpentadecane	57, 71, 85, 99	226	MMA
S9	45.96	3-methylpentadecane	57, 71, 85, 99	226	MMA
S10	47.12	hexadecane	57, 71, 85, 226	226	A
S11	49.53	2-methyl hexadecane	57, 71, 85, 197	240	MMA
S12	49.81	3-methyl hexadecane	57, 71, 85, 197	240	MMA
S13	50.64	8-heptadecene	55, 57, 69, 83	238	U
S14	50.91	heptadecane	57, 71, 85, 99	240	A
S15	52.52	8-methyl heptadecane	57, 71, 85, 112	254	MMA
S16	54.28	1-octadecene	55, 57, 83, 97	252	U
S17	54.52	octadecane	57, 71, 85, 99	254	A
S18	54.67	5-octadecene	55, 57, 83, 97	252	U
S20	56.70	2-methyl-octadecane	57, 71, 85, 97	268	MMA
S21	56.97	5-methyl-octadecane	57, 71, 85, 97	268	MMA
S22	57.95	nonadecane	57, 71, 85, 99	268	A
S23	61.05	1-nonadecene	55, 69, 83, 97	266	U
S24	61.24	eicosane	57, 71, 85, 99	282	A
S25	61.36	3-eicosene	55, 57, 83, 97	280	U
S26	61.82	5-eicosene	55, 57, 83, 97	280	U
S27	64.36	heneicosane	57, 71, 85, 99	296	A
S28	67.19	10-heniocosene	55, 57, 69, 97	294	MMA
S29	67.37	docosane	57, 71, 85, 99	310	A
S30	67.48	1-docosene	55, 57, 83, 97	308	U
S31	67.94	docosene (unspecified isomer)	55, 57, 83, 97	308	U
S32	70.23	tricosane	57, 71, 85, 99	324	A
S33	72.98	tetracosane	57, 71, 85, 99	338	A
S34	75.63	pentacosane	57, 71, 85, 99	352	A
S35	78.19	hexacosane	57, 71, 85, 99	366	A
S36	78.74	1-hexacosene	55, 57, 83, 97	364	U
S37	80.64	heptacosane	57, 71, 85, 99	380	A
S38	83.01	octacosane	57, 71, 85, 99	394	A
S39	83.48	cholestane	55, 217, 357, 372	372	Ste
S40	83.97	cholest-4-ene	55, 57, 355, 370	370	Ste
S41	84.27	2-methylene-cholestan-3-ol	69, 81, 83, 97	400	Ste
S42	85.30	nonacosane	57, 71, 85, 100	408	A
S43	87.51	triacontane	57, 71, 85, 99	422	A
S44	89.65	hentriacontane	57, 71, 85, 99	437	A
S45	89.84	hop-17,21-ene	191, 207, 367, 410	410	Hop
S46	91.75	dotriacontane	57, 71, 85, 99	451	A
S47	94.02	tritriacontane	57, 71, 85, 99	465	A
S48	96.62	tetratriacontane	57, 74, 85, 99	479	A
S49	99.63	pentatriacontane	57, 74, 85, 97	493	A
S50	103.2	hexatriacontane	57, 74, 85, 97	507	A

The even-carbon dominance they show is indicative of a bacterial origin (Davis, 1968; Han & Calvin 1969; Walker & Colwell, 1976; Nishimura & Baker, 1986; Ambles et al*.,* 1989; Grasset & Ambles 1998; Aloulou et al., 2010). Even-over-odd preferences have been observed from the HyPy of bacterial cultures (Love et al., 2005) and also biofoulants from other stages of the treatment plant from which the present biofoulant sample was sourced (Berwick, 2010; Berwick et al., 2010a). This pattern contrasts the strong odd-over-even *n*-alkane signatures of terrestrial plants (Farrington & Tripp, 1977; Brassell et al., 1978; Wakeham & Farrington 1980; Doskey & Talbot 2000; Chikaraishi & Naroaka, 2003; Zhou et al., 2003).

The detection of C_{11}–C_{20} mono-methyl alkanes (MMAs, **S3-S5**, **S7-S9**, **S11-S12**, **S15**, **S20-S21** and **S28**, Table 2.4) is also consistent with a bacterial source. MMAs are normally attributed to bacterial lipids (Koster et al*.,* 1999), although they can also be sourced from cyanobacteria and algae (Summons et al., 1988). On the basis of $\delta^{13}C$ signatures, a series of C_{23} to C_{31} MMAs detected in torbanites of varying ages (Audino et al*.,* 2001; Grice et al*.,* 2001) were attributed to the heterotrophic reworking of the biomass and/or race A of *Botryococcus braunii* living under glacial conditions (Audino et al., 2001; Grice et al., 2001).

Hopanes are in general microbial biomarkers, commonly derived from the bacteriohopane polyols (BHPs) present in the cell membranes of bacteria (Ourisson et al., 1982 & 1984), but are also produced by algae (e.g., Moldowan et al., 1985). Berwick et al. (2010b) previously showed that MSSV and HyPy produced C_{27}–C_{33} hopanes from a membrane biofoulant of this treatment plant. HyPy was shown to yield higher concentrations of higher MW and biogenic $\beta\beta$-hopane isomers—reflecting softer and more selective bond cleavage (Berwick et al*.,* 2010b). Specific hopane isomers have been attributed to different stages of maturation, with $\beta\beta$-hopanes the least mature and $\alpha\beta$-hopanes the most mature (Ensminger et al., 1977; Ourisson & Rohmer, 1992). Hopanes and MMAs were also detected by GCMS in the present study (Table 2.4) although not at sufficiently high concentration for CSIA (Section 4.4.2.2). Nevertheless, their presence in these samples implies the biofoulant has a significant microbial element which has possibly reworked some of the available organic substrate which will be discussed further in 4.4.2.2.2. Microbial detritus and exudates, in the form of EPS and Soluble Microbial Products (SMPs), have been identified as major contributors to biological fouling (Characklis, 1981; Lappin-Scott & Costerton; 1989; Davey & O'Toole, 2000; Jarusutthirak & Amy, 2006; Liang et al., 2007).

It is therefore plausible that fatty acids from bacterial sources, both indigenous to membrane and of exogenous origin, are a major structural component of the biofoulant.

4.4.1.2 Aromatic Compounds

The GCMS distributions of the aromatic fractions from the HyPy of all foulant samples were also similar. A representative TIC ($Insol_{BC}$) of the aromatic fraction obtained following HyPy of the biofoulant is shown in Fig. 4.2.

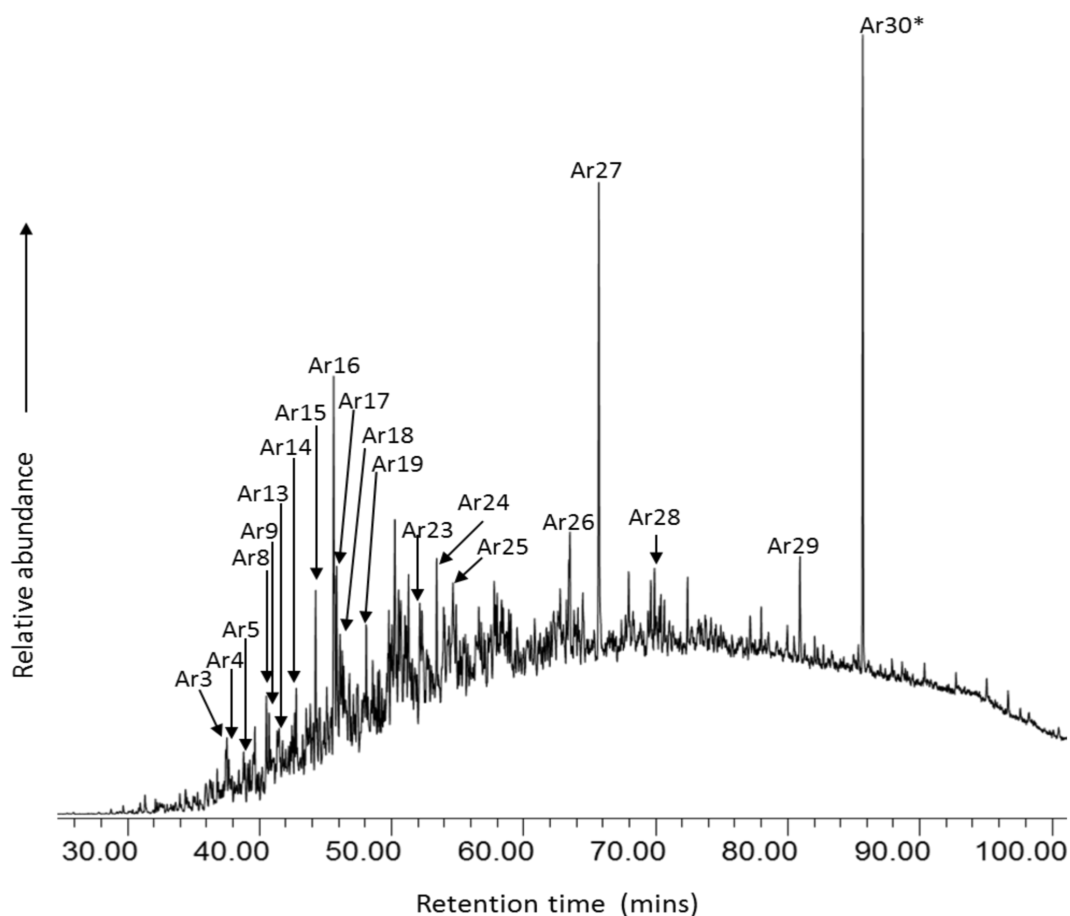


Figure 4.2 Partial TIC from GCMS analysis of the aromatic fraction from the HyPy of the $Insol_{BC}$ sample. Peak assignments correspond to products listed in Table 4.2.

The aromatic products were less well resolved than the aliphatics. A broad Unresolved Complex Mixture (UCM) was present in the aromatic fraction of all membrane foulants (e.g., Fig. 4.2) which may be related to the biodegradation (i.e., microbial reworking) of hydrocarbon compounds (Han & Calvin, 1969; Eganhouse & Kaplan, 1982). A list of 30 major products identified on the basis of their mass spectra (via previously published material and NIST and Wiley MS libraries) is shown in Table 4.2.

Table 4.2 Major aromatic (Ax) products detected by GCMS analysis of aromatic fraction from HyPy of membrane biofoulant sample m/z of four main fragment ions, molecular weight (MW) and product class details are provided. * = Unspecific isomer.

Peak No.	RT (min)	Compound ID	*m/z*	MW	Compound Class
A1	34.51	1-methyl-napthalene	142, 141, 115, 139	142	Naphthalene
A2	37.27	5-ethyl-1,2,3,4-tetrahydro napthalene	131, 115, 145, 160	160	Naphthalene
A3	38.66	1,2,3,4-tetrahydro-5,6-dimethylnapthalene	145, 117, 115, 160	160	Naphthalene
A4	38.91	2-ethylnapthalene	141, 156, 115, 131,	156	Naphthalene
A5	39.13	1-ethylnapthalene	141, 156, 115, 131,	156	Naphthalene
A6	39.42	dimethylnapthalene*	156, 141, 155, 115	156	Naphthalene
A7	40.00	dimethyl napthalene*	156, 141, 155, 115,	156	Naphthalene
A8	40.08	hexahydro-indacene	158, 130, 157, 115	158	Cyclic
A9	40.20	dimethylnapthalene*	156, 141, 155, 115,	156	Naphthalene
A10	40.41	unidentified pyrrole	145, 91, 119, 120	145	Pyrrole
A11	40.59	dimethylnapthalene*	156, 141, 155, 115	156	Naphthalene
A12	40.90	dimethylnapthalene*	156, 141, 155, 115	156	Naphthalene
A13	41.22	1,2,3,4-tetrahydro-6-propyl	145, 174, 131, 117	174	Naphthalene
A14	42.76	acenapthalene	153, 154, 152, 76	154	Naphthalene
A15	46.10	hexahydro-benz(e)indene	172, 144, 129, 91	172	Cyclic
A16	47.32	1-(2-propenyl)-napthalene	168, 153, 167, 164	168	Naphthalene
A17	47.52	diphenylmethane	168, 167, 165, 152	168	phenyl
A18	47.76	4-methyl-1,1-biphenyl	152, 165, 167, 168	168	phenyl
A19	49.53	2-ethylbiphenyl	167, 182, 165, 152	182	phenyl
A20	50.105	*n*-decylbenzene	91, 92, 218, 133	218	Benzene
A21	51.48	4,4'-dimethyl1,1'-Biphenyl	167, 182, 165, 152	182	Phenyl
A22	52.408	2,3'- dimethyl 1,1'-Biphenyl	167, 182, 165, 152	182	Phenyl
A23	53.31	1,2,3,4-tetrahydrophenanthrene	182, 154, 141, 165	182	Phenanthrene
A24	54.32	phenanthrene	178, 176, 179, 177	178	Phenanthrene
A25	55.64	1-butylnonylbenzene	91, 147, 105, 260	260	Benzene
A26	63.15	1,2,3,6,7,8-hexahydropyrene	208, 165, 207, 179	208	Pyrene
A27	65.34	pyrene	202, 200, 201, 101	202	Pyrene
A28	69.13	9-phenyl-benzo cyclo heptene	218, 217, 203, 202	218	Cyclic
A29	79.04	3-methyl benzo- phenanthrene	242, 241, 239, 226	242	Phenanthrene
A30	85.31	2,6,10,14,18,22-tetracosahexaene	69, 81, 95, 137	410	Contaminant

The main products were aromatics (e.g., benzenes, indenes, naphthalenes, phenanthrenes, phenols) and cyclic unsaturates (e.g., alkyl substituted cyclo-hexenes). A distinctive series of long chain alkyl ($C_{6\text{-}9}$) phenols were detected. These analytes had been previously detected by MSSV pyrolysis of biofoluant from this treatment plant (Berwick, 2010) and were recognised to be anthropogenics sourced from linear alkylbenzenesulfonates of common detergents (Eganhouse et al., 1983; Takada & Ishiwatari, 1987).

4.4.1.3 Polar Compounds

GCMS analysis of the derivatised polar fraction from the HyPy treated foulants showed a number of tri-methylsilyl (TMS) ester products (Table 4.3). The TIC profile (Fig. 4.3) of the $Insol_{BC}$ sample shows the major product was the tri-TMS ester of levoglucosan (**P34**, the 1,6-anhydride of glucose, Simoneit et al., 1999).

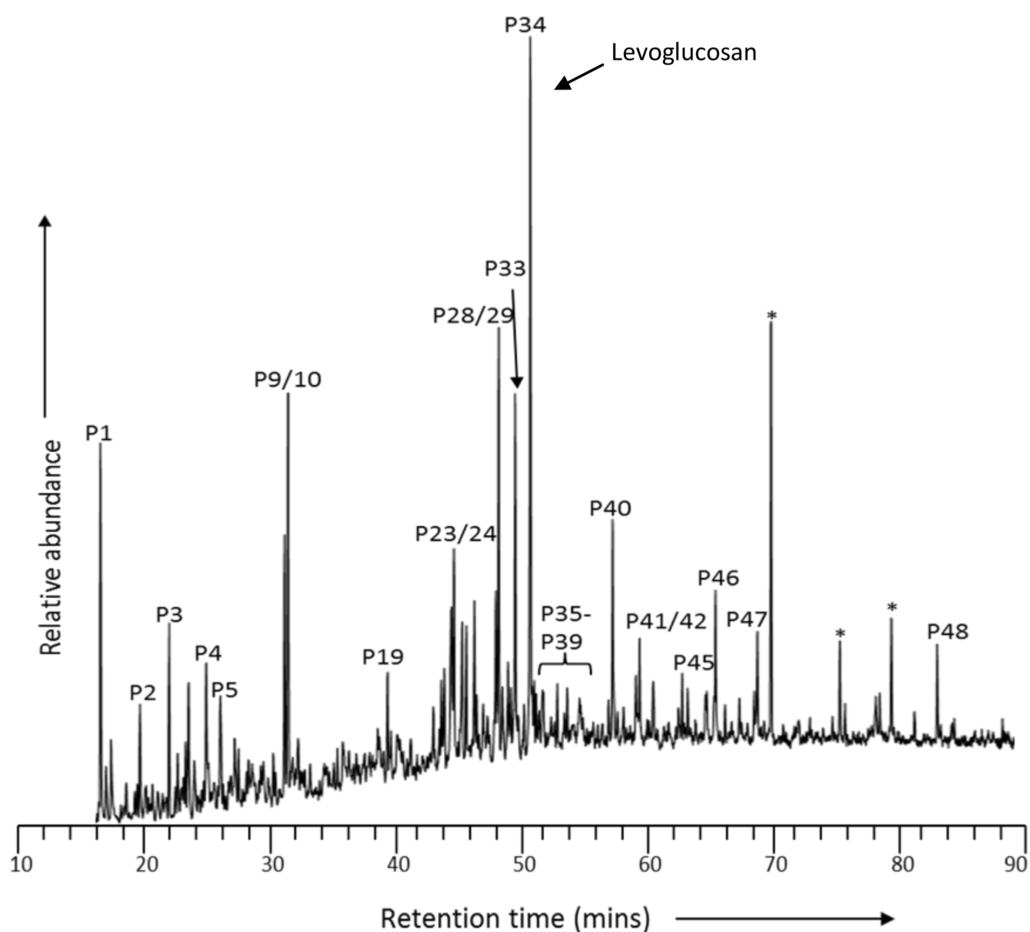


Figure 4.3 Partial TIC of derivatised polar fraction HyPy $Insol_{BC}$. *= Contaminants. Peak assignments correspond to products listed in Table 4.3.

Table 4.3 Major polar (P_x) products detected by GCMS analysis of derivatised polar fraction from HyPy of membrane biofoulant sample.

Peak	RT (min)	Compound ID	*m/z*	MW
P1	16.58	2 methyl-4 phenyl pyrimidine	170, 77, 120, 200	170
P2	18.54	boric acid TMS triester	221, 73, 263, 222	278
P3	20.18	decamethyltetrasiloxane	207, 73, 299, 208	310
P4	26.27	2-furancarboxylicacid TMS ester	125, 169, 95, 184	184
P5	26.36	3-hydroxypyridine 1 TMS ester	152, 167, 73, 153	167
P6	27.86	butanoic acid, 2-methyl-3-(TMS(oxy))-TMS ester	73, 147, 117, 218	262
P7	28.80	3-hydroxy-6-methylpyridine TMS	166, 181, 73, 150	181
P8	29.04	alpha-D-galactoside,methyl tetrakis-TMS ester	73, 147, 188, 204	482
P9	29.53	3-trimethylsilyoxy-6-methylpyridine	166, 181, 167, 73	181
P10	30.06	thiocyanicacid -TMS ethylester	160, 74, 116, 73	175
P11	31.48	substituted azasilaboroline	166, 180, 139,	181
P12	32.92	tetramethyl 5TMS 3,7dioxa-2,8-disilanonane	73, 147, 205, 218	308
P13	33.73	hexanoic acid, 2-[(trimethylsil)oxy]-TMS ester	73, 159, 157, 89	276
P14	33.88	5-methyl-2,3-dihydro-1H-benzazepin-2-one	73, 173, 129, 75	173
P15	34.75	diethylmalonate-TMS	73, 117, 75, 147	232
P16	35.44	2-ethylthio-2-methyl-3-phenylprop-2-ene-1-ol	179, 73, 186, 112	208
P17	36.15	1-*p*-tolylcyclopentene	73, 117, 143, 75	158
P18	38.61	D-ribofuranose,1,2,3,5-tetrakis-O-(TMS)	217, 73, 147, 218	438
P19	38.78	1H-Indole-3-acetic acid-TMS-ethylester	73, 217, 147, 215	275
P20	43.68	beta D-glucopyranoside,methyl 236-tris-O-methyl-2,6-bis-O-(TMS)	73, 129, 147, 204	452
P21/P22	44.37	*N*-[2(5-hydroxy-1H-indol-3-yl)ethyl] acetamide	159, 146, 73, 217	150
P23	46.83	methyl 6-deoxy-2,3,5-tris-O-(TMS)-alpha-L-	73, 159, 147, 187	394
P24	46.96	3-O-methyl-1,2,4-tris-O-(TMS)-xylopyranose	217, 73, 146, 131	380
P25	47.36	substituted TMS ester of galactopyranose	73, 217, 146,147	424
P26	47.57	substituted TMS ester of xylopyranose	73, 217, 146, 205	380
P27	47.97	glucopyranose,4,6-di-O-methyl-1,2,3-tris-O-(TMS)-	73, 204, 147, 205	206
P28	49.20	alpha-D-galactofuranose,1,2,3,5,6-pentakis-O(TMS)-	217, 73, 204, 147	540
P29	49.17	alpha.-D-ribofuranoside 2,3,5 -tris-O(TMS)-	217, 73, 204, 147	380
P30	49.35	unidentified	85, 101, 151, 73	-
P31	49.62	arabinose, 2,3,4,5-tetrakis-O-(TMS)-	73, 217, 204, 147	438
P32	49.76	arabinopyranose,tetrakis-O-(TMS)-	73, 217, 204, 147	438
P33	49.97	alpha-D-arabinopyranose tetrakis-O-(TMS)-	73, 217, 204, 147	438
P34	50.72	Levoglucosan - Tri TMS	73, 204, 217, 333	378
P35	50.82	substitued TMS sugars	73, 217,147,191	438
P36	50.92	substitued TMS sugars	73, 217,147,191	438
P37	51.02	substitued TMS sugars	73, 217,147,191	438
P38	51.31	substitued TMS sugars	73, 217,147,191	438
P39	52.06	substitued TMS sugars	73, 217,147,191	438
P40	54.78	3-ethyl-4-methyl-5-(methylthio)isothiazole	131, 73, 173, 129	173
P41/P42	59.84	ethyl 6,7-(1,3-dimethytrimethylene)-4-ethylindole-2-carboxylate	285, 73, 117, 75	285
P43-47	62-71	series of fatty acid TMS esters	313, 117, 73, 75	range
P48	87.54	hexamethyl-3,26-dioxa-2,27-disilaoctacosane	117, 73, 149,207	514

Levoglucosan is a common pyrolysis product of; cellulose (Shafizadeh, 1984; Simoneit et al., 1999), carbohydrates (Pouwels et al., 1987; Pastorova et al., 1994) and environmental samples impacted by these organic sources (e.g., peat; Boon et al., 1996; Simoneit et al., 1999; Gravitis et al., 2001). It has also been reported from the thermal degradation of bacterially produced EPS (Zamora et al., 2002). Levoglucosan has also been shown to enhance the growth of micro-algae (Luyen et al., 2007) and may have a similar effect on the biota fouling the membranes.

As many of the compounds presented in Table 4.3 were only tentatively identified, however, a more meaningful presentation of the results, by way of main product groupings observed in the derivatised polar fraction of the membrane foulants is shown in Figure 4.4.

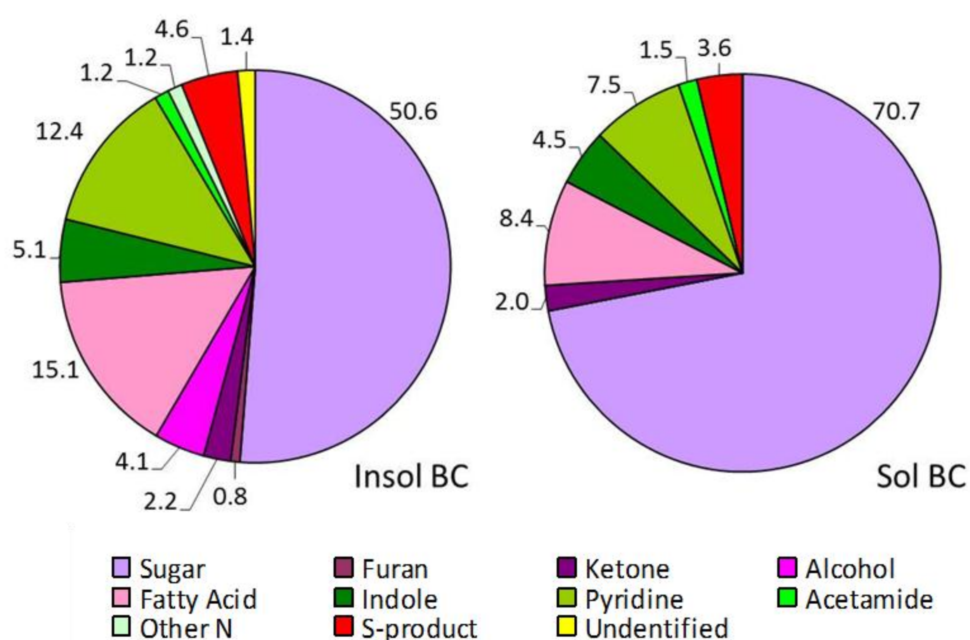


Figure 4.4 Comparison of major product groupings in the derivatised polar fraction from HyPy $Insol_{BC}$ and Sol_{BC} biofulants

As the isotopic characteristics of derivatised polar products in both the soluble and insoluble foulant fractions (described in Section 4.3.1) are discussed in some detail in Section 4.4.2.2.3, their molecular profiles are both presented here to give an indication of the different foulant constituents.

Sugars were the dominant fraction—over 50 % of products detected in the polar fractions of the HyPy foulants were TMS-esters of sugars (this was even greater, 70 %, in the soluble

fraction, Fig. 4.4). The remaining compounds were a mixture of carbohydrate precursor-derived products (e.g., ketones and furans), a range of nitrogen containing products (e.g., indoles, pyrimidines and an acetamide) and sulfur containing products (e.g., isothiazole). The sugar component was the least dominant in the insoluble fraction, which contained greater abundances of fatty acids and N- containing products, particularly pyridines.

These findings are consistent with those of Croué et al. (2003a) who investigated membrane foulant material from a different stage of the same treatment plant. Through FTIR, ^{13}C-NMR and Py-GCMS analysis Croué et al. (2003a) concluded that the insoluble foulant material was more enriched in nitrogenous structures whereas the soluble fraction comprised a greater abundance of carbohydrate material. It follows therefore, that in the present study, the second most abundant grouping in the insoluble biofoulant fraction was N-products. Previous MSSV pyrolysis of biofoulants revealed high concentrations of acetamide products (Berwick et al., 2010b) which derive from N-acetyl amino sugars (Stankiewicz et al., 1996). Previous ^{13}C-NMR and FTIR spectral characterisation of membrane biofoulants (Croué et al., 2003a; Croué et al., 2006) also indicated an abundance of N-acetyl amino sugars. These pre-cursors are likely to contribute towards products determined in the present study. The molecular results of the present study however shed little additional information into the nature of the biofoulant than previous studies. Isotopic analysis (Section 4.4.2) however provided more insightful findings.

4.4.2 Isotopic Characterisation of Membrane Biofoulants

4.4.2.1 Determination of Bulk Isotope Values

The bulk δ^{13}C values from the EA-irMS analysis of the four membranes are shown in Table 4.4. Values ranged from -27.5 ‰ to -28.6 ‰ which are within the -23 ‰ to -28 ‰ range previously reported for aquatic NOM analysis (Malcolm, 1990; Leenheer et al., 2003) and also consistent with earlier bulk analysis of a membrane biofoulant of this treatment plant (-27.0 ‰ for water soluble (Sol) material and -27.9 ‰ for water insoluble (Insol) material Greenwood et al., 2006). The soluble fraction was more enriched in ^{13}C by ~0.7 to 1 ‰ relative to the insoluble fraction—analyses of foulants from this treatment plant showed a similar 0.5 ‰ differential between soluble and insoluble fractions (Greenwood et al., 2006).

Table 4.4 $\delta^{13}C$ values for the four foulant fractions. $^{(n)}$ = number of replicates from which average values and standard deviations (sd) were measured.

Sample	$\delta^{13}C$ ‰	sd
Sol_{BC}	-27.5	$0.10^{(3)}$
$Insol_{BC}$	-28.5	$0.13^{(3)}$
Sol_{AC}	-27.9	$0.08^{3)}$
$Insol_{AC}$	-28.6	$0.02^{(3)}$

Croué et al. (2003a) reported that the analysis of a membrane foulant, from the same treatment plant, showed that the insoluble fraction was more enriched in proteinaceous structure, including phenyl alanine, representing relatively recent deposition. In the present study, the n-C_{14} to n-C_{34} n-alkane profile (Fig.4.1) displays a strong even-over-odd predominance, also indicating a dominant bacterial input—bacterially produced n-alkanes ranging from C_{14} to C_{31} (with maxima at either one or two n-Cs) are commonly reported (e.g., Han & Calvin, 1969; Nishimura & Baker, 1986; Grimalt & Albaiges, 1987; Elias et al., 1997). A similar even-over-odd was additionally observed in n-alkanes (chain lengths $>n$-C_{19}) in biofoulant taken from the same treatment plant (Berwick, 2010; Berwick et al., 2010a). Collectively these similar findings suggest that the insoluble fraction isolated from the biofoulant comprises freshly deposited material, including ^{13}C depleted lipids of bacteria. On the other hand, the soluble fraction is believed to be rich in sugars and EPSs (Croué et al., 2003a), which tend to be more ^{13}C enriched (Leavitt & Long, 1982; Badeck et al., 2005, Table 4.5). The membrane cleaning process had little influence on the bulk isotopic values of the fractions.

4.4.2.2 Compound Specific $\delta^{13}C$ characteristic of the Membrane Foulants

4.4.2.2.1 Suitability of Fractions for GC-irMS

The resolution and concentrations of the n-alkanes of the aliphatic fraction and several of the major derivatised products of the polar fraction were conducive to CSIA. The aromatic fraction however, comprised a high abundance of closely eluting compounds and a large UCM which limited the product resolution necessary for CSIA. For this reason the aromatic fraction was not subjected to CSIA here.

If more time had been available the aromatic fraction could have been separated into less complex fractions. An example of such a fractionation method is further column

chromatography separation with incremental increases in proportion of DCM to *n*-pentane (e.g., by 2.5 % adjustments in the range 25–35 %) resulting in a PAH (polyaromatic hydrocarbon mixture; Bastow et al., 2007) fraction. Other commonly obtained simplified aromatic fractions include alkyl benzenes and mono-aromatic steranes.

4.4.2.2.2 Aliphatics (n-alkanes)

Baseline GC resolution and the sufficiently high concentrations of the *n*-C_{16} to *n*-C_{32} *n*-alkanes supported the measurement of their $\delta^{13}C$ values (Fig. 4.5). The *n*-alkanes were generally depleted in ^{13}C relative to the bulk foulant values by -0.3 ‰ to -2.9 ‰. The lipid fraction of biological samples is typically more depleted by -3 ‰ to -11 ‰ compared to the values of the bulk material (De Niro & Epstein 1977; Monson & Hayes 1982; Blair et al., 1985; Hayes 1993; Abraham et al., 1998; Boschker et al., 1999).

The level of ^{13}C depletion seen in the foulant *n*-alkanes is just below this range, indeed some *n*-alkanes (*n*-C_{26} and *n*-C_{18}) were slightly ^{13}C enriched relative to the bulk (Fig. 4.4). This may be due to:

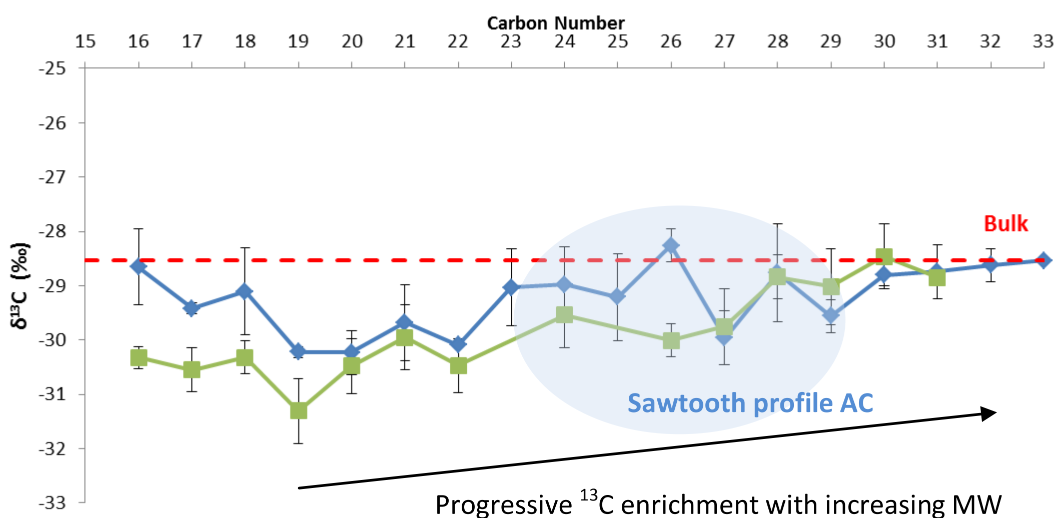
1) Dominance of carbohydrate rich EPS providing a relatively ^{13}C enriched substrate on which a complex biofilm community grows, itself progressively becoming more ^{13}C-enriched.
2) Species-specific variation of ^{13}C depletion in lipids (DeNiro & Epstein, 1977) i.e., LMW *n*-alkanes (reflecting algal/bacterial source inputs) have different $\delta^{13}C$ values than HMW *n*-alkanes (reflecting terrestrial plant source inputs).

Although the microbial community of biofoulants is extremely complex (Bereschenko et al., 2008 & 2010), particular bacterial species (e.g., *Sphingomonas*) have been strongly implicated in biofilm establishment and growth on the membranes used for water treatment *(*Bereschenko et al., 2008 & 2010). *Sphingomonas* are Gram -ve *alphaproteobacteria* which possess monosaccharide and tetrasaccharide-type glycosphingolipid instead of the more usual lipopolysaccharide in their cellular lipids (Kawasaki et al., 1994).

The $\delta^{13}C$ values of *n*-alkanes or the fatty acid fractions of glycosphingolipid and lipopolysaccharide have not previously been compared. However, it is likely that the differing number of carbons involved in the different glycolipid precursors results in

opportunities for differing ^{13}C discrimination — based on differences observed between *iso, anteiso* and *n*-alkanes with different fatty acid precursors (e.g., PLFAs measured in Chapter 3; also reported by Grice et al., 2008; Zhou et al., 2010).

a) Insol. *n*-alkanes = BC = AC



b) Sol. *n*-alkanes BC = AC =

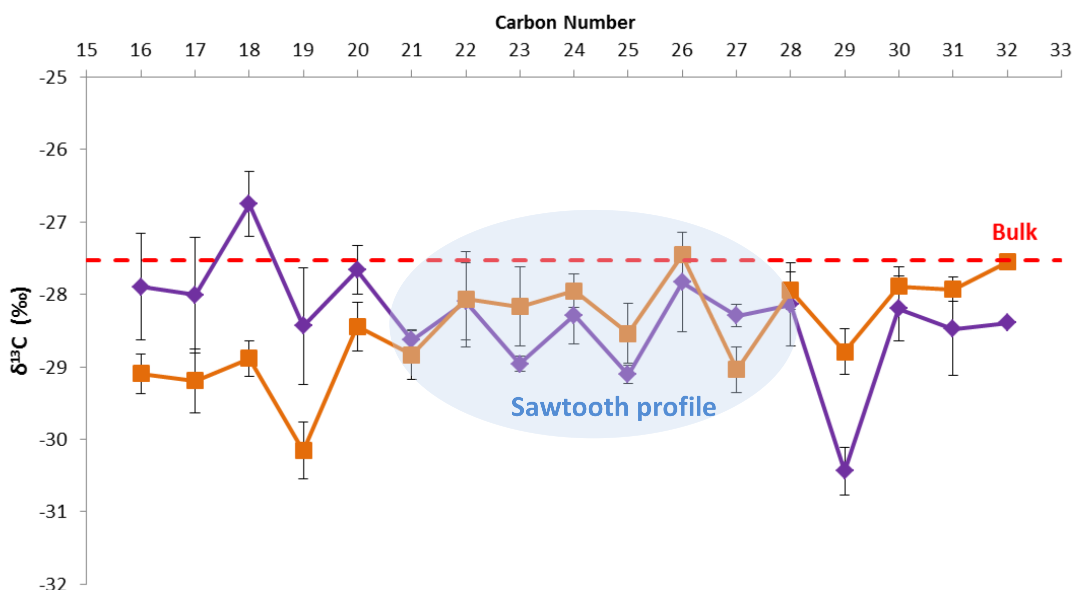


Figure 4.5 CSIA $\delta^{13}C$ profiles of n-alkanes from (a) $Insol_{BC}$ (green squares) and $Insol_{AC}$ (blue diamonds) foulant fractions; and (b) Sol_{BC} (orange squares) and Sol_{AC} (purple diamonds) foulant fractions. Bulk values shown by red dashed line. Error bars represent standard deviation indicated in Table 4.4. The blue oval in (a) highlights the distinctive 'saw tooth profile'.

The heavier $\delta^{13}C$ values of *n*-alkanes from the Sol. fractions compared to the Insol. fractions were closer to the bulk $\delta^{13}C$ values (Table 4.4). Insol. *n*-alkanes ranged from -31.3 ‰ to -28.8 ‰ (Fig. 4.5a), with both pre- and post-cleaned fractions showing a progressive

enrichment in ^{13}C with MW. This trend, not seen in the $\delta^{13}C$ *n*-alkane profiles of the Sol. fractions (Fig. 4.5b), is thought to reflect a complex fraction containing both indigenous and newly synthesised biochemicals (e.g., cellular lipids) as well as some biodegraded material (e.g., EPSs). The soluble fraction however, may be a simpler fraction containing a higher proportion of LMW biodegraded organic moieties (e.g., EPS and SMPs). It is also possible that an unknown degree of isotopic fractionation may have occurred during the solubility separation step, as this was not assessed.

As biodegradation can lead to the opposite trend of decreasing $\delta^{13}C$ values with increasing C-number (e.g., Pearson et al., 2007), it is possible that this effect may counteract that of the progressive enrichment in ^{13}C with MW (occurring simultaneously), resulting in the apparent 'homogenised' effect seen with the *n*-alkane $\delta^{13}C$ values of the soluble fraction.

In general, the even-number *n*-alkanes were more enriched in ^{13}C than neighbouring odd-numbered *n*-alkanes, particularly between *n*-C_{24} and *n*-C_{30}. In several samples *n*-C_{19} was the most ^{13}C depleted *n*-alkane (with the exception of Sol_{AC}) and *n*-C_{18}, *n*-C_{26} or *n*-C_{30} the most ^{13}C enriched *n*-alkane. As the treatment plant feed-water is surface-derived, it is possible that these *n*-alkanes may be from a plant source, although the likelihood of this is reduced given the extensive pre-treatment of the feedwater (Ventresque et al., 2000; Houari et al., 2010). This represents the inverse of the 'saw-tooth' $\delta^{13}C$ trend of heavier odd-numbered *n*-alkanes commonly detected in organic extracts and attributed to even-numbered fatty acid precursors (Collister et al., 1994; Huang et al., 1995; Grice et al., 1997 & 2001; Chikarashi & Naraoka, 2003; Grice & Brocks, 2011). As previously discussed in Section 4.4.1.1, the carbon number products of HyPy directly reflect the carbon-number of their free or bound carboxylic acids, ether and alcohol precursors (Love et al., 2005). The differences in fatty-acid precursor depletion can be explained by two distinct biosynthetic pathways which result in the production of ^{13}C-depleted even-number fatty acid precursors and ^{13}C-enriched odd number fatty acid precursors (from which the *n*-alkanes derive via reduction during HyPy).

A biosynthetic pathway pertaining to the more common isotopic pattern of isotopically heavier odd-numbered *n*-alkanes has recently been proposed by Zhou et al. (2010). The basis of the pathway has been described in Monson and Hayes (1980), Chikarashi and Naraoka (2003) and Grice et al. (2008). Briefly, the formation of even-chain *n*-alkanes

occurs via decarboxylation of the corresponding (*n*+1) odd-chain-*n*-fatty acids (which are themselves synthesised by the repetitive extension and elongation of propionyl-CoA with 2-carbon units derived from malonyl-ACP; see 3.2.8.3).

Of particular relevance to the present findings is the formation of propionate by the enzymatic reduction of the relatively ^{13}C enriched carboxyl group of pyruvate (DeNiro & Epstein, 1977; Monson & Hayes, 1980, 1982; Zhou et al., 2010). This propionate goes on to function as a precursor to odd-chain-*n*-fatty acid and the resultant even-chain *n*-alkanes are relatively enriched in ^{13}C. Zhou et al. (2010) proposed that the reduced pyruvate is then esterified using Coenzyme A thiol (CoSH), resulting in the formation of propionyl-CoA, and that competition in the pyruvate pool (preferentially used for propionate) leaves the relatively depleted ^{13}C for the formation of acetate. As opposed to the odd chain-*n*-fatty acid precursor, the even-chain-*n*-fatty acids precursor is the relatively ^{13}C depleted acetate. Although this biosynthetic pathway is proposed for plants, it may similarly account for the saw-tooth pattern observed in the biofoulants. It also implies preferential utilisation of ^{12}C in the microbial fatty acid biosynthesis of the foulants.

The δ^{13}C values of the *n*-alkanes show this "inverse saw-tooth profile" over a wider MW range, between *n*-C_{18}–*n*-C_{30} (Fig. 4.5b), but do not reflect any obvious change with molecular weight, consistent with a more homogeneous matrix from microbial reworking (i.e., biodegradation). Differences in C-*n* range of the "inverse saw-tooth profile" evident in the soluble and insoluble fractions may reflect differences in the contribution of their source inputs, particularly ^{13}C enriched EPS (<*n*-C_{24}) and ^{13}C depleted microbial lipids (>*n*-C_{24}).

The microbial complexity of biofilms and foulants in water treatment is only beginning to be understood (Chen et al., 2004; Pang et al., 2007; Bereschenko et al., 2007 & 2008). Bereschenko et al. (2007 & 2008) observed a distinct bacterial community with a consistent dominance of *Sphingomonas* spp. present in the biofoulant of a spiral-wound membrane (*Cf.* the treatment plant feedwater) and proposed, as in the present study, that there is active formation of biofilm on the membrane surface itself.

Planktonic-form *Sphingomonas* (travelling from upstream biofilm deposits within the plant) were identified in the early formed biofilm and attributed to colonisation on the membrane surfaces. Once attached, an initial EPS layer (i.e., ^{13}C-enriched polysaccharide material) was

formed (within four days) and other microbially complex and distinct EPS layers developed subsequently (over 32 days). The relatively ^{13}C-enriched *n*-alkane data obtained for the soluble fraction (comprising mainly heavy sugars, some of which had been newly synthesised), may indicate the presence of an established microbial community, hence the homogeneous profile seen in the sol. fraction *n*-alkanes. The more ^{13}C-depleted fraction (insol.) additionally contains depleted bacterial lipids to indigenous and reworked sugars, and may reflect the inputs of a newly established microbial community.

The overall relatively ^{13}C-enriched *n*-alkane data (-0.3 ‰ to -2.9 ‰ *Cf.* bulk foulant) compared to more typical level of ^{13}C depletion seen in *n*-alkanes (-3 ‰ to -11 ‰ *Cf.* bulk foulant; De Niro & Epstein 1977; Monson & Hayes 1982; Blair et al*.,* 1985; Hayes 1993; Abraham et al*.,* 1998; Boschker et al*.,* 1999) may be due to dominance of carbohydrate rich EPS providing a relatively ^{13}C enriched substrate on which a complex biofilm community grows, itself progressively becoming more ^{13}C-enriched. Thus this explanation (i.e., based on findings obtained in the present study) is in keeping with the insight gained by molecular-biology and the sophisticated imaging techniques of Bereschenko et al. (2007 & 2008).

Sphingomonas produce prolific sticky EPS consisting of the 'sphingans' (gellan, welan, and rhamsan) and other carbohydrate-rich material (Pollock, 1993; Fialho et al., 1999; Pollock & Armentrout, 1999; Azeredo & Oliveira, 2000; Johnsen et al., 2000; Fialho et al., 2008). The EPS produced by *Sphingomonas* isolated from drinking water treatment membranes are an ongoing field of active research (e.g., Bereschenko et al., 2011; Tala et al., 2012). Greater understanding of these compounds (for example the determination of δ^{13}C values for gellan, welan, and rhamsan of cultures organisms) will provide useful fundamental background information which would help provide a more accurate interpretation of the CSIA data generated in the present study.

Whilst enzymatic cleaning of the membrane biofoulant samples made little difference to the bulk stable carbon isotopic composition of the respective sample fractions (Table 4.4), it did lead to differences in the δ^{13}C profiles of the *n*-alkanes, highlighting the greater detail provided by CSIA. After cleaning the lower MW *n*-alkanes ($<C_{21}$) were 1 to 2 ‰ heavier in δ^{13}C, whereas some of the higher MW *n*-alkanes showed lighter δ^{13}C values, possibly reflecting the removal of an unidentified fraction during cleaning.

4.4.2.2.3 Sugars in the Polar Fraction

Table 4.5 shows the $\delta^{13}C$ values of sugar derived products detected in the polar fraction. Insufficient GC resolution prevented the $\delta^{13}C$ measurement of the TMS-esters of galactofuranose, ribofuranose, arabinose and arabinopyranose, so their combined value (average of 4 sugars, Table 4.5) was measured, the average value of these four products in the insoluble fractions ranged from -15.8 ‰ to -37.2 ‰. Concentrations of these products were too low in the soluble fraction to allow $\delta^{13}C$ measurement. Their concentration in the insoluble fractions suggests an EPS source.

Table 4.5 Corrected $\delta^{13}C$ values of derivatised polar fractions of the biofoulants determined by GC-irMS. (n) = number of replicates from which average values and standard deviations were measured. * = galactofuranose, ribofuranose, arabinose and arabinopyranose, these components were not resolved to baseline level and an average value of the four were taken.

	$\delta^{13}C$ ‰			
	$Insol_{BC}$	$Insol_{AC}$	Sol_{BC}	Sol_{AC}
Average of 4 sugars*	-21.9 ±0.82$^{(3)}$	-34.9 ±1.0$^{(3)}$	Not detected	Not detected
levoglucosan	-15.8 ±1.0$^{(3)}$	-31.4 ±0.46$^{(3)}$	-23.0 ±1.0$^{(3)}$	-37.2 ±0.2$^{(3)}$
Bulk biofoulant	-28.5	-28.6	-27.5	-27.9

'Before cleaning', these sugars were ^{13}C enriched relative to the bulk, as is typical of biological organisms. However 'after cleaning' they were ^{13}C depleted relative to the bulk. Levoglucosan was the only sufficiently isolated product for which $\delta^{13}C$ values could be measured and it was able to be measured in both insoluble and soluble samples. Levoglucosan was more ^{13}C enriched in the insoluble fraction, this may indicate more freshly deposited sugar material, or may simply be due to isotopic fractionation between insoluble and soluble material. As the provided biofoulant samples were separated into water-soluble and water-insoluble fractions shortly after initial sampling (at an earlier instance, in a different institution, refer Section 4.3.1 and Croué et al., 2003a), isotopic fractionation during the separation step was not investigated.

The cleaning process did however lead to a substantial and consistent depletion in ^{13}C of 13–15.8 ‰ in levoglucosan of both samples, mirroring the $\delta^{13}C$ trend of the combined sugars. These data trends may indicate preferential removal of ^{13}C-enriched sugars. The protease based cleaning solution (Croué, personal communication) might also effectively

hydrolyse the EPS matrix, which could contribute to the significant isotopic fractionation measured, or again, may be due to isotopic fractionation occurring during the cleaning process. Further research is necessary in order to determine if isotopic fractionation during the cleaning, or solubility-separation processes occurred.

Brant et al. (2010) showed enzymatic cleaning removes proteins, lipids, hydrocarbons and sugars. Existing commercial cleaning procedures however, are typically more effective in the removal of protein material than polysaccharide material (Doumeche et al*.,* 2007; Houari et al*.,* 2010), and usually fail in completely removing the complex biofilm communities responsible for fouling, resulting in repeat biofouling (Flemming, 2002; Bereschenko et. al., 2011). The effect of different cleaning solutions on foulant EPS is an ongoing issue of interest, and CSIA has the potential to help distinguish the relative removal efficiency of different product groups.

A combination of CSIA with contemporary molecular biology and microscopic techniques on material generated from model-treatment plants (e.g., Bereschenko et. al., 2008, 2010 & 2011) may provide a more detailed picture of carbon-dynamics involved in fouling and cleaning processes.

4.5 Conclusions

Recovery of the HyPy product and its separation into fractions by subsequent column chromatography and derivatisation was partially successful in achieving the required degree of baseline separation for CSIA. Whilst *n*-alkanes in the saturate fraction and derivatised sugar precursors in the treated polar fraction were able to be successfully analysed, most constituents of the aromatic fraction and derivatised polar fraction were not sufficiently resolved for CSIA. However the analytical results provided insight into potential biosynthetic processes, organic sources and carbon dynamics with examples given below:

- Even-number *n*-alkanes of relatively high abundance and MMAs in the aliphatic fraction; and sugar products (possibly from EPS) in the derivatised polar fraction were indicative of a microbial source.
- The *n*-alkane $\delta^{13}C$ profile showed an inverse saw-tooth profile with the odd-carbon numbered *n*-alkanes lighter than the even-carbon-numbered *n*-alkanes, reflecting the differing biosynthetic pathways of even and odd fatty acid precursors. This is

consistent with the proposal of Zhou et al. (2010) that the reduced pyruvate is esterified using (CoSH), resulting in the formation of propionyl-CoA, and that competition in the pyruvate pool (preferentially used for propionate) results in the preferential utilisation of ^{12}C during fatty acid biosynthesis.

- The n-C_{19} alkane (formed by biosynthetic decarboxylation of the C_{20} FA) was repeatedly the most ^{13}C depleted n-alkane of all the foulant samples, consistent with a relatively ^{13}C depleted acetate fatty acid precursor consistent with a lipid source. The n-C_{26} alkane (formed by biosynthetic decarboxylation of the C_{27} FA), was frequently the most ^{13}C enriched, consistent with a relatively ^{13}C enriched propionate fatty-acid precursor resulting from the enzymatic reduction of the ^{13}C enriched carboxyl group of pyruvate.
- The bulk value of the water soluble fraction of the membrane biofoulant material was slightly (< 1 ‰) more ^{13}C depleted than the corresponding insoluble fraction. At the compound specific level, the heavier $\delta^{13}C$ value of the insoluble fraction suggests relatively ^{13}C enriched EPS (i.e., levoglucosan -15.8 ‰), although this fraction also contained some ^{13}C depleted microbial lipids (n-C_{16}–n-C_{22} alkanes). The slightly less ^{13}C-enriched soluble fraction was rich in 'reworked' EPS and biosynthesised non-indigenous SMPs with lighter $\delta^{13}C$ signatures (i.e., levoglucosan -23.0 ‰). This isotopically light signature determined for the biofoulant as a whole may be explained by microbial reworking (producing ^{13}C depleted carbohydrate SMP). Further work is required to decipher if the observations were due to simple isotopic fractionation occurring during the isolation process.
- The isotopic insights provided by the present research compliment the molecular findings of Croué et al. (2003a) which showed components of the water insoluble foulant fraction represented more recent deposition. In the present study, the strong even-over-odd predominance of n-C_{14} to n-C_{34} n-alkane profiles indicates a dominant bacterial input, extending this, CSIA of the n-alkanes showed a progressive ^{13}C enrichment with increasing MW indicating the generation of new (relatively ^{13}C enriched) material.
- The isotopic insights provided by the present research compliment the insight gained by molecular-biology and the sophisticated imaging techniques of Bereschenko et al. (2007 & 2008) which proposed the active formation and

proliferation of biofilm on the spiral wound membrane surface itself (not from the material from treatment plant feedwater). In the present study, the overall relatively ^{13}C-enriched *n*-alkane data (-0.3 to -2.9 ‰ *Cf.* bulk foulant) compared to more typical level of ^{13}C depletion seen in *n*-alkanes (-3 to -11 ‰ *Cf.* bulk foulant) was attributed to dominance of carbohydrate rich EPS providing a relatively ^{13}C enriched substrate on which a complex biofilm community grows, itself progressively becoming more ^{13}C-enriched.

Whilst the methodology requires further optimising, CSIA can extend the molecular analysis of organics in drinking water systems. Although demonstrated here on membrane foulants, this analytical technology could be applied to the characterisation of aquatic NOM in source water inputs providing sufficient quantities of natural organic matter could be isolated.

Chapter 5

Characterisation of NOM in Two Source Waters

5.1 Overview

Drinking water sources have been traditionally selected for their pristine nature, and regulation by water utilities strives to protect them from anthropogenic chemicals representing a potential health risk. Managing microbial loads and types is one important aspect of the multi-barrier approach typically practiced. This case study investigates the practical utility of the SPME and PLFA molecular and stable isotopic characterisation by application of these analyses to Western Australian drinking water sources. The separate molecular and stable isotopic analysis of highly-semi volatile aquatic organics by SPME and PLFAs, biomarkers of the viable microbial community, may help monitor organic and microbial dynamics in aquatic environment, respectively.

The NOM of two West Australian reservoirs (Bolganup Creek Reservoir and Harding Reservoir) with contrasting catchments were separately isolated and characterised by SPME, PLFA and established analytical methods (outlined in Chapter 1). The acquired analytical data was also integrated with general information from previous assessment studies of the catchments (Garbin et al., 2010). This work aims to explore the extent to which CSIA may practically complement contemporary NOM characterisation protocols. A specific objective was to ascertain whether the $\delta^{13}C$ signatures of the volatile and semi-volatile component of the DOC, detected by SPME, and the microbiota living in the source waters, detected by PLFA analysis, was indicative of the primary organic input of the source water.

5.2 Background

5.2.1 Sample Sites

Bolganup Creek Reservoir and Harding Dam Reservoir differ markedly in geographical location and environmental factors including climate and source input. Rainfall and surface runoff provide the main recharge in both catchments (Marchesani, 1993). Significantly,

the C3 dominant catchments of Bolganup Creek and C4 dominant catchment of Harding Dam Reservoirs should be reflected by quite distinctive $\delta^{13}C$ signatures.

5.2.2 Location of Sampling Sites

Fig. 5.1 shows the locations of Harding and Bolganup Reservoirs.

Figure 5.1 Satellite image of Western Australia showing Bolganup and Harding Reservoir locations and nearby townships. Image obtained from Google Earth 18/09/2010.

5.2.3 Bolganup Creek Reservoir

Bolganup Creek Reservoir is located on the southern coast of Western Australia, approximately 40 km north of Albany and 20 km east of Mount Barker (Fig. 5.1). It presently functions as a non-potable water supply to the nearby Porongurup settlement, and was previously used to supplement the Lower Great Southern Town Water Supply Scheme

(Water Corporation, 2004a; DoW, 2008). An aerial photograph of the reservoir and surrounding forest is shown in Fig. 5.2. The catchment covers an area of 5 km^2 most of which falls within the confines of Porongurup National Park, although some is also located on a freehold farm. It is a predominantly native forest catchment, dominated by Karri (*Eucalyptus diversicolor*), small native shrubs and Lichens (Water Corporation, 2005a). This site was selected to represent a strongly C3 dominant system with extensive vegetation coverage (Fig. 5.3).

Figure 5.2 Aerial photograph of Bolganup Creek Reservoir. Image obtained from Google Earth 26/11/2009.

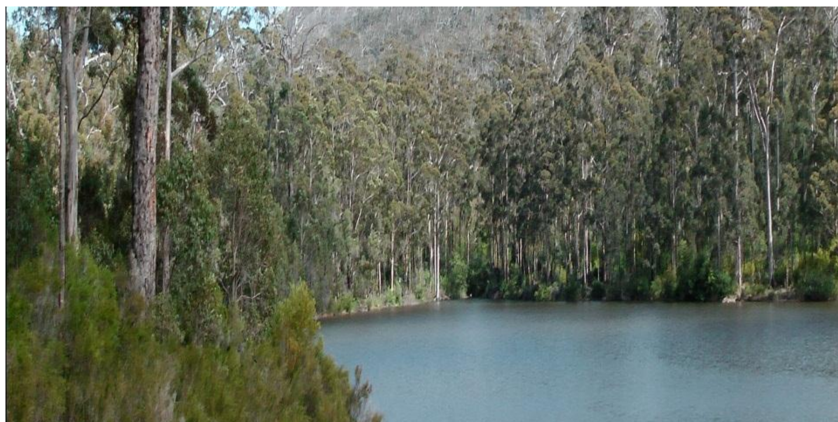


Figure 5.3 Photograph of Bolganup Creek Reservoir (Taken by Author, 2009).

The Dam is an earth fill structure, constructed in 1957 (DoW, 2008). The reservoir has a capacity of 225 Mega Litres (ML), a surface area of 0.04 km^2 and is 13.7 m deep (DoW, 2008). The reservoir experiences stratification in the summer months (November to April), when a thermocline develops at a depth of 2–3 m, and the water column usually returns to homogeneity by June (Garbin et al., 2010) .

The main water input to the reservoir is *via* surface runoff, with average inflows of 300 ML per year (Marchesani, 1993). The average annual rainfall for 1971–2000 was 692.6 mm, most of which falls in the winter months between May and August.

The Bolganup Creek Catchment Area is under the protection of the Country Areas Water Supply Act (1947), however recreation and conservation activities are supported within the catchment. The potential for these and other land use activities to contaminate the water (DoW, 2008) led to the establishment of a drinking water source protection plan by the Department of Water in 2008.

5.2.4 Harding Dam Reservoir

Harding Dam Reservoir is located in the Pilbara region, in the north of Western Australia, approximately 40 km southeast of Karratha (Fig. 5.1). The dam was constructed between 1983–1984 and is the primary potable supply of the West Pilbara Water Supply Scheme. The Reservoir has a capacity of 64 Giga Litres (GL), a surface area of 14 km^2 and a maximum depth of 24 m (WRC, 1999). An aerial photograph of the reservoir and catchment is shown in Fig. 5.4. The catchment area spans 1100 km^2, and is dominated by Spinifex hummock grasslands on slopes and Mulga shrublands and grasslands on depositional plains (shown in Fig. 5.6), although some trees, including River Gums (*Eucalyptus camaldulensis*), Coolabahs (*Eucalyptus victrix)* and Cadjebuts (*Melaleuca argentea*), are found along the major creeks and drainage lines (Water Corporation, 2004b). Water inflow into the dam is irregular and dependent upon intense rainfall events (typical totals of more than 100 mm; BOM, 2010) which occur during cyclones and thunderstorm events in summer and which contributes to frequent associated flooding. The Pilbara region experiences the greatest frequency of cyclones than anywhere else in Australia (BOM, 2010).

Harding Dam Reservoir experiences stratification for most of the year and 'overturn' of the dam usually occurs between May and June (WRC, 1999). Watercourse flows in the region

are highly seasonal and dry up for at least part of each year. Evaporation losses are also significant.

In the absence of a significant recharge event, the full reservoir has a capacity to supply approximately 15 GL water per year for two years. Seasonal cyanobacterial blooms in the Dam represent an on-going management concern (Water Corporation, 1997 & 2005b; WAEPA, 2007; WQRA, 2009; Garbin et al., 2010).

Figure 5.4 Aerial Photograph of Harding Dam Reservoir. Image obtained from Google Earth 26/11/2009.

Figure 5.5 Photograph of Harding Dam Reservoir (Water Corporation, 1997) showing Spinifex dominance of adjacent catchment.

5.3 Methodology

5.3.1 Sample Collection, Treatment and Storage

Approximately 100 L of water were collected from both the Bolganup Creek and Harding Dam Reservoirs in May 2009. The water was initially stored in 10 x 10 L polyethylene containers rinsed three times prior to filling for courier to Curtin University where they were kept chilled for up to 5 days until analytical processing. The samples were filtered using prewashed 0.45 µm glass fibre filters (GF/F, 43 mm, Whatman) under vacuum to remove particulate material and the filtrate was transferred to a 100 L stainless steel drum. The samples were stored refrigerated at 4°C.

5.3.2 Determination of Basic Water Characteristics

Basic water properties of DOC concentration, UV absorbance at 254 nm and 400 nm were measured for both raw (100 L) and concentrated (8 L) waters. DOC analysis was carried out as described in Section 2.3.5.1. UV absorbance was determined using a Shimadzu Pharmaspec UV-1700 UV-Visible spectrophotometer. A 1 cm quartz cell was used to determine absorbance at 254 nm and a 5 cm quartz cell was used to determine absorbance at 400 nm. UV absorbance at 254 nm was used to calculate SUVA (the UV_{254} absorption in m^{-1} divided by the DOC concentration in mg L^{-1}) and UV absorbance at 400 nm was used to determine total colour units (TCU) by calibration with colour standards.

5.3.3 Sample Concentration

80 L of both filtered waters were pre-concentrated ~10 fold using reverse osmosis (RO; at 414 kPa) and continual recycling of the retentate through the RO system to a suitable volume for the resin separation (~8 L) was reached. The RO system was pre-cleaned with sodium bicarbonate (5 L) and Milli-Q (5 L) water and cleaned between sample preparations. The effect of RO on introducing potential isotopic fractionation was investigated by analysis of standards before and after RO concentration, and no fractionation was evident. An additional 20 L of both waters was concentrated by RO for PLFA and SPME analysis.

5.3.4 Isolation of NOM

The isolation and fractionation of each NOM sample was carried out on a pre-cleaned, 500 mL sequential XAD resin system as schematically illustrated in Fig. 5.6. The system consisted of non-ionic macroporous styrene-divinyl benzene resins XAD-8 and XAD-4

(Amberlite, Rohm and Haas, column capacity 33 L). The procedure was based on that described by Croué (2004).

The resins were cleaned using Milli-Q water (2 L), followed by Milli-Q:acetonitrile (2 L, 25:75 v/v), then Milli-Q water until the DOC concentration of the eluent was below 0.05 mg L^{-1}. The resins were then preconditioned using hydrochloric acid (0.1 M, 1 L) prior to the introduction of each sample.

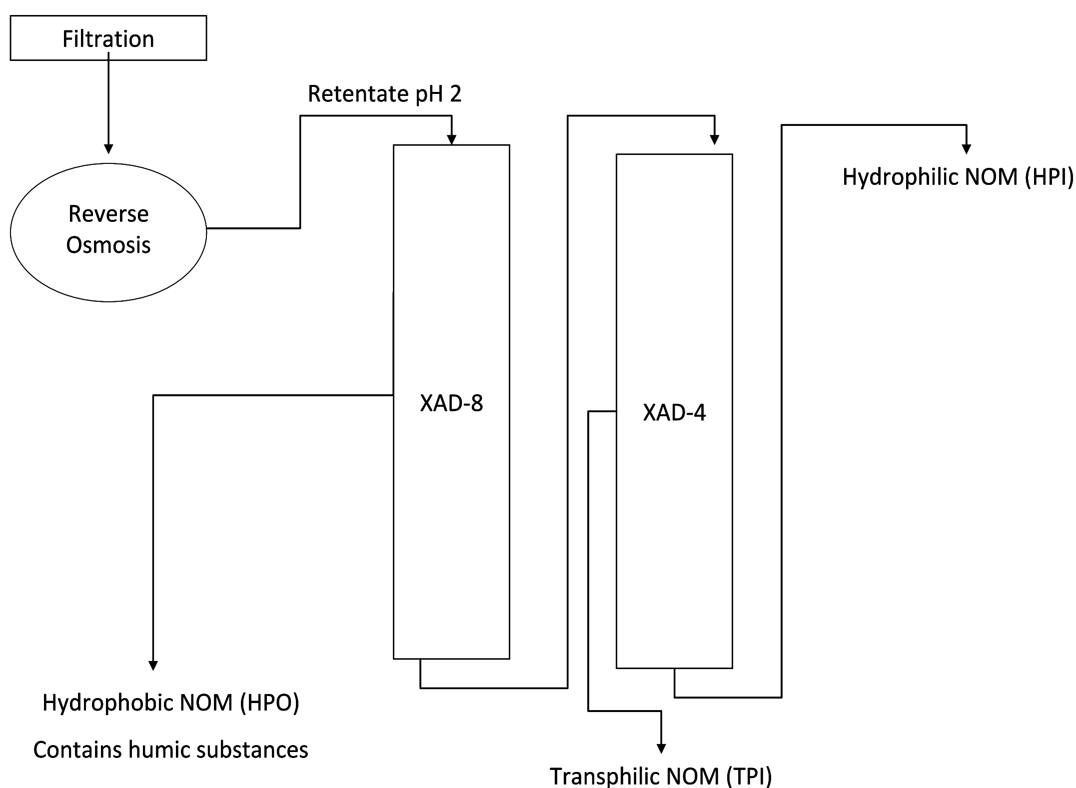


Figure 5.6 Schematic of XAD-4 and XAD-8 resin column series used for NOM isolation and fractionation. HPI fraction not analysed further.

Both waters (pre-concentrated to 8 L) were acidified to pH 2, in 4.5 L batches, with hydrochloric acid (1 M) immediately before the XAD fractionation. The acidified water samples were peristaltically pumped through the XAD-8 resin to adsorb the HPO fraction. The eluent of the XAD-8 resin containing the transphilic (TPI) and hydrophilic (HPI) fractions was then pumped through the XAD-4 resin, which adsorbed the TPI fraction. The eluent from the XAD-4 resin (the HPI fraction) was not analysed further.

The adsorbed NOM fractions were eluted from the resins by first adjusting the resins to pH 2 with formic acid (0.1 M) and pumping through aqueous formic acid (1300 mL) until the

conductivity of the effluent was the same as the influent. This was followed by elution with Milli-Q:acetonitrile (25:75 v/v; 500 mL), until the sample was clear). The eluents (intensely coloured) were collected in pre-annealed glass bottles, the acetonitrile was removed by rotary evaporation (Labrota 4001, Heidolph instruments GmbH & Co.) and the remaining fractions frozen, then lyophilised (Freezone freeze dry system 77500-61, Labconco) to yield the HPO fraction (Harding, 166 mg; Bolganup, 617 mg). Only trace amounts (<1 mg) of the TPI fraction and none of the HPI fraction of both samples could be retrieved. This finding is consistent with previous reports that both Harding and Bolganup Dam NOM were dominated by very hydrophobic acids (VHA; Garbin et al., 2010). Only the HPO fraction was obtained in sufficient quantity for detailed characterisation by application of the full suite of analytical procedures.

5.3.5 Molecular and Stable Isotopic Characterisation of HPO-NOM

5.3.5.1 Bulk Isotopic Characterisation

Bulk $\delta^{13}C$ analysis was performed at ANU, Canberra, as described in Section 2.3.5.4. C and N content were determined using the same equipment.

5.3.5.2 FTIR Analysis

FTIR spectra of HPO fractions were acquired using a Perkin Elmer Spectrum 100 fitted with a single-bounce diamond attenuated total reflection (ATR) accessory. Four background and four sample scans were collected, with an optical resolution of four wave-numbers. An ATR correction was used to compensate for the variation of penetration depth with wavelength that is inherent with ATR techniques. All FTIR spectra were scanned between 4000 and 700 cm^{-1} and data was acquired and processed with OPUS software. Detector resolution was maintained at 4 cm^{-1} for all analyses.

5.3.5.3 ^{13}C -NMR Analysis

Solid-state ^{13}C Cross Polarisation Magic Angle Spinning Analysis (CPMAS) NMR was conducted at a frequency of 50.3 MHz on a Varian Unity 200 spectrometer. The HPO samples were packed in a cylindrical zirconia rotor (7 mm diameter) fitted with Kel-F end-caps, and spun at 5000 ± 100 Hz using a MAS probe (Doty Scientific). Spectra were acquired using a 1 ms contact time, a 1 s recycle delay, and collection of between 24000–61000 scans. The free induction decays (FID) were acquired with a sweep width of 40 kHz and 1216 data points were collected over an acquisition time of 15 ms. The FID was zero-filled

to 32768 data points and 50 Hz Lorentzian line broadening and 0.01 s Gaussian broadening were used for processing. The methyl resonance of hexamethylbenzene (17.36 ppm) was used as an external reference for the chemical shift and the background signal of the acquired spectra was corrected by subtracting the signal of an empty rotor under the same acquisition conditions. The strength of the NMR signal of the HPO sample was calibrated against that of a Glycine reference standard, the details of which are described in Smernik & Oades (2000a & 2000b). The NMR analyses were conducted at the University of Adelaide.

5.3.5.4 Flash Pyrolysis

Approximately 0.5 mg of the HPO fractions were separately loaded into pre-annealed quartz pyrolysis tubes, each containing a pre-annealed quartz rod together with a small amount of analytical grade quartz wool (Part No. 1001-0345, CDS Analytical) to avoid sample loss. Flash pyrolysis was conducted using a Chemical Data Systems analytical pyroprobe 5250, fitted with a pyrolysis auto-sampler, at 600°C for 20 s whilst the pyrolysis chamber was held isothermal at 300°C.

An Agilent 6890 GC fitted with a DB5-MS column (60 m x 0.25 mm i.d. x 0.25 µm, J&W Scientific), coupled to an Agilent 5973 MSD was used for GCMS analysis. A split system set at 40:1 with a UHP helium carrier gas at 131 kPa head pressure was used for the analyses. The GC oven was initially held at -20°C (using liquid CO_2 cryogenic control) for 1 minute, followed by an 8°C min^{-1} increase to 40°C, then a 4°C min^{-1} increase to 310°C which was held isothermal for a final 20 mins.

Full scan acquisition was performed over the range *m/z* 50–550 at four scans s^{-1}. An electron energy of 70 eV, source temperature of 250°C and transfer line of 310°C were used. Peak identifications were based on correlation of GC elution position and mass spectral data with previously published data and mass spectral libraries (NIST 05 and Wiley 275).

5.3.5.5 Micro Scale Sealed Vessel Pyrolysis

Microscale sealed vessel pyrolysis (MSSVpy) represents a more controlled thermal treatment strategy that has proven useful in establishing structures and source inputs of aquatic NOM (Greenwood et al., 2006; Berwick et al., 2007, 2010a&b.). The traditional method of analytical (flash) pyrolysis is largely limited to the detection of thermally 'labile', apolar products (Saiz-Jimenez, 1994). MSSVpy can promote the reduction of polar NOM moieties and significantly increase the yields of GC amenable products (Greenwood et al.,

2006; Berwick et al., 2007; Berwick et al., 2010a&b) thus representing a valuable compliment to Flash-py.

Small amounts of HPO fraction (~0.3 mg) were loaded into angled annealed glass tubes (5 cm long x 5 mm i.d.), which had been pre-filled to the middle with annealed glass beads. Glass beads were also added above the sample to within 2 mm from the top of the tube to fill the void volume. The loaded tubes were then sealed by an air-acetylene flame, with care, ensuring that the samples were not heated directly.

The sealed vessels were then heated off-line in an oven (300°C, 72 hrs). For analysis, the thermally treated sealed vessels were loaded into a purpose-built injector port (300°C) installed on the top of the GC oven, and cracked using a plunger. The released pyrolysate products were transferred with the helium carrier gas (UHP, BOC gases) to the GC column (50 m x 0.32 mm i.d. x 0.25 µm DB5-MS J & W scientific capillary column), where they were cryo-focused using liquid nitrogen (BOC gases) prior to GCMS analysis. GCMS analysis of the volatile MSSV pyrolysates was carried out on an Agilent 6890 GC coupled to an Agilent 5973 MSD. The GC oven conditions were the same as those described for the Flash-py analysis (5.3.5.4).

5.3.5.6 SPME-GCMS and CSIA of Volatile DOC Fraction

SPME-GCMS of the concentrated and raw waters was conducted as described in Section 2.3.5.3. Analyses were conducted in duplicate for each fibre type, using pre-conditioned PDMS and PA fibres. Blanks and standard mixes were run every six samples. Single fibres were used for GCMS analysis and multiple (x 4) fibres as described in Section 2.4.1.3 were used for CSIA. The effect of the RO concentration step on molecular and isotopic fractionation was evaluated by separately spiking samples with thymol before and after concentration. GCMS and CSIA oven conditions were as described in Section 2.3.5.3.

5.3.5.7 GCMS and CSIA Analysis of PLFAs

Two litres of RO concentrated waters from both the Bolganup and Harding Dams were run through GF/F filter paper. The paper was ultrasonicated and PLFA extractions were prepared as described in Section 3.3.1.1 and 3.3.2. Additionally, five litres of the raw waters (not concentrated) were passed through GF/F filter paper and PLFA extractions prepared to evaluate for any molecular or isotopic fractionation of the RO process. GCMS and CSIA conditions were as described in Section 3.3.3 and 3.3.5.

5.3.5.8 Statistical Analysis of PLFAs

Non-metric multidimensional scaling (MDS), cluster and Analysis of Similarity (ANOSIM) analysis were conducted using Plymouth Routines In Multivariate Ecological Research E-software version 6 (PRIMER-E v6) as mentioned in Chapter 3 (Section 3.3.4) and detailed in Appendix A. Analysis of Variance (ANOVA) was performed using IBM® SPSS® Statistics 21 statistical software, detail is presented in Appendix B.

5.4 Results and Discussion

5.4.1 Basic Water Characteristics

DOC concentration, 254 nm UV absorbance, SUVA and colour measurements for the Bolganup and Harding raw waters prior to RO concentration are given in Table 5.1.

Table 5.1 Basic character of Bolganup Creek and Harding Dam Reservoirs source (Raw) waters.

	Bolganup Creek	Harding Dam
DOC (mg L^{-1})	9.8	3.6
UV_{254} (cm^{-1})	0.420	0.115
SUVA (L mg^{-1} m^{-1})	4.3	3.2
Colour (TCU)	55	7

The DOC of the Bolganup Creek water was almost 3 times higher than the Harding Dam water. The aromatic character and colour indicated by UV_{254} and SUVA were also greater for the Bolganup water. These data are generally consistent with previous water quality data (personal communication, Drinking Water Quality Branch, Water Corporation, 2010) and reported data for these two reservoirs (Garbin et al., 2010). The different character of the two waters is also consistent with the dominant vegetation of the respective catchments.

The DOC values and percentage recoveries from the RO concentration and XAD fractionation of the source waters are shown in Table 5.2. DOC recovery from the RO concentration was greater than 92 %, consistent with the 80–99 % range generally recovered from waters with this separation procedure (Serkiz & Perdue 1990; Abbt-Braun & Frimmel., 1999; Maurice et al., 2002; Croué, 2004; Song et al., 2009).

The higher HPO percentage measured for Bolganup (97 %) compared to Harding (92 %) was consistent with previous HPO levels inferred from SUVA values (Garbin et al., 2010) —which

generally increase with the proportion of the HPO fraction (Croué et al., 1999a; Croué, 2004).

Table 5.2 Organic carbon content of Bolganup and Harding source waters, RO concentrate and XAD fractions. ND = not determined because of very low amount recovered. The total XAD recovered was calculated from the amount applied to the resins i.e., after RO concentration.

	Bolganup	Harding
DOC raw (mg L-1)	9.8	3.6
DOC conc (mg L^{-1})	95.0	33.1
RO Recovery (%)	97	92
HPO (mg)	617.2	166.7
HPO (% DOC)	81.2	62.9
TPI (mg)	27.4	ND
TPI (% DOC)	3.6	ND
Total XAD Recovery (%)	84.8	62.9

A photograph of the HPO samples showing visible differences in yield and colour is shown in Fig. 5.7.

Figure 5.7 Photograph of HPO fractions isolated from Harding Dam and Bolganup Creek - higher yield of Bolganup is evident.

The non-humic TPI fraction of Bolganup comprised only 3.6 % of concentrated DOC of the concentrated sample, whilst 15.2 % of the DOC was lost during XAD separation (Table 5.2) which is typical of this procedure (Croué et al., 1998). Only a trace amount (estimated <10 mg) of the TPI fraction of the Harding water was recovered. Neither of the TPI fractions were further characterised given the very low amounts recovered.

Fractionation yields indicated a large proportion of humics (HPO) in both waters, with the HPO fraction of Bolganup (81 %) larger than Harding (63 %) probably due to significant allochthonous input in Bolganup, with a greater non-humic input in Harding comprising the remaining 37 %. The non-isolation of TPI from Harding however, was a little unexpected in light of its history of algal blooms which typically contribute to this fraction (Section 5.2.4), but may be due to limitations of the XAD fractionation process. Previous researchers have reported unexpected low recoveries of DOC during combined XAD-8/XAD-4 fractionation. For example, using XAD-8 and XAD-4 in series, Croué et al. (1998) reported a recovery of only 57.7 % of the DOC for some waters and a recovery of only 18 % (upon further processing) of the DOC that did not initially adsorb to the XAD columns, suggesting that the hydrophilic and ultra-hydrophilic NOM fractions were the ones which were lost. Such losses were attributed to irreversible sorption of some classes of DOC onto the exchange resins, through the formation of precipitates, volatilisation or via foam formation during vacuum evaporation steps (Croué et al., 1998).

The colloid fraction may be significant in Harding water but was not isolated for analysis in the present study. Ultrafiltration could be used to recover the loss-vulnerable ultra-hydrophilic humic acid fraction which frequently comprises organic colloids (Leenheer et al.1995; Croué et al.,1998) but it was not conducted in the present study due to time limitations.

5.4.2 Characterisation of HPO fractions

5.4.2.1 Bulk Carbon Isotope Values and Carbon and Nitrogen Composition

The $\delta^{13}C$ values measured for the Bolganup (-28.1 ‰) and Harding (-24.0 ‰) HPO fractions (Table 5.3) are consistent with the respective contributions of C3 and C4 plant sources (O'Leary, 1981). C4 plants such as spinifex have a more significant impact on the isotopic composition of H-HPO although a value of -24 ‰ indicates some contribution from C3 plants such as Cadjebuts and River Gums identified at several locations in the catchment (Section 5.2.4).

Other influential organic sources include the seasonal cyanobacterial blooms in Harding (e.g., bi-annual peaks of phytoplankton, each of over 350000 cells mL^{-1}; Garbin et al., 2010. Harding also receives major runoffs during monsoonal rains (Section 5.2.4 and Garbin et al., 2010). The N and C compositions are similar for each of the HPO samples, though the N levels slightly higher and C levels slightly lower for Harding.

Table 5.3 Isotopic and Elemental data from EA-irMS analysis of HPO fractions.

	B-HPO	H-HPO
$\delta^{13}C$ (‰)	-28.1	-24.0
% N	1.6	2.1
% C	51.5	49.2
C:N ratio	32	23

5.4.2.2 FTIR Analysis

The FTIR spectra of the Bolganup and the Harding HPO fractions are shown in Fig. 5.8. The FTIR spectra of both samples were very similar, with both showing strongest absorptions over 1820–1660 cm^{-1}. These FTIR profiles are similar to those reported for the HPO-fractions of classical NOM systems (e.g., Great Salt Lake; Leenheer et al., 2004; Neversink Reservoir, Wershaw et al., 2005). The most intense absorption at 1720 cm^{-1} was typical of the C=O asymmetric stretch of carboxylic acid groups (Stevenson, 1982), and is usually prominent in the spectra of aquatic NOM and related materials such as Suwannee River fulvic acid (e.g., Elkins & Nelson, 2001). This absorbance was slightly more intense in B-HPO than H-HPO.

Other prominent spectral features include:

- Bands near 1650 cm^{-1}, 1560 cm^{-1} and 500 to 700 cm^{-1} (amides).
- Broad bands centred at 3400 cm^{-1} and from 1000 to 1150 cm^{-1} (alcohols).
- Prominent bands at 2960, 2930, 1460, and 1380 cm^{-1} (aliphatic hydrocarbons).

The broad absorption observed in the 3400–2400 cm^{-1} region, slightly higher in B-HPO, reflects the hydroxyl groups of phenols and other alcohols or acids. Its co-occurrence with the intense 1720 cm^{-1} absorption was indicative of the carboxylic acids (Stevenson, 1982) of carbohydrates (Bloom & Leenheer, 1989; Leenheer, 2004). Minor broad shoulders at 2750–2500 cm^{-1} and a 1250–1300 cm^{-1} absorbance are also typical of the carboxylic acid functional groups of fulvic acids (Cabaniss, 1991; Elkins & Nelson, 2001). The slightly greater 3650–3000 cm^{-1} absorbance of B-HPO than H-HPO was consistent with a more quantitatively significant input of carbohydrate from Eucalyptus leaves (Bernhard-Reversat, 1993, 1999) or a higher input of aromatic lignin carbon in the forested catchment.

The spectra of both fractions also showed a strong absorption shoulder at 1650–1560 cm^{-1} (Fig. 5.8), although it was more prominent in Bolganup. This absorption may be indicative of

primary and secondary amines and amides, or aromatic carbon and carbonyl groups which also absorb in this region (Stevenson, 1982).

In both HPO-fractions a distinctive doublet in the 3000-2800 cm^{-1} region centred on 2960, cm^{-1} and 2900 cm^{-1} represent C–H absorptions (Stevenson, 1982), the latter generally specific to the stretching of methyl (CH_3) groups (Zou et al.,2010).

Minor absorption bands at 1460 cm^{-1} and 1380 cm^{-1} are characteristic of the symmetric and asymmetric C–H bending of methyl and methylene groups (Stevenson, 1982). Together with the presence of OH groups they indicate either a carbonyl and/or hydrocarbon character (Perdue & Gjessing., 1990). The absorbance was slightly higher in B-HPO, consistent with absorbance of the 3650–3000 cm^{-1} region, suggesting carbonyl groups.

Overall, FTIR spectroscopy provided little distinction between the two HPO fractions. B-HPO had a slightly higher response and exhibited slightly greater proportion of carbonyl and carboxyl signals which may be indicative of higher proportions of polyphenols, sugar, or amino sugars which are all rich in forest biomass. The lignin/tannin-diagnostic methoxy ($O–CH_3$) groups (e.g., 1127 cm^{-1}, Robinson, 1991; Leenheer, 2009) showed only a very minor increase in the forested Bolganup over the more sparsely vegetated Harding.

The greater aromatic signal seen in the FTIR of Bolganup is consistent with its higher SUVA value (Table 5.1), although FTIR was not as sensitive as SUVA (2x variation) to this difference. Previous FTIR studies have similarly reflected limitations in quantitatively analysing heterogeneous amorphous, highly functionalised material such as NOM (Stevenson, 1982; Maurice et al.,2002; Croué, 2004; Wang et al.,2004; Gonzalez-Perez et al.,2004). The general similarity in FTIR spectra may reflect the regularity of the major biochemical precursors of environmental water bodies and possibly also similar diagenetic processes in the reservoirs, an issue which is investigated further in the assessment of the other analytical data (Section 5.4.3.3).

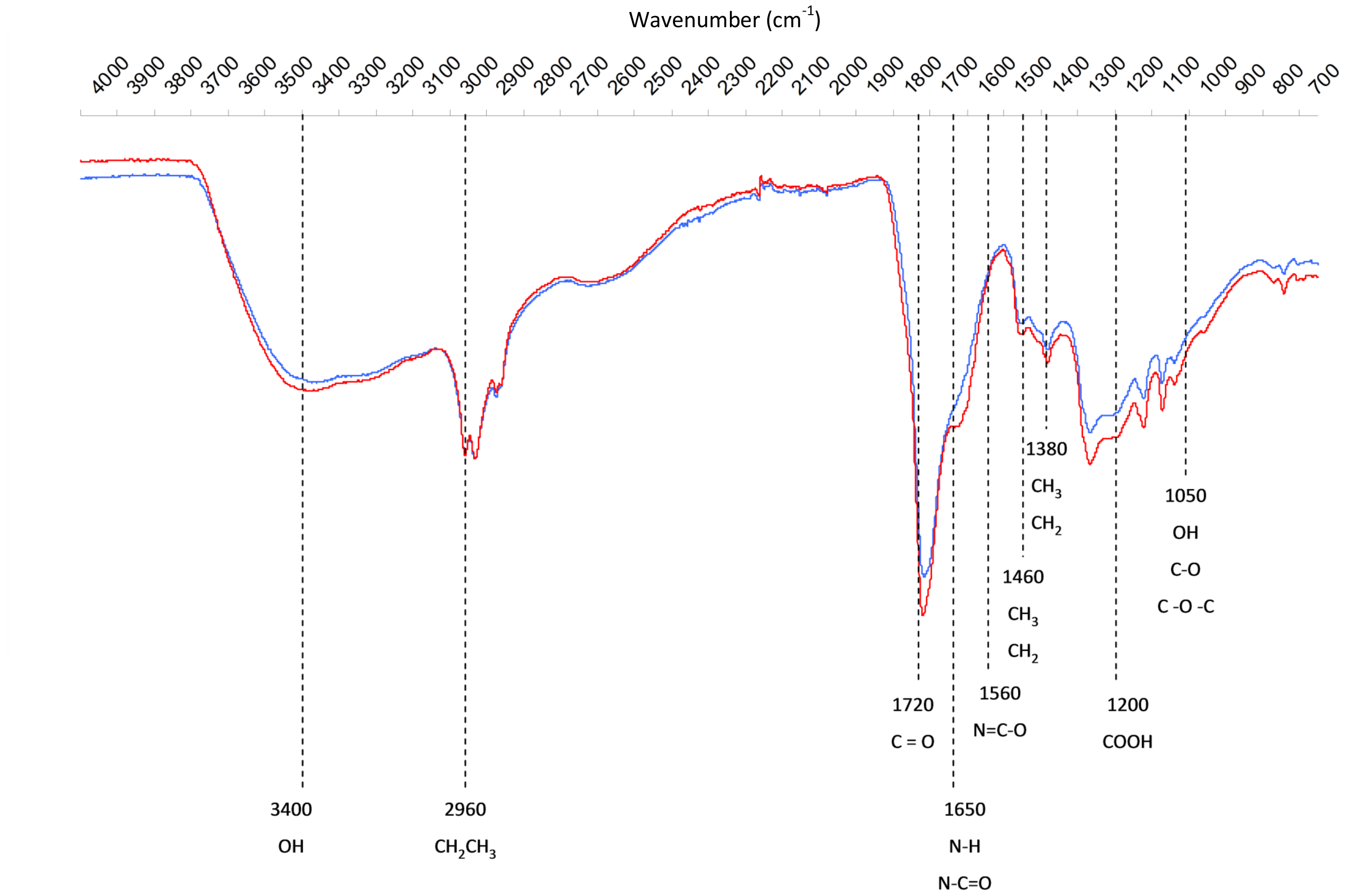


Figure 5.8 FTIR spectra of Harding (Blue) and Bolganup (Red) HPO NOM fractions.

5.4.2.3 ^{13}C-NMR Analysis

Both HPO fractions also showed relatively similar NMR spectra (Fig. 5.9). Several spectral features could again be attributed to the main functional groupings of common biological precursors. For example, O/N-alkyl groups reflect the presence of amides or carbohydrates (Malcolm, 1990), and carbonyl C indicates carboxylic acids, amides or esters (Croué et al.,2000; Hatcher et al.,2001; Keeler et al.,2006; Malcolm, 1990). The small peak at 120 ppm (indicative of aryl carbon) may be due to a spinning side-band (SSBs) artefact common to solid state NMR analysis. These can occur every 60 ppm and are usually evident at ~115 ppm (personal communication, Smernik, 2010). The small peak at 130 ppm has been ascribed to substitution of an aromatic ring with C–C linkages (Leenheer & Rostad 2004). Leenheer and Rostad (2004) also reported that small peaks at 140–160 and 105–120 ppm, such as also evident here, indicated phenolic structures.

The ^{13}C NMR CPMAS spectra were integrated over five spectral regions, and the relative abundances of these groupings are shown in Table 5.4. Both HPO samples were highly aliphatic. H-HPO contained a slightly higher alkyl (45–0 ppm) signal, with moderate levels of aromaticity (B-HPO>H-HPO) and carboxylic units.

The non-branched aliphatic signal at 30 ppm was more distinctive in H-HPO whereas branched alkyl aliphatics (40 ppm) and quaternary aliphatic carbons (50 ppm) were more distinctive in the B-HPO spectrum. High quaternary carbon signals have been previously attributed to microbial degradation products of terpenoid precursors (Leenheer et al.,2003), and represent a non-humic input—discussed further in Section 5.4.4.

The other major absorbance of both samples was O/N-alkyl carbon (110–45 ppm), indicative of alcohol (van Heemst et al., 2000), ether or ester (Leenheer & Rostad, 2004) functional groups sourced from polysaccharide or other carbohydrate precursors (Leenheer & Rostad, 2004). B-HPO showed the higher signals of O/N-alkyl (110–45 ppm) and aryl carbon (165–110 ppm), indicative of aromatic and/or other unsaturated organic compounds which could also be indicative of terpenoids (Leenheer et al., 2004).

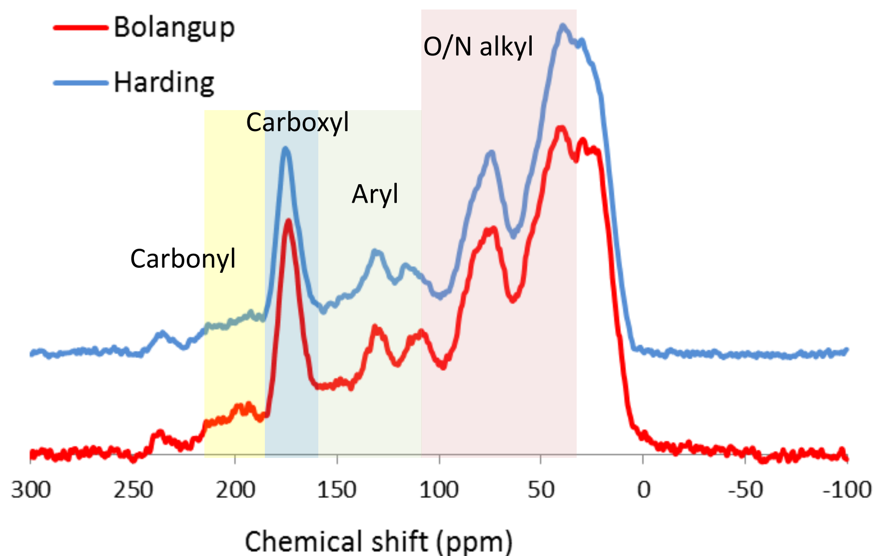


Figure 5.9 Solid state ^{13}C CPMAS NMR spectra of Harding and Bolganup HPO fractions (ppm = parts per million). Pale pink shading (110–45 ppm) indicates O/N alkyl carbon. Pale green shading (165–110 ppm) represents Aryl carbon. Pale blue shading represents Carboxyl carbon (185–165 ppm). Pale yellow shading represents Carbonyl carbon (220–185 ppm).

Table 5.4 Proportion of carbon types in the solid state ^{13}C-CPMAS NMR spectra of the Harding HPO and Bolganup HPO-NOM fractions (expressed as percentage of total signal).

Sample	Carbonyl 220–185 ppm	Carboxyl 185–165 ppm	Aryl 165–110 ppm	O/N-Alkyl 110–45 ppm	Alkyl 45–0 ppm
B-HPO	4.1	9.8	16.4	37.1	32.6
H-HPO	4.0	10.1	14.8	35.0	36.0

The high abundance of aromatic (aryl in Table 5.4) and O-alkyl structures is consistent with the relatively higher SUVA (and DOC) value of Bolganup water (Table 5.1) and the predominance of carboxyl or phenolic groups measured by FTIR analysis (Fig. 5.8) although NMR analysis showed a modest abundance of carboxyl carbon (185–165 ppm). SUVA correlates strongly with the aromatic character of DOC (Traina et al., 1990, Novak et al., 1992), but not necessarily with other functional groups, which is better provided by NMR and FTIR (Weishaar et al., 2003).

A signal (small shoulder) at 58 ppm can be indicative of tannin methoxy groups (identified in the FTIR spectra—Fig. 5.8), however an absence of signals at 153 and 156 ppm suggests negligible tannin and related phenolic OH input (Wilson & Hatcher, 1988, Benner et al.,

1990; Preston & Sayer, 1992, Preston et al., 1997; Leenheer & Rostad, 2004, McIntyre et al., 2005). A small alcohol peak evident at 60–90 ppm is probably related to the minor peak at 105 ppm indicative of ketal anomeric carbons of carbohydrates (Leenheer & Rostad, 2004). Carbonyl C was also present in both samples, indicated by the sharp and prominent band at 172 ppm and the smaller broad signal from 185–220 ppm.

Other NOM fractions (Berwick et al., 2010b; Leenheer et al., 2004) have shown generally similar ^{13}C NMR spectra which have been largely attributed to terpenoid biochemicals from autochthonous sources. These must be quantitatively significant even in the Bolganup Reservoir, where the organic load from the forested catchment would be high (Fig. 5.2).

Both HPO fractions showed minimal lignin-derived methoxy aromatics and plant-derived condensed tannins. Methoxy phenols diagnostic of lignin (e.g., guiaicol and syringol) are not very easily distinguished by ^{13}C-NMR spectroscopy (personal communication, Smernik, 2010). Pyrolysis may offer greater delineating power of these particular compounds (presented in the following sections) which in particular would be expected in Bolganup considering the lush forest vegetation of its catchment. Nor did Bolganup show an enhancement of aromatic C, which might be reflective of lignin-derived phenols (Perdue & Ritchie, 2004). Both NOM samples appear richer in aromatics than algal terpenoids (as described in Leenheer et al., 2003; Berwick et al., 2010b).

Another limitation of ^{13}C-CPMAS NMR analysis is that although it is a solid state analytical technique, it still requires a certain amount of analyte solubility, limiting accurate measurement to polar NOM enriched in carboxyl and OH groups, with relatively short alkyl chains (Smernik, 2009, personal communication).

5.4.2.4 Flash Pyrolysis

The Flash-Py of the HPO fractions of both waters produced a broad distribution of pyrolysates. Partial pyrograms, highlighting the retention time regions containing the vast majority of diagnostic pyrolytic products (RT 28 to 44 min) are shown in Fig. 5.10. The major products were non-source specific unsaturated aliphatic (**F20, F21, F22**) and alkyl benzenes (compounds **F38, F41, F43, F51, F59**). Carbohydrate derived furans and ketones (e.g., 4-methyl-1,3-isobenzofurandione, **F80**) were also prominent. Their identity and relative abundances of eighty seven pyrolysate products quantified in the NOM samples are shown in Table 5.5.

Two plasticisers (pthalic anhydride and dibutyl-phtalate) were also detected in consistently high concentrations in both samples. These artefacts were not present in control analyses, strongly implying they are from the polypropylene containers used for sampling (Leenheer & Rostad 2004). They have not been included in the product quantifications.

The pyrolysates were grouped into several precursor or structural groupings, and the relative abundances of these groups are shown in Figure 5.11. Both fractions show similar product group distributions, dominated by undiagnostic products, which are examined in greater detail in Section 5.4.2.5.

The most significant differences between the two waters was a greater proportion of carbohydrate derived pyrolysates in H-HPO (*Nb*. not reflected by FTIR or NMR data) and alkyl phenol pyrolysates (e.g., **F48, F49, F50, F53, F57** and **F58**) in B-HPO *(Nb.* consistent with NMR data). Alkyl phenols can derive from a variety of biological sources, thus preventing unequivocal source assignment (Guo et al., 2003). Possible sources include phenolic moieties (e.g., tyrosine) in protein (Simmonds et al., 1969; Wilson et al., 1983; van Heemst et al., 1999), polycarboxylic acids (Bracewell et al., 1980), algal derived poly phenols (van Heemst et al., 1996) and hydrolysed polysaccharide/protein mixtures (van Heemst et al., 1999).

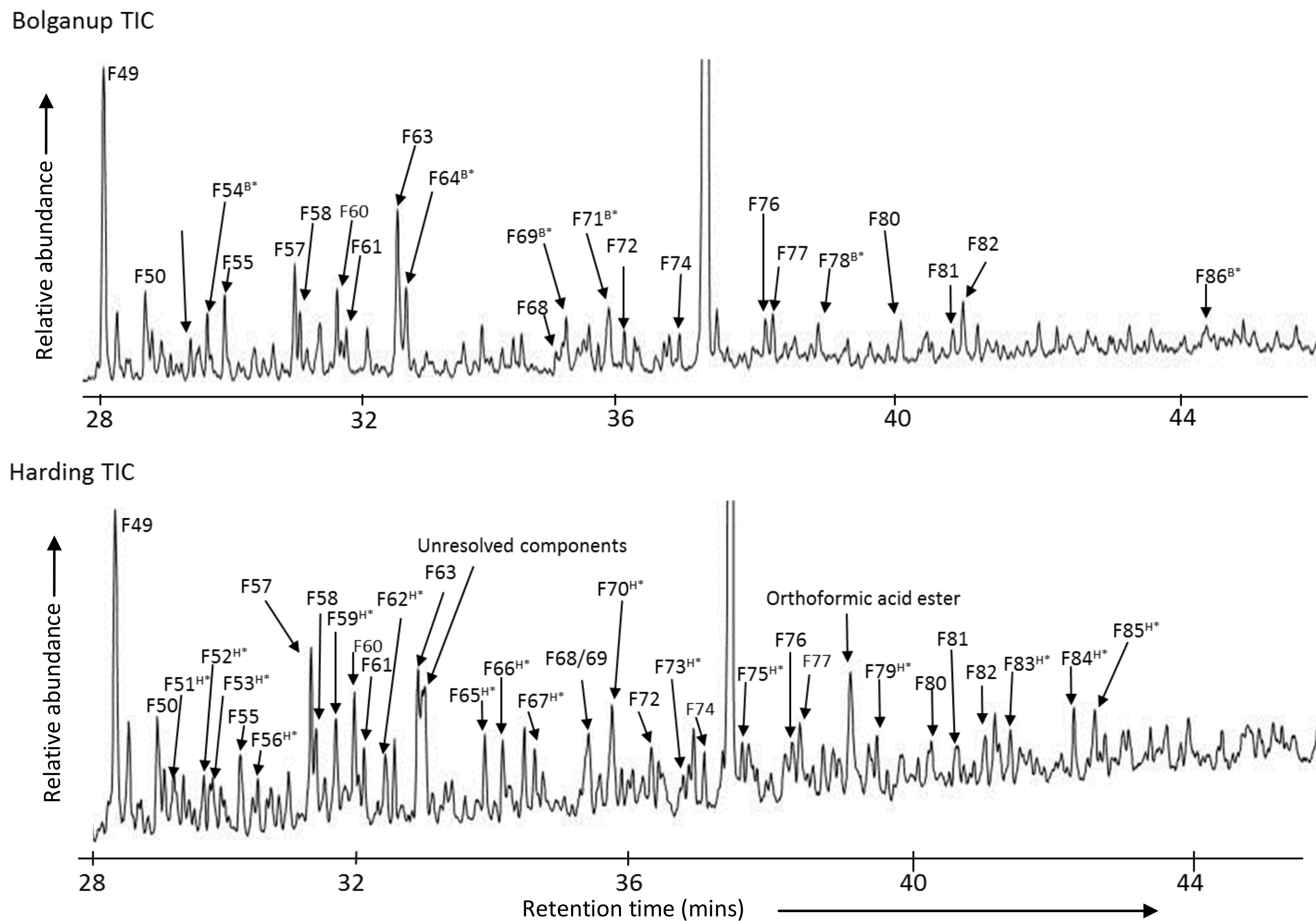


Figure 5.10 Partial TIC from the Flash-Py GCMS of Bolganup (top) and Harding (bottom) HPO fractions. Peak assignments correspond to products listed in Table 5.5. B* = Detected only in B-HPO, H* = Detected only in H-HPO.

Table 5.5 Relative abundances of the major products (F1-87) from the Flash-Py GCMS of Bolganup and Harding HPO fractions. RT = retention time; MW = molecular weight; *m/z* - 4 main mass spectral ions. B* = Detected in B-HPO only; H*= H-HPO only. F1 = flash pyrolysis product 1.

Peak i.d.	RT	Compound i.d.	*m/z*	MW	Precursor
F1	8.47	2-methyl propanal	43, 41, 72, 27	72	Carbohydrate
F2	9.24	3-methyl butene H*	55, 42, 70, 39	70	-
F3	9.39	2,3-butanedione	43, 86, 42, 44	86	Carbohydrate
F4	9.48	2-butanone	43, 72, 29, 57	72	Carbohydrate
F5	9.58	2-methyl furan	82, 53, 81, 39	82	Carbohydrate
F6	10.69	acetic acid	43, 45, 60, 42	60	Carbohydrate
F7	10.86	cyclohexadiene B*	79, 80, 77, 78	80	-
F8	11.07	3-methyl cyclopentene H*	67, 82, 39, 41	82	-
F9	11.38	benzene	78, 77, 51, 52	78	-
F10	11.63	2-methyl butanal H*	41, 57, 58, 86	86	Carbohydrate
F11	11.82	3-methyl butene-2-one H*	43, 41, 69, 84	84	Carbohydrate
F12	12.30	2-pentanone H*	43, 86, 71, 58	86	Carbohydrate
F13	12.71	2-methyl pentanone H*	43, 57, 29, 100	100	Carbohydrate
F14	12.93	2,5-dimethylfuran	96, 95, 43, 53	96	Carbohydrate
F15	13.24	2,4-dimethylfuran	96, 95, 67, 53	96	Carbohydrate
F16	14.22	3-methyl-1,2,3-hexatriene H*	79, 77, 94, 91	94	-
F17	14.50	2-methyl-1,2,3-hexatriene H*	79, 77, 94, 91	94	-
F18	14.57	pyrrole	67, 28, 41, 39	67	N-product
F19	15.18	toluene	91, 92, 65, 39	92	-
F20	16.03	2-ethyl-1-hexene H*	70, 55, 41, 112	112	-
F21	16.36	3,4-dimethyl-2-hexene H*	55, 83, 41, 112	112	-
F22	16.80	3-methyl-2-heptene	70, 55, 41, 112	112	-
F23	17.03	2,3,5-trimethyl-furan H*	110, 109, 95, 67	110	Carbohydrate
F24	17.75	furfural	96, 95, 39, 28	96	Carbohydrate
F25	17.92	2-methyl-pyrrole H*	80, 81, 28, 53	81	N-product

F26	18.25	unspecified methyl pyrroles H*	80, 81, 28, 53	81	N-product
F27	18.91	tetramethylcyclopentene H*	109, 67, 124, 41	124	Possibly terpenoid
F28	18.99	ethylbenzene H*	91, 106, 77, 65	94	-
F29	19.17	5-methyl-furanone H*	55, 43, 98, 27	98	Carbohydrate
F30	19.36	*p*-xylene	91, 106, 105, 77	106	-
F31	19.42	*o*-xylene	91, 106, 105, 77	106	-
F32	20.75	2-methyl-2-cyclopenten-1-one	67, 96, 53, 39	96	Carbohydrate
F33	21.99	5-methyl-2-furanone H*	55, 43, 83, 98	98	Carbohydrate
F34	22.24	dihydro-3-methylene-2,5- furandione	68, 39, 40, 112	112	Carbohydrate
F35	22.84	2-ethyl hexanal	57, 72, 43, 128	128	Carbohydrate
F36	23.11	5-methyl-2-furancarboxaldehyde	110, 109, 53, 81	110	Carbohydrate
F37	23.21	3-methyl-2-cyclopenten-1-one	96, 67, 53, 39	96	Carbohydrate
F38	23.33	1-ethyl-2-methyl benzene H*	105, 120, 77, 91	120	-
F39	23.58	3-methyl-2-furanone H*	41, 69, 98, 39	98	Carbohydrate
F40	23.74	phenol	94, 66, 65, 39	94	Phenol
F41	24.59	1,3,5-trimethyl benzene	105, 120, 91, 77	120	-
F42	25.36	succinic anhydride	28, 56, 27, 100	100	Carbohydrate
F43	25.72	1,2,3-trimethyl benzene H*	105, 120, 77, 119	120	-
F44	25.89	2-ethyl-1-hexanol	57, 41, 55, 83	130	-
F45	25.98	3,4-dimethyl-2,5-furandione	39, 54, 82, 126	126	Carbohydrate
F46	26.20	2,3-dimethyl-2-cyclopenten-1-one	67, 110, 39, 95	110	Carbohydrate
F47	26.37	4-methyl-5-furan-2-one H*	69, 41, 39, 98	98	Carbohydrate
F48	26.79	2-methylphenol	108, 107, 79, 90	108	Phenolic
F49	27.63	4-methylphenol	107, 108, 77, 79	108	Phenolic
F50	28.26	2-methoxyphenol	108, 124, 81, 53	124	Lignin
F51	28.50	1-methyl-4-(1-methylethenyl)-	117, 132, 115, 91	132	Possibly terpenoid
F52	28.95	2,3-dimethyl-4-hydroxy-2-butenoic	55, 83, 112, 39	112	Carbohydrate
F53	29.08	2,6-dimethylphenol H*	122, 107, 77, 91	122	Phenolic

F54	29.20	levoglucosenone B*	98, 96, 68, 126	126	Carbohydrate
F55	29.50	triethylphosphate	99, 155, 127, 182	182	-
F56	29.76	3,5,5-trimethyl-2-cyclohexen-1-one	82, 138, 54, 95	138	Carbohydrate
F57	30.55	3,5-dimethylphenol	107, 122, 121, 77	122	Phenolic
F58	30.62	2,3-dimethylphenol	107, 122, 121, 77	122	Phenolic
F59	30.92	1-ethyl-2,3-dimethyl benzene H*	119, 134, 91, 105	134	Possibly terpenoid
F60	31.20	4-ethylphenol	107, 122, 77, 91	122	Phenolic
F61	31.34	3,4-dimethyl phenol	122, 107, 121, 77	122	Phenolic
F62	31.66	dimethyl phenol, unspecified isomers	122, 107, 121, 77	122	Phenolic
F63	32.15	1,2-benzenediol	110, 64, 63, 81	110	Tannin
F64	32.24	2,4-dimethyl phenol B*	122, 107, 121, 77	122	Phenolic
F65	33.14	2,3-dihydro-benzofuran H*	120, 91, 119, 65	120	Carbohydrate
F66	33.41	4,7-dimethyl-benzofuran H*	146, 145, 115, 131	146	Carbohydrate
F67	33.89	2,4,6- trimethyl-phenol H*	121, 136, 91, 77	136	Phenolic
F68	34.53	3-methyl-1,2-benzenediol	124, 78, 123, 51	124	Tannin
F69	34.69	3-methoxy-1,2-benzendiol B*	140, 125, 97, 51	140	Tannin
F70	35.04	1,3-dimethyl-1-indene H*	129, 128, 144, 115	144	Carbohydrate
F71	35.33	1,3-benzenediol (resorcinol) B*	110, 82, 81, 53	110	Tannin
F72	35.63	4-methyl-1,2-benzenediol	124, 78, 123, 77	124	Tannin
F73	36.26	2,4,6-trimethylphenol H*	121, 136, 91, 77	136	Phenolic
F74	36.41	1-methyl-napthalene	142, 141, 115, 63	142	-
F75	36.98	methyl-napthalene (unspecified	142, 141, 115, 63	142	-
F76	37.62	2-methyl-1,4-benzenediol	124, 123, 77, 78	124	Tannin
F77	37.84	2,6-dimethoxy-phenol	154, 139, 91, 111	154	Lignin
F78	38.53	2-methyl-1,2 benzenediol B*	124, 123, 77, 78	124	Tannin
F79	38.99	1,2,3-trimethylindene H*	143, 158, 128, 115	158	Carbohydrate
F80	39.80	4-methyl-1,3-isobenzofurandione	162, 90, 118, 134	162	Carbohydrate
F81	40.60	2,4-dimethyl-napthalene	156, 141, 115, 155	156	-

F82	40.75	dimethyl napthalene (unspecified	156, 141, 115, 155	156	-
F83	40.98	benzo-pyran-2-one H*	118, 90, 89, 63	146	Carbohydrate
F84	41.92	isoindole-1,3-dione H*	147, 76, 104, 50	147	Carbohydrate
F85	42.24	1,3-dihydro-2-indol-2-one H*	133, 104, 105, 78	133	Carbohydrate
F86	44.46	4-hydroxy-3-methoxy-benzoic acid B*	168, 153, 97, 125	168	Tannin
F87	49.06	benzoic acid 2-ethylhexyl ester H*	105, 70, 112, 123	234	-

Alkyl phenols are also commonly associated with lignin and tannin (Bracewell & Robertson 1976; Philp et al., 1982; Wilson et al., 1983; Bracewell et al., 1989). However, they can only reliably be ascribed to fresh lignin sources when co-occurring with methoxy phenols (Bracewell et al., 1989; Saiz-Jimenez; 1994; van Heemst et al., 1999; Guo et al., 2003). Only trace amounts of methoxy phenols (**F50** and **F77**) were evident from the pyrolysis of both B-HPO and H-HPO (B-HPO -2.8 % *Cf*. 1.7 % H-HPO; Fig. 5.10–12 and Table 5.5). Furthermore, ^{13}C-NMR (Fig. 5.9) showed negligible influence of lignin methoxy compounds.

Figure 5.11 Relative abundances of common product groups detected from Flash-Py of B-HPO and H-HPO

The absence of high abundances of diagnostic lignin products in the Flash-Py of the HPO fractions, excludes lignin as a significant organic input to either source water. It is possible that extensive lignin degradation occurred during periods of stratification in the reservoirs

(discussed in more depth in Section 5.4.3.3), but there is no evidence for this phenomenon, and it is unlikely that lignin is the primary source of the alkyl phenols.

Research by van Heemst et al. (2000) and Guo et al. (2003) described abundant alkyl phenols, with an absence of significant abundances of methoxy phenols, in aquatic NOM fractions of riverine systems and attributed these to polysaccharide and protein precursors on correlation to representative standards (van Heemst et al., 1999). More specifically the source of the alkyl phenols were attributed to the macromolecular remnants of proteinaceous material which had been hydrolysed in microbially mediated processes in the water column.

It is also possible that some of the alkyl phenols arise from the cyclisation and aromatisation of the aliphatic terpanes (Simoneit et al., 1986; Hayatsu et al., 1990; Hartgers et al., 1994a&b, Berwick et al., 2010b) of algae, bacteria and plants. Terpenoid inputs were significant in NOM isolated from the surface waters of the Great Salt Lake (Leenheer et al.,2004) and the Colorado River, USA (Leenheer et al.,2003) and terpenoids were also identified as a major source of HPO-NOM isolated from North Pine Dam, Australia (Berwick et al.,2010b). NOM produced by the biodegradation of terpenoids are characterised by aliphatic quaternary carbons, branched methyl groups and ring structures (Leenheer et al., 2003). Compounds **F27, F51** and **F59** (Table 5.5) showing branched alkyl structural moieties are possible terpenoid derived products.

5.4.2.5 MSSV Pyrolysis

Partial TICs from the MSSVpy GCMS analysis of B-HPO and H-HPO are shown in Fig. 5.12. Table 5.5 lists 131 compounds detected in either of the two fractions and the relative abundance of these were subsequently used to determine product group distributions (Fig. 5.13) which show a high proportion of aromatic and O-containing products. MSSVpy promotes the reduction of polar NOM moieties, identified as being significant in both B-HPO and H-HPO by FTIR and ^{13}C NMR analyses, and increases the GC yields of NOM products (Greenwood et al., 2006; Berwick et al., 2007; Berwick et al., 2010a).

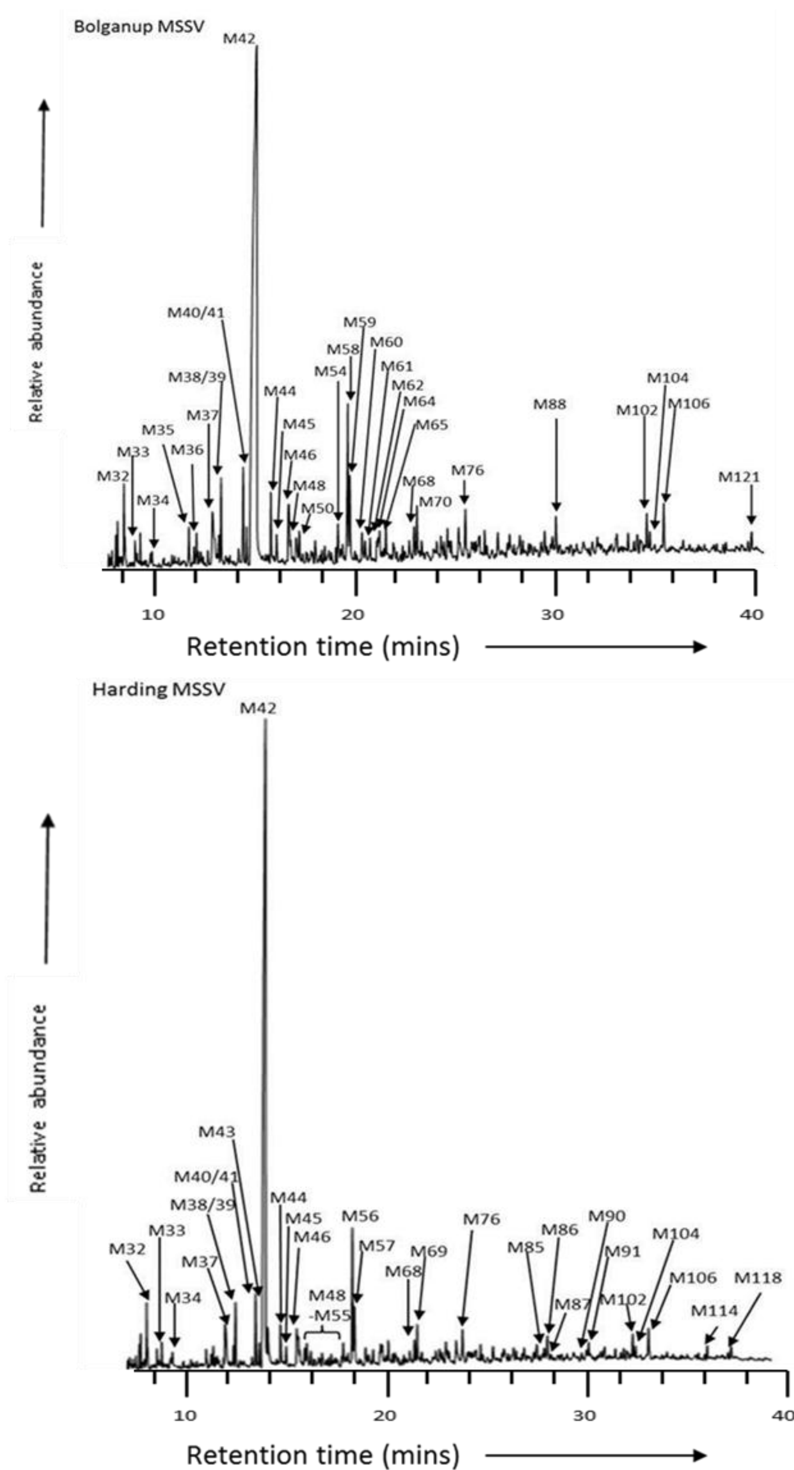


Figure 5.12 Partial TIC from the MSSVpy GCMS analysis of B-HPO and H-HPO. Peak assignments correspond to products listed in Table 5.6.

Table 5.6 Major products (Mx) and associated precursors from the MSSVpy GCMS of Bolganup and Harding HPO fractions, with retention time, MW and m/z details of 4 main fragment ions indicated. B* = detected in B-HPO only; H*= detected in H-HPO only

Peak	RT	Compound i.d.	*m/z*	MW	Grouping	Precursor
M1	2.25	1,2-dimethyl *cis* cyclopropane H*	55, 70, 53, 67	70	Aliphatic	-
M2	2.46	cyclopentane H*	67, 68, 53, 66	68	Aliphatic	-
M3	2.53	4-methyl-2-pentene H*	69, 84, 55, 56	84	Aliphatic	-
M4	2.66	3-methyl pentane H*	57, 56, 55, 71	86	Aliphatic	-
M5	2.71	2-methyl-1-pentene H*	56, 55, 69, 84	86	Aliphatic	-
M6	2.80	methylhydrazone acetaldehyde H*	72, 57, 71, 73	72	Aldehyde	Carbohydrate
M7	2.84	3-methyl furan H*	82, 53, 81, 54	91	Furan	Carbohydrate
M8	2.88	methyl pentene - unspecified isomers H*	69, 84, 55, 56	91	Aliphatic	-
M9	3.02	methyl pentene - unspecified isomers H*	69, 84, 55, 56	91	Aliphatic	-
M10	3.14	2,3-dimethyl-2-butene H*	69, 84, 55, 67	87	Aliphatic	-
M11	3.42	1-methyl cyclopentene H*	67, 82, 81, 53	81	Aliphatic	-
M12	3.51	3-ethyl-1-pentene H*	69, 70, 55, 56	80	Aliphatic	-
M13	3.56	benzene H*	78, 77, 51, 52	78	Alkyl benzene	-
M14	3.96	methyl-cyclohexane H*	83, 55, 98, 56	98	Aliphatic	-
M15	4.01	2-pentanone H*	86, 71, 58, 55	86	Aliphatic and cyclic	Carbohydrate
M16	4.08	3-ethyl-2-pentene H*	69, 55, 98, 83	98	Aliphatic	-
M17	4.16	heptane H*	71, 57, 56, 100	100	Aliphatic	-
M18	4.30	2,5-dimethyl furan H*	96, 95, 53, 81	96	Furan	Carbohydrate
M19	4.37	1,4-dioxane H*	88, 58, 57, 69	88	Other O products	-
M20	5.27	1-methylethylidene cyclobutane H*	81, 96, 67, 79	96	Heterocyclic	-
M21	5.31	5-methyl-3-heptene H*	55, 83, 112, 67	112	Aliphatic	-
M22	5.35	ethylidenecyclopentane H*	67, 96, 81, 79	96	Heterocyclic	-
M23	5.51	1,2,3-trimethylcyclopentene H*	95, 67, 110, 79	110	Aliphatic	-

M24	5.59	toluene	91, 92, 65, 39	94	Alkyl benzene	-
M25	5.73	3-methylheptane	57, 85, 56, 84	114	Aliphatic	-
M26	6.20	3-methyl-2-heptene	70, 55, 112, 83	112	Aliphatic	-
M27	6.37	1,3-dimethyl-1-cyclohexene H*	95, 110, 67, 55	110	Aliphatic	-
M28	6.44	2,3-dimethyl-3-hexene	55, 69, 70, 112	112	Aliphatic	-
M29	6.76	2-methyl-4-methylene-hexane	70, 55, 112, 69	112	Aliphatic	-
M30	6.95	3,4,5-trimethylpyrazole H*	110, 109, 95, 67	110	N-product	Amino acid
M31	8.44	ethylbenzene H*	91, 106, 65, 77	106	Alkyl benzene	-
M32	8.79	*p*-xylene	91, 106, 105, 77	106	Alkyl benzene	-
M33	9.54	*o*-xylene	91, 106, 105, 77	106	Alkyl benzene	-
M34	10.02	benzaldehyde H*	105, 106, 77, 51	106	Aldehyde	Carbohydrate
M35	12.00	2-ethylhexanal	57, 72, 55, 56	128	Aldehyde	Carbohydrate
M36	12.26	1-ethyl-2-methylbenzene H*	105, 120, 91, 77	120	Alkyl benzene	-
M37	13.17	phenol	94, 66, 65, 55	94	Phenols	-
M38	13.33	1,2,3,4,5-pentamethylcyclopentene H*	123, 81, 138, 95	138	Aliphatic	Terpenoid
M39	13.59	1, 2, 3-trimethylbenzene	105, 120, 119, 77	120	Alkyl benzene	-
M40	14.68	1, 3, 5-trimethylbenzene	105, 120, 119, 77	120	Alkyl benzene	-
M41	14.69	1-methyl-4-(1-methylethyl)benzene H*	119, 134, 91, 117	134	Alkyl benzene	-
M42	15.35	2-ethyl-1-hexanol	57, 55, 70, 83	130	O products - Alcohols	-
M43	15.14	2,3-dimethyl-2-cyclopenten-1-one H*	67, 110, 82, 95	110	Aliphatic and cyclic	Carbohydrate
M44	16.05	2-methylphenol	107, 108, 77, 79	108	Phenols	Lignin
M45	16.33	3-(1-methylethyl)-2-cyclopenten-1-one	109, 82, 81, 124	124	Aliphatic and cyclic	Carbohydrate
M46	16.75	4-methylphenol	107, 108, 77, 79	108	Phenols	-
M47	16.93	3-methylphenol H*	108, 107, 79, 77	108	Phenols	-
M48	17.09	1-methyl-2-(1-methylethyl)benzene	119, 134, 91, 77	134	Alkyl benzene	-
M49	17.30	1-phenyl-1-butene H*	117, 132, 91, 77	132	Aliphatic	-
M50	17.41	2,3,4-trimethyl-2-cyclopenten-1-one	109, 124, 81, 96	124	Aliphatic and cyclic	Carbohydrate
M51	17.46	3-ethyl-2,4-dimethyl-pyrrole H*	57, 108, 123, 107	123	N-product	Amino acid

M52	18.01	2,6-dimethyl phenol H*	122, 107, 121, 77	122	Phenols	-
M53	19.12	1,3-dimethyl-5-(1-methylethyl)-benzene H*	133, 148, 91, 105	148	Alkyl benzene	-
M54	19.37	2-ethylphenol B*	107, 122, 77, 79	122	Phenols	-
M55	19.37	1-methyl-2-(2-propenyl)benzene H*	117, 132, 115, 131	132	Alkyl benzene	-
M56	19.60	acetic acid, 2-ethylhexyl ester H*	70, 57, 55, 83	172	Other O products	Carbohydrate
M57	19.72	1-ethyl-2,4-diemethylbenzene H*	119, 134, 91, 77	134	Alkyl benzene	-
M58	19.86	2,4-dimethylphenol B*	122, 107, 121, 77	122	Phenols	-
M59	19.96	1-ethyl-2,4-dimethylbenzene B*	119, 134, 91, 107	134	Alkyl benzene	-
M60	20.56	4-ethylphenol	107, 122, 77, 79	122	Phenols	-
M61	20.72	3,5-dimethylphenol	122, 107, 121, 77	122	Phenols	-
M62	20.96	2,3-dimethylphenol	122, 107, 121, 77	122	Phenols	-
M63	21.10	2-ethyl-3,4,5-trimethyl-pyrrole H*	122, 137, 107, 121	137	N-Product	Amino
M64	21.43	2,3-dihydro-1,6-dimethyl-Indene B*	131, 146, 91, 115	146	Heterocyclic aromatic	-
M65	21.74	2,3-dihydro-1,2-dimethyl-Indene B*	131, 146, 91, 115	146	Heterocyclic aromatic	-
M66	21.50	2,4-dimethylphenol H*	122, 107, 121, 77	122	Phenols	-
M67	22.90	2-ethyl-4-methylphenol	121, 136, 91, 77	136	Phenols	-
M68	23.14	2,3,6-trimethylphenol	121, 136, 91, 77	136	Phenols	-
M69	23.03	1-butanol-3-methylpropanoate H*	57, 70, 55, 43	144	Other O products	-
M70	23.28	1,3,6-trimethyl phenol B*	121, 136, 91, 77	136	Phenols	-
M71	24.00	2-ethenyl-1,3,5-trimethylbenzene	131, 146, 91, 115	146	Alkyl benzene	-
M72	24.20	2,4,6-trimethylphenol H*	121, 136, 91, 77	136	Phenols	-
M73	24.26	2-ethenyl-1,3,5-trimethylbenzene	131, 146, 91, 115	146	Alkyl benzene	-
M74	24.78	butanoic acid, pentyl ester B*	71, 70, 57, 112	200	Other O products	Carbohydrate
M75	25.34	hexamethylbenzene	147, 162, 91, 77	162	Alkyl benzene	-
M76	25.67	trimethylphenol (unspecifiedisomers)	121, 136, 91, 77	136	Phenols	-
M77	26.63	2-ethenyl-1,3,5-trimethylbenzene	131, 146, 91, 115	146	Alkyl benzene	-
M78	26.74	2,5,6-trimethylbenzimidazole	160, 159, 145, 91	160	N-Product	Protein/base
M79	27.00	4-(2-butenyl-)1,2-dimethylbenzene B*	145, 160, 146, 130	160	Alkyl benzene	-

M80	27.59	1-(2-hydroxy-5-methylphenyl)-ethanone H*	135, 150, 107, 77	150	Aliphatic and cyclic	Carbohydrate
M81	27.86	2,3,4,6-tetramethylphenol B*	135, 150, 91, 149	150	Phenols	-
M82	28.37	1,2,3-trimethylindene	143, 158, 129, 141	158	Heterocyclic benzene	-
M83	28.52	1,1,3-trimethylindene	143, 158, 129, 141	158	Heterocyclic benzene	-
M84	28.65	1-(2-butenyl)2,3-dimethyl benzene H*	145, 130, 160, 91	160	Alkyl benzene	-
M85	29.60	1,3-dimethylnapthalene H*	156, 141, 155, 115	156	Alkyl napthalene	-
M86	29.88	2,3-dimethyl napthalene H*	156, 141, 155, 115	156	Alkyl napthalene	-
M87	29.98	1,2-dihydro-1,4,6-trimethylnapthalene	157, 172, 142, 128	172	Alkyl napthalene	-
M88	30.15	2,6-dimethyl napthalene B*	156, 141, 155, 115	156	Alkyl napthalene	-
M89	30.88	1,2,3,4-tetrahydro-5,6,7,8-	132, 173, 188, 117	188	Alkyl napthalene	-
M90	31.67	1,2-dihydro-3,5,8-trimethyl napthalene	157, 172, 142, 128	172	Alkyl napthalene	-
M91	31.96	1,2-dihydro-3,5,6-trimethyl napthalene B*	157, 172, 142, 128	172	Alkyl napthalene	-
M92	32.20	tetrahydro tetramethylnapthalene	132, 173, 188, 117	188	Alkyl napthalene	-
M93	32.40	tetrahydro tetramethylnapthalene	132, 173, 188, 117	188	Alkyl napthalene	-
M94	32.88	tetrahydro tetramethylnapthalene	132, 173, 188, 117	188	Alkyl napthalene	-
M95	32.90	trimethyl napthalene (unspecified isomers)	155, 170, 115, 128	170	Alkyl napthalene	-
M96	33.17	tetrahydro tetramethylnapthalene	132, 173, 188, 117	188	Alkyl napthalene	-
M97	33.45	trimethyl napthalene (unspecified isomers)	155, 170, 115, 128	170	Alkyl napthalene	-
M98	33.72	5-ethyl-1-indole-2,3-dione H*	132, 147, 77, 104	175	N-product	Protein/amino
M99	33.73	trimethyl napthalene (unspecified isomers)	155, 170, 115, 128	170	Alkyl napthalene	-
M100	34.18	trimethyl napthalene (unspecified isomers)	155, 170, 115, 128	170	Alkyl napthalene	-
M101	34.33	1,4,6-trimethyl napthalene H*	155, 170, 115, 128	170	Alkyl napthalene	-
M102	34.65	1,6,7-trimethyl napthalene B*	155, 170, 115, 128	170	Alkyl napthalene	-
M103	34.77	2-ethyl-3-methoxypyrazine H*	138, 107, 119, 139	138	N-product	Protein/bases/ amino sugar
M104	34.80	2,3,6-trimethyl napthalene B*	155, 170, 115, 128	170	Alkyl napthalene	-
M105	35.19	1,6,7-trimethylnapthalene H*	155, 170, 115, 128	170	Alkyl napthalene	-
M106	35.49	1,4,6-trimethyl napthalene B*	155, 170, 115, 128	170	Alkyl napthalene	-

M107	36.16	1-methyl-7-(1-methylethyl)napthalene H*	169, 184, 128, 115	184	Phenol	-
M108	37.50	4-(1-methyl-1-phenylethyl) phenol B*	197, 212, 91, 198	212	Phenol	-
M109	37.82	1,6-dimethyl-4-(1-methylethyl) napthalene	183, 198, 168, 153	198	Alkyl napthalene	-
M110	38.11	dimethyl-4-(1-methylethyl) napthalene	183, 198, 168, 153	198	Aliphatic	-
M111	38.43	ethyl-1,4-dimethyl azulene - unspecified	184, 169, 128, 153	184	Azulene	-
M112	38.56	4,5,5-trimethyl-1,3-cyclopentadien-1-yl	184, 169, 128, 115	184	Alkyl benzene	-
M113	39.10	ethyl-1,4-dimethyl azulene - unspecified	184, 169, 128, 153	184	Azulene	-
M114	39.54	tetramethyl napthalene - unspecified	184, 169, 153, 128	184	Alkyl napthalene	-
M115	39.66	tetramethyl napthalene - unspecified	184, 169, 153, 128	184	Alkyl napthalene	-
M116	39.84	tetramethyl napthalene - unspecified	184, 169, 153, 128	184	Alkyl napthalene	-
M117	40.07	phenylmethylbenzene - unspecified dimethyl	181, 196, 165, 182	196	Alkyl benzene	-
M118	40.16	phenylmethylbenzene - unspecified dimethyl	181, 196, 165, 182	196	Alkyl benzene	-
M119	41.27	phenylmethylbenzene - unspecified dimethyl	181, 196, 165, 182	196	Alkyl benzene	-
M120	41.39	phenylmethylbenzene - unspecified dimethyl	181, 196, 165, 182	196	Alkyl benzene	-
M121	42.16	1-methylethyl napthalene - unspecified	183, 198, 153, 184	198	Alkyl napthalene	-
M122	42.35	dimethyl-4-(phenylmethyl)-benzene	181, 196, 165, 182	196	Alkyl benzene	-
M123	42.78	1-methyl-3-(4-methylphenyl)benzene B*	167, 182, 165, 183	194	Alkyl benzene	-
M124	43.87	1-methylethyl napthalene - unspecified	183, 198, 153, 184	198	Alkyl napthalene	-
M125	48.33	3, 6-dimethyl phenanthrenes B*	206, 191, 189, 205	206	Alkyl phenanthrene	-

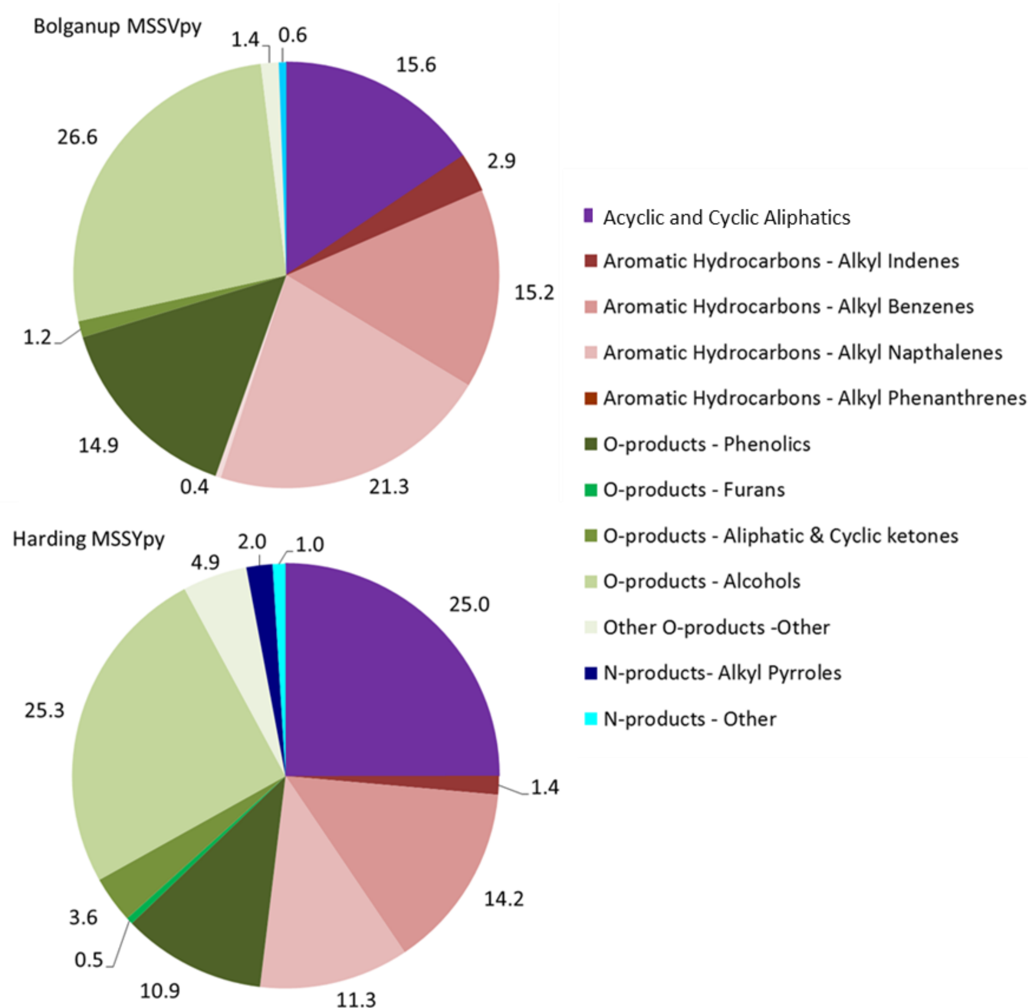


Figure 5.13 Product group distribution of pyrolysis products from MSSVpy analysis of B-HPO (top) and H-HPO (bottom).

5.4.2.5.1 MSSVpy of Bolganup HPO

Sixty-eight products were identified with good confidence in the B-HPO, representing 46 % of the total pyrolysate. The most abundant peak was attributed to 2-ethyl hexanol, which comprised 27 % of the total pyrolysate signal, but it may possibly be an anthropogenic contaminant (discussed later).

Oxygen product groups (green shaded sections of pie chart, Fig. 5.13) showed the greatest percentage distribution (43 %) and included phenols, other alcohols, furans and ketones. High concentrations of alky-phenols were similarly detected by Flash-Py (21 %), and are also consistent with; the large alkyl-O signal measured by ^{13}C NMR, and the high O–H signal measured by FTIR.

Common sources of environmentally occurring alkyl phenols include lignin, tannin protein and algal biopolymers (Section 5.4.2.4). Only trace amounts of dihydroxy or methoxy aromatic structures typical of wood lignin or tannin were detected by MSSVpy, at concentrations even lower than detected by Flash-Py. Alkyl phenols may also arise from the cyclisation and aromatisation of aliphatic terpanes (Simoneit et al., 1986; Hayatsu et al., 1990; Hartgers et al., 1994a&b, Berwick et al., 2010b) of algae, bacteria and plants. Both of the case study waters are subject to algal blooms, particularly cyanobacteria, which likely contribute high loadings of aliphatic terpanes.

Alkyl substituted aromatics (pink shadings, Fig. 5.13) included alkyl-benzenes (15 %), naphthalenes (21 %), phenanthrenes (<0.5 %) and indenes (3 %). These product groups were detected in higher abundance in B-HPO (39.4 %) than H-HPO (27 %). Relatively high aromatic signals were consistently detected in B-HPO by SUVA, FTIR and ^{13}C NMR analysis of the HPO fractions.

Aliphatic and cyclic hydrocarbons (purple shadings, Fig. 5.13) of mainly methylated alkenes and alkanes were measured at 16 % of the total pyrolysis signal. This relatively low level (*Cf*. Harding, 25 %) is again consistent with the findings of NMR and FTIR.

N-containing products (i.e., other N-products, light blue shadings, Fig. 5.13) were the least abundant product groups in B-HPO comprising just 0.6 % of the total signal measured, very different from H-HPO (Section 5.4.2.5.2) the exclusive product detected was 2,5,6-trimethylbenzimidazole (a fungicide, probably originating from the catchment).

5.4.2.5.2 MSSVpy of Harding HPO

Ninety products were tentatively identified in H-HPO, representing 47 % of the total MSSV pyrolysate signal of this sample. LMW compounds were particularly prominent.

O-products (green shading, Fig. 5.13) were again abundant (40 % of identified), with non-source specific polymethyl phenolic compounds well represented (11 %) but less than in B- HPO (15 %). Flash-Py similarly showed a higher phenol content for the forested B-HPO.

H-HPO showed slightly higher proportion of alkyl furans and cyclic ketones (4.1 % of identified) than B-HPO (1.2 %). These O-containing products are largely attributed to carbohydrate precursors and may arise from simple sugars or polysaccharides such as cellulose, hemicellulose and pectin (Pouwels et al., 1987; Pastorova et al., 1994; Almendros et al., 1997; Berwick et al., 2010b). H-HPO also contained several aldehyde products not

detected in B-HPO. SPME analysis similarly identified aldehydes in Harding water but not Bolganup water (Section 5.4.3.1). Collectively, these species may contribute to the moderate carbonyl NMR signal of H-HPO. NMR carbonyl signals were comparable for both HPO fractions (~4 %, Table 5.4), however the carbohydrate speciation differences evident in the MSSV data suggests contributions from different sources. Trace concentrations (<1 %) of furans (e.g., 3-methyl furan, 2,5 -dimethyl furan) were detected in H-HPO, however none were detected in B-HPO .

Aromatic hydrocarbons (pink shadings, Fig. 5.13) comprised the next most abundant grouping, collectively accounting for 27 % of the identified peaks. This is less than the 34 % aromatic hydrocarbons measured for B-HPO, and consistent with the more aliphatic nature of H-HPO indicated by FTIR and NMR analysis. The alkyl napthalenes (11 %) and alkyl benzenes (11 %) were equally represented in H-HPO, unlike in B-HPO which was dominated by alkyl napthalenes (21 %). In particular, the high H-HPO proportion of tetramethyl benzenes (Fig. 5.15) may be indicative of microbial or algal carotenoids (Hartgers et al., 1994a,b&c; Pedentchouk et al., 2004) and the more frequent occurrences of algal blooms in the Harding Dam (WQRA, 2009; Water Corporation, 2005b, Garbin et al., 2010).

Aliphatic and cyclic hydrocarbons (purple shading, Fig. 5.13) comprised 25 % of the assigned products. This was notably greater than B-HPO (16 %), and also consistent with the high alkyl signal from FTIR and ^{13}C NMR analysis. This group consisted of low relative abundances (<1 %) of many low molecular weight cyclanes, branched alkanes and alkenes, including 3-methyl hexane, 2,3-dimethyl-3-hexene and 2-methyl-4-methyl-hexane which may be related to the terpenoids of algae or plants (Leenheer et al., 2003).

N-Products (blue shading, Fig. 5.13) were determined to be 3 % in H-HPO, also notably higher than B-HPO (0.6 %). These consisted of alkyl pyrroles (2 %) and other specific N-products such as 3,4,5-trimethylpyrazole and 2-ethyl-3-methoxypyrazine. The C:N ratios of 23 for H-HPO, and 32 for B-HPO (Table 5.3), and the relatively high N-absorbances measured by ^{13}C NMR and FTIR were also indicative of a higher N-organic content in the Harding water. Notably, higher concentrations of N-organics in Harding were not evident in the Flash-Py data. Flash-Py is not as sensitive as MSSVpy to low MW alkyl substituted N-heterocycle (e.g., pyrrole) products of aquatic NOM (Berwick et al., 2007; Berwick et al., 2010a). These low MW N-organic species are typically attributed to diagenetically altered proteins (Berwick et al.,2010a), although other potential biochemical sources of N-organics include microbially sourced N-acetylaminosugar polymers (e.g., peptidoglycan; Leenheer et

al., 2003) or aliphatic monoterpenes (Nimmagadda & McRae, 2007; Berwick et al., 2010a&b). The latter precursor would also contribute to the high alkyl carbon signal measured by NMR.

Suspected anthropogenic contaminant

2-Ethyl hexanol was detected in very high abundance (25–27 % of integrated signal) in the HPO fraction of both NOM waters. It has previously been detected by MSSVpy of NOM (Berwick et al., 2010a), but is not typically a major pyrolysis fragment. It can be naturally produced by bacteria and fungi (Nalli et al., 2006; Korpi et al.,2009) but is also a microbial metabolite of the often detected environmental contaminant 2-ethylhexyl phthalate (Nalli et al., 2006), used in the production of poly-vinyl chloride plastic (Staples et al., 1997; Fromme et al., 2002; Horn et al., 2004). It may contribute to the large alkyl-O signal measured by FTIR and ^{13}C NMR analysis of both HPO fractions, but was not a significant Flash-Py product.

5.4.3 Characterisation of Source Waters Concentrated to aid Stable Isotope Analysis.

To test the utility of isotopic signatures for complementary source characterisation of aquatic NOM, CSIA via the developed SPME (Chapter 2) and PLFA (Chapter 3) approaches were applied to concentrated aliquots of the Bolganup and Harding waters. Concentrated aliquots were used to counter the relatively low sensitivity of CSIA (compared to GCMS). These samples were first analysed by GCMS for product identification.

5.4.3.1 Characterisation of VOC and SVs by SPME-GCMS

Complementary characterisation of VOC and SVOC products were conducted *via* separate analyses using the PDMS and PA fibres since they respectively favour non-polar analytes (e.g., benzenes) and polar compounds (e.g., phenols) as discussed in Sections 2.4.1.1–2 (Chapter 2).

The profiles of the VOCs and SVOCs obtained with each fibre were similar, with most differences reflected by minor products. More products were detected using the PDMS fibre. The TIC from (PDMS) SPME-GCMS analysis of Harding is shown in Figure 5.14. The identity (tentative) and relative abundances of 26 major products detected by SPME GCMS of Harding or Bolganup are given in Table 5.7.

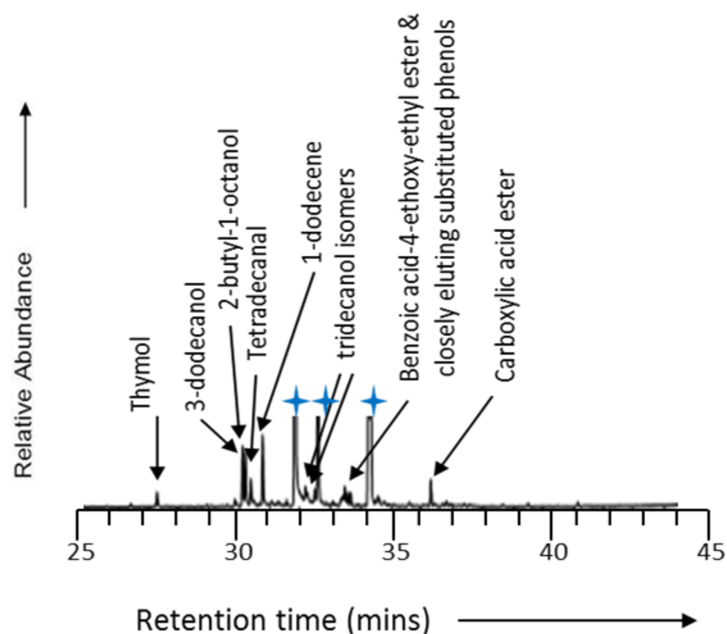


Figure 5.14 Partial TIC from the SPME (PDMS fibre) GCMS analysis of Harding water. The largest peaks are contaminants represented by blue crosses, and their full abundance is not portrayed in the zoomed region shown.

The SPME product profiles of the source waters are quite different to the SPME-GCMS of the Chapter 2 leachates. Leachate products **29**, **33**, **53** and **83** of Table 2.5 (Chapter 2) were the only ones detected in the source waters. Product **53** is a suspect contaminant and **83** may derive from bacteria, although there are other potential sources of this product so neither of these two products are particularly source diagnostic. However products thymol (**29, CSa**) and 2-methoxy-3-ethyl pyrazine (**33, CSb**) are biologically derived. Thymol (i.e., 2-isopropyl-5-methylphenol) is a monoterpene phenol derivative of cymene which occurs in *Eucalyptus* leaves (Zini et al., 2003) and is thus indicative of C3 plant input. The N-product 2-methoxy-3-ethyl-pyrazine was detected in Bolganup, but not Harding water. Biological sources of this compound include amino sugars (possibly from cellular walls; Koehler et al., 1969; Muller & Rappert, 2010). 2-Methoxy-3-ethyl-pyrazine (**33, CSb**) was also detected in plant leachates (Chapter 2) and may derive from Eucalyptus leaves (e.g., Silou et al., 2009) which are in plentiful supply in the Bolganup catchment. Methoxy-substituted alkyl pyrazines recently detected in water supplies have attracted some concern. For example, 2-methoxy-3,5-dimethyl-pyrazine was linked to complaints of malodours in water supplies from NW Spain (Ventura et al., 2010).

Of the new products detected in the source waters (i.e., not previously evident in the leachates), three products, cyclododecane (**CSi**), the ester of 2,2,4-trimethyl-1,3-

pentanediol di*iso*butyrate (**CSt**) and 2,5 *bis* 1,2-dimethyl ethyl phenol (**CSm**), were particularly dominant (Fig. 5.14). Cyclodocecane was consistently (*Nb*. both fibres) the most abundant product of both source waters.

Table 5.7 Major products and their chemical group from the SPME analysis of Bolganup and Harding waters. *m/z* = details of 4 main fragment ions (B) = Detected in Bolganup only (PDMS Fibre). (H) = Detected with Harding only (PDMS Fibre). Bold Numbers in brackets correspond to products detected from the Chapter 2 leachates and listed in Table 2.5 with corresponding isotopic values in Table 2.8).

Peak i.d.	RT (min)	Compound i.d.	*m/z*	MW	Grouping
CSa	27.25	thymol **(29)**	135, 150, 191, 77	150	Phenol
CSb	28.90	2-methoxy-3-ethylpyrazine	138, 137, 123, 95	138	N-product
CSc	29.82	5-dodecanol (H)	69, 32, 55, 41	186	Alcohol
CSd	30.07	3-dodecanol	59, 83, 55, 69	186	Alcohol
CSe	30.17	2-butyl-1-octanol	57, 43, 71, 55	186	Alcohol
CSf	30.34	tetradecanal (H)	57, 82, 96, 140	212	Aldehyde
CSg	30.73	1-dodecene	43, 55, 71, 83	186	Alkene
CSh	31.55	unidentified substituted	95, 32, 151, 165	-	Ester
CSi	31.80	cyclododecane	43, 55, 71, 83	168	Cycloalkane
CSj	32.14	6-tridecanol (H)	55, 69, 83, 101		Alcohol
CSk	32.21	tridecanol (unspecified	69, 55, 83, 43	200	Alcohol
CSl	32.45	2-tridecanol (H)	69, 55, 83, 43	200	Alcohol
CSm	32.56	2,5 *bis* 1,2-dimethyl ethyl	191, 206, 192, 57	206	Phenolic
CSn	33.05	benzoic acid-4-ethoxy-ethyl	121, 149, 194, 138	194	Ester
CSo	33.36	unidentified alcohol (H)	69, 55, 41, 83	-	Alcohol
CSp	33.44	trimethyl-alcohol (H)	57, 55, 69, 97	-	Alcohol
CSq	33.53	unidentified substituted	135, 107, 136, 57	206	Unknown
CSr	33.63	4 -(1,2,3,3-tetramethylbutyl)	135, 107, 32, 191	206	Phenolic
CSs	34.23	unidentified substituted	57, 70, 97, 71	214	Unknown
CSt	34.31	2,2,4-trimethyl-1,3-	71, 97, 159, 143	286	Ester
CSu	34.54	unidentified carboxyllic acid	71, 43, 143, 69	-	Ester
CSv	36.28	hexadecanoic acid methyl	28, 43, 74, 87, 143		Ester

Cyclododecane is used as an intermediate in the manufacture of polyamides, polyesters, lubricating oils and nylon (IJC, 1983) so possibly derive from the plastic containers used for sampling. The 2,2,4-trimethyl-1,3-pentanediol di-*iso*butyrate ester is also a known plasticiser marker whilst phenols such as 2,5 *bis* 1,2-dimethyl ethyl phenol are common in many industrial products (e.g., synthetic resin, plastics, rubbers, oils, dyes, surfactants). The similarly high quantities detected in each of the waters are consistent with a common source, such as plastic containers used for sample collection.

Phthalates and highly *bis*-substituted phenols are common artefacts of SPME analysis (Demyttenaere et al., 2003) and were not detected in the procedural controls (run every four samples). These three contaminant products were detected in exceptionally high relative abundances in two remote source waters, with pristine catchments and exposure to minimal industrial activity, although there is some viticulture and other agriculture in vicinity of Bolganup Creek (DoW, 2008). Contamination of this scale is likely related to water sampling or storage and their high signals may also be influenced by a very high affinity to the SPME fibres. The plastic containers used during water sampling and storage are the leading suspect for these contaminants. Such containers have been long used for traditional analysis such as metal speciation, traditional chlorine demand studies (Water Corporation, 2003) and even previous SPME water studies (Zander, 2008) without apparent incident. However they may not be appropriate for the trace organic analyses performed here.

The overwhelming abundances of the three anthropogenic products in the concentrated surface waters may severely limit the molecular information obtained from SPME-GCMS analysis. The absorption of products in high concentrations can reduce the extraction efficiency of products with a lower propensity for absorption (Pfannkoch et al., 2003; Shirey, 1999).

Products thought to be indigenous to the Bolganup and Harding water were classified by functional groups to help interpretations (Fig. 5.15). In Bolganup water, the most abundant indigenous products were esters of mainly carboxylic acids (30 %; Fig. 5.15), many of which were previously seen in the plant leachates (Chapter 2). Esters were significantly less abundant in the Harding water (11 %; Fig. 5.15). Methyl ester carboxylic acids are common in aquatic systems (Carabias-Martinez et al., 2003), deriving from either natural or anthropogenic sources (Thurman, 1985). Natural sources include bacterial fatty acid catabolism (Mas-Castella & Guerrero, 1995) or photochemical degradation of high molecular weight DOC (Bertilsson & Tranvik, 2000). These compounds are preferentially utilised by bacteria during prolonged periods of stratification (Christian & Lind, 2007) and their high concentrations in Bolganup may reflect stratification conditions in the reservoir.

Alcohols were the major products in Harding (52 %; Fig. 5.15), whereas alcohols represented just 12 % the measured products of Bolganup. There are multiple biochemical sources of alcohols, making them difficult to distinguish on the basis of molecular distribution alone. Phenols, particularly thymols (also derived from Eucalyptus leaves; Silou

et al., 2009), were more abundant in Bolganup than Harding, consistent with the pyrolysis and other spectroscopic data.

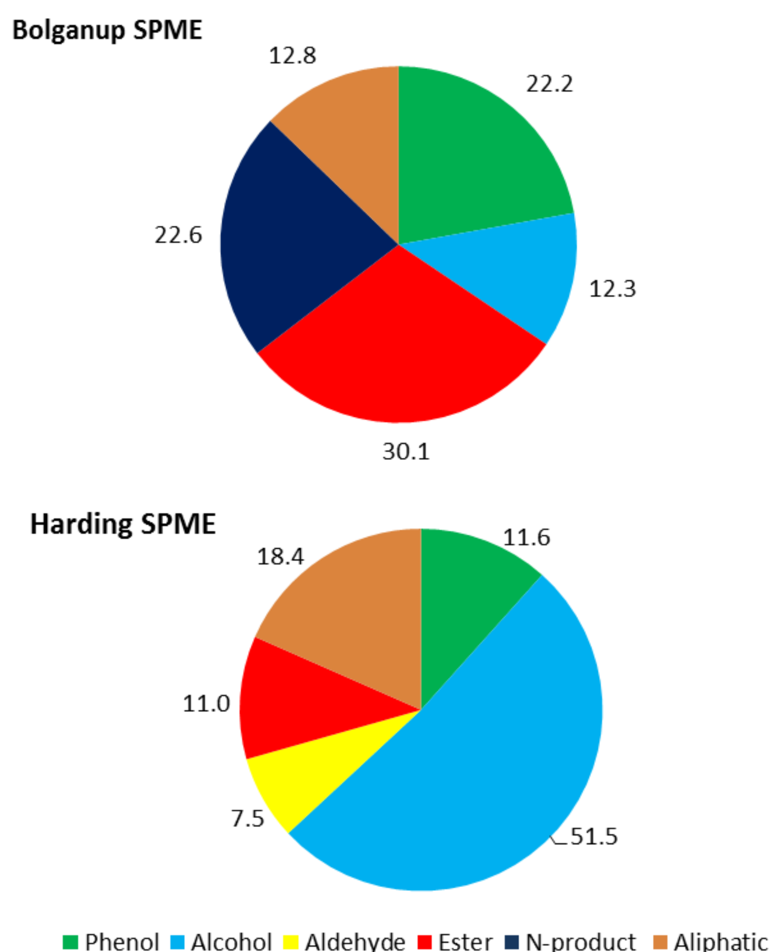


Figure 5.15 Distribution of product groups detected by SPME GCMS (PDMS fibre) of Bolganup and Harding source waters (suspected contaminants were not included).

The aldehyde tetradecanal, a bacterial metabolite (Ulitzur & Hastings, 1979; Riendeau & Meighen, 1985), was detected in Harding but not Bolganup water and is the sole contributor to the aldehyde group abundance show in Fig. 5.15. Widely detected in natural waters, carbonyl compounds are also common photodegradation products of DOM (Mopper & Stahovec, 1986; Kieber et al., 1990).

The significant algal, and to a lesser extent bacterial, content of Harding Reservoir (Garbin et al., 2010) may also account for the higher aliphatic (i.e., dodecene) signal measured (18 % Harding *Cf*. 13 % Bolganup), which was also largely consistent with the other spectroscopic data acquired (Section 5.4.2). Again, however, alkenes such as this are not particularly source diagnostic.

5.4.3.2 Isotopic Characterisation by SPME-CSIA

The $\delta^{13}C$ values of several individual products of the SPME analysis of the concentrated Bolganup and Harding source waters. But $\delta^{13}C$ CSIA data could only be acquired for the six most abundant volatile products of Bolganup and four largest products of Harding (Table 5.8). These include the three suspected contaminants which dominated the molecular profile, and three other indigenous compounds. Nevertheless, SPME CSIA may help distinguish petroleum based anthropogenic from natural products, although the $\delta^{13}C$ of petroleum based components falls within the broad isotopic range of C3-derived molecular components.

Table 5.8 $\delta^{13}C$ values and standard deviations of volatiles species measured by (PDMS) SPME-CSIA of concentrated Bolganup and Harding waters. (n) = number of replicated analysis. *= compound close to detection threshold (Nb. contribute to a high standard deviation).

Compound ID	$\delta^{13}C$ (‰) Harding PDMS	$\delta^{13}C$ (‰) Bolganup PDMS
Bulk	-24.0	-28.1
thymol	-27.4 ±0.62$^{(2)}$	-
2-methoxy-3-ethylpyrazine*	-	-34.9 ±1.91$^{(2)}$
cyclododecane	-31.3 ± 0.47$^{(3)}$	-31.3 ± 0.47$^{(3)}$
2,5 *bis* 1,2-dimethyl ethyl phenol	-24.8 ± 0.82$^{(3)}$	-23.7 ± 0.82$^{(3)}$
benzoic acid-4-ethoxy-ethyl ester	-	-27.2 ± 0.62$^{(2)}$
2,2,4-trimethyl-1,3-pentanediol di-*iso*butyrate	-16.5 ± 1.01$^{(2)}$	-15.5 ± 0.55$^{(2)}$
unspecified carboxylic acid ester	-34.5 ± 0.61$^{(2)}$	-33.6 ±0.56$^{(2)}$
hexadecanoic acid methyl ester	-	-28.9 ±0.63$^{(2)}$

Most $\delta^{13}C$ values measured were generally within the range typical of C3 plant source, with the exception of 2,2,4-trimethyl-1,3-pentanediol di-*iso*butyrate (-16.5 ‰), which was heavier than the normal C3 source range. The $\delta^{13}C$ of cyclododecane was the same in both waters, consistent with a common contamination source. The $\delta^{13}C$ values of the four other compounds measured were all very similar but provide little insight to any specific natural sources. Of note however, the $\delta^{13}C$ of the thymol detected in Harding water was 3.4 ‰ more depleted than the bulk $\delta^{13}C$ of the parent H-HPO fraction value (Table 5.2). The respective $\delta^{13}C$ values of the bulk Marri (Chapter 2) and thymol leached from it showed an almost identical trend (i.e., thymol 3.2 ‰ more depleted; Table 2.11). Hexadecanoic acid methyl ester (16:0) in Bolganup water had a $\delta^{13}C$ value of -28.9 ‰ which was similar to the bulk B-NOM value (-28.1 ‰; Table 5.3), typical of a C3 source.

Overall, however, the present CSIA data of several VOC and SVOC of the concentrated source waters did not facilitate any clear source information, suggesting minimal value of

these analyses at its present stage of development and application to water, particularly with respect to contamination and sensitivity issues limiting the number of analytes for which $\delta^{13}C$ could be measured.

5.4.3.3. PLFAs of Source Water Microbiota

A representative TIC from GCMS analysis of Harding (H) PLFA fraction is shown in Fig. 5.16.

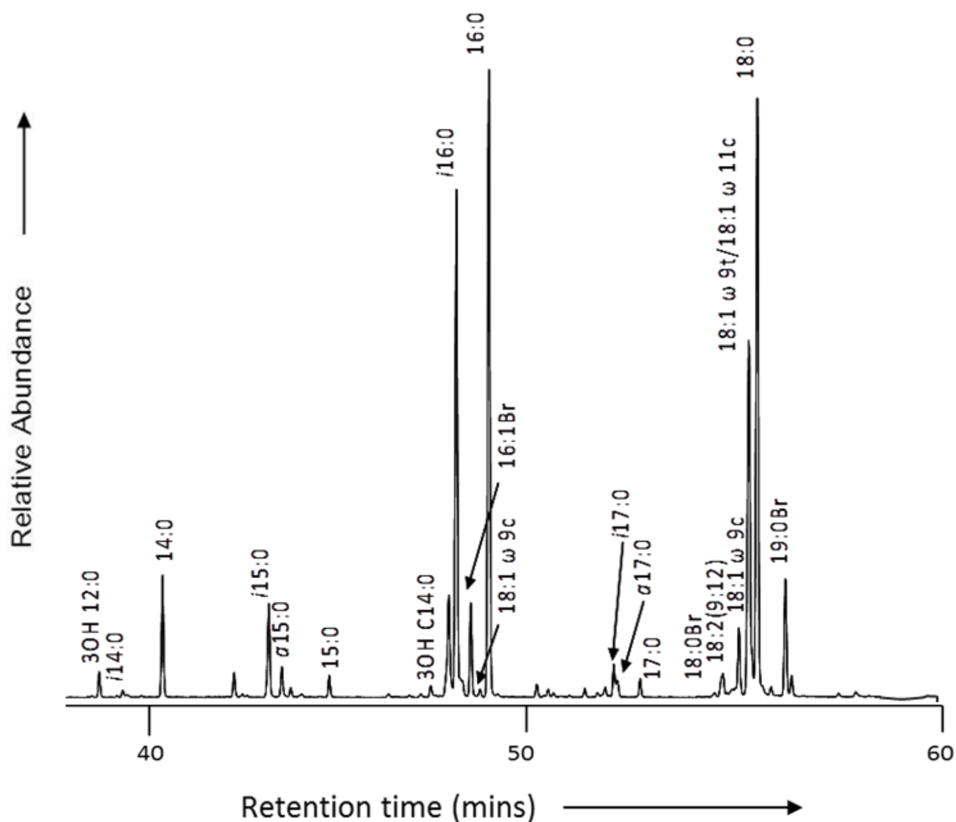


Figure 5.16 Partial TIC (RT 40–50 min) from GCMS analysis of H-PLFA. Peak assignments correspond to products listed in Table 5.9.

The 28 major PLFAs used in the quantification of the leachates (Chapter 3, Table 3.6) were again prominent and their concentrations are shown in Table 5.9. The PLFAs of greatest concentration for H-PLFA and Bolganup (B) PLFA were 16:0, 18:0, *i*16:0 and 18:1ω9*t*-18:1 ω11*c* (i.e., co-eluting peak). The relative proportions of PLFA groupings are shown in Fig. 5.17. The PLFAs of both waters were similarly dominated by saturated straight chain (Nsat) PLFAs (43.7 % B, and 43.2 % H) with terminally branched (TB) PLFAs (22.0 % B, and 24.8 % H) and monounsaturated (MU) PLFAs (17.0 % B, and 21.4 % H) also abundant.

Table 5.9 PLFA concentrations (ng mL^{-1}) determined for raw Harding and Bolganup Reservoir waters. nd = not detected.

PLFA	PLFA	Bolganup	Harding
12:0(3OH)	OHsat	0.44	0.06
*i*14:0	TBsat	nd	0.26
14:0	Nsat	0.14	0.26
*i*15:0	TBsat	0.16	0.24
*a*15:0	TBsat	0.13	0.07
15:0	Nsat	0.03	0.05
14:0(3OH)	OHsat	0.92	0.30
*i*16:0	TBsat	1.02	1.26
Br Me -16:0	MCBsat	0.08	nd
Br Me -16:0	MCBsat	nd	0.21
16:1ω9*c*	MUnsat	nd	0.02
16:1ω7*c*	MUnsat	0.13	0.21
16:0	Nsat	1.09	1.50
Br Me -17:0	MCBsat	nd	0.03
*i*17:0	TBsat	0.05	0.02
*a*17:0	TBsat	0.04	0.01
cy17:0	Cyclo	0.07	0.04
17:0	Nsat	0.06	0.04
Br Me -18:0	MCBsat	0.20	0.09
18:2(9,12)	PUsat	0.06	0.18
18:1ω9*c*	MUnsat	0.07	0.28
18:1ω9t/18:1ω11*c*	MUnsat	0.70	1.03
18:0	Nsat	1.29	1.51
Br Me -19:0	MCBsat	0.04	0.05
cy19:0	Cyclo	0.03	0.05
20:0	Nsat	0.04	0.01
Total PLFA concentration in raw water (ng mL^{-1})		6.81	7.77

Bolganup contained a higher proportion (10.1 %) of hydroxyl branched (OH) PLFAs – 12:0(3OH) and 14:0(3OH) – than Harding (4.4 %). Cyclopropyl (cyclo)-PLFAs were also more slightly more prevalent in Bolganup (1.9 % *Cf.* 1.3 % Harding). Both cyclo and OH PLFAs are both indicative of Gram -ve sources (Cavigelli et al., 1995, Ratledge & Wilkinson, 1988; Zelles, 1997). Differences were also evident in the mid chain branched (MCB) PLFAs, which in B-PLFA (4.2 %) were almost twice those of H-PLFA (2.3 %).

Dowling et al. (1986) suggested that MCB PLFAs may be representative of anaerobic microbes, although they have been generally regarded as non-source specific (e.g., potentially from detrital plankton, bacteria or zooplankton; Wakeham, 1995). Branched

and hydroxyl PLFAs however, have been correlated to the exhaustion of easily biodegraded substrate levels and increasing temperatures (Barjea et al., 2008).

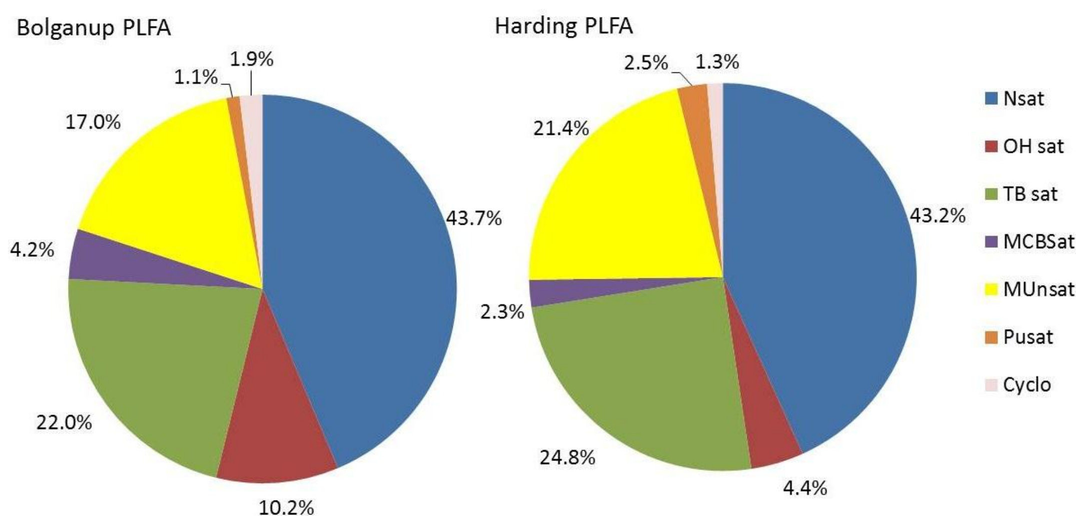


Figure 5.17 Relative abundances of PLFA groups detected in Bolganup (left) and Harding (right) waters.

Cyclo-PLFAs have been observed to respond to changes in environmental conditions. The ratio of cy17:0 to its monoenoic isomer (16:1ω7*c*) increases as bacteria respond to changes in environmental conditions, representing a shift to slower rates of growth (Jacques & Hunt, 1980; Guckert et al., 1986; Navarrete et al., 2000). This relative abundance of cy17:0 compared to 16:1ω7*c* was over double in B-PLFA (cy17:0/16:1ω7*c* = 0.54) than H-PLFA (cy17:0/16:1ω7*c* = 0.19). This PLFA data may reflect the increased anoxia associated with the more intensive stratification impacting Bolganup. Bolganup Reservoir often experiences significant stratification, usually between November and April (Garbin et al., 2010), with bottom waters experiencing anoxic reducing conditions. Harris and Antennucci (2008) reported an increase in concentrations of both 'labile' and refractory DOC (proposed to be due to diffusion), under stratified conditions for Bolganup Reservoir (flux rates of *ca*. 0.0002 mg C L^{-1} hr^{-1}, maximum levels occurring during maximum periods of stratification). The mobilisation process responsible is not clearly understood and is often discussed in combination with the reductive dissolution of metal oxyhydroxides (e.g., Grybos et al., 2007; Bjorkvald et al., 2008; Butler, 2011). Similarly, Garbin et al. (2010) reported the mobilisation of iron (and manganese) during stratification of Bolganup Reservoir. Bacterial Fe (III) reduction has been well described in stratified lakes (Mortimer, 1941 & 1942; Davison et al., 1981; Verdouw & Dekkers, 1980; Jones et al., 1983). During stratified periods in Bolganup Reservoir, Fe (III) may provide an alternative substrate to DOC, which may

stimulate the growth of niche bacterial communities capable of reducing Fe (III) (e.g., Gram -ve *Proteobacteria*; Coates et al., 1998; Coates et al., 2001). Hence, anaerobic and facultative anaerobic bacteria may flourish at the sediment- water interface during stratification.

In the present study, the water was sampled at the tail end of the stratification period, when the easily biodegraded substrate (i.e., detrital remains of zooplankton, algae, faecal matter and leaf material deposited in the last mixing period) is likely close to exhaustion. Towards the end of a stratified period, microbial organisms that previously flourished, may begin to undergo nutrient and temperature stresses associated with increased bottom water anoxia. This would results in an increase in the relative abundances of cyclo-PLFAs (Guckert et al., 1986; Pinkart et al., 1995; White et al., 1997; Green & Scow, 2000; Smith et al., 2000) and unsaturation (Dowling et al., 1986). It also explains the relatively high abundances of branched and hydroxyl PLFAs which have been correlated with the consumption of easily biodegraded substrates, as well as increasing temperatures (Barjea et al., 2008).

Stratification has also occurred in Harding (the dense bottom layer, i.e., hypolimnion, forming at 4–10 m depth; Garbin et al., 2010), but varies in frequency from annual to biannual (WRC, 1999; Water Corporation, 2005b; Garbin et al., 2010; WQRA, 2009). Mixing is promoted by ambient temperature, typically occurring when the surface temperature drops <20°C (Harris & Antennuci 2009; Garbin et al., 2010).

Both source waters have been historically impacted by algal blooms which high stratification can favour (e.g., Smayda, 1997), although these blooms have been more significant in Harding (bi-annual peaks of phytoplankton, each of over 350000 cells mL^{-1}; Garbin et al., 2010) than Bolganup (peaks of 30000 cells mL^{-1}; Garbin et al., 2010). The total concentrations and distribution diversity of PLFAs were slightly higher in Harding than Bolganup (Table 5.9), despite the lower DOC concentration determined for Harding (Table 5.1). PLFA concentrations reportedly (Boissier & Fontevieille, 1993; Søndergaard & Worm, 2001; Servais et al., 2003; Søndergaard & Middelboe, 1995) correlate more strongly with biodegradable DOC (BDOC) which is a significant proportion of the DOC in Harding and Bolganup (Garbin et al., 2010). BDOC, more meaningfully expressed as % DOC available, was 24–28 % for Harding and 17–21 % for Bolganup (based on values determined for CRCWQT Project No. 2.0.2.3.2.1, these values are from a different sampling instance and are therefore provided here for illustration purposes only).

Algal sources also contribute to 'labile' DOC, and a relationship between the occurrences of blooms and BDOC levels have been observed at Harding (Garbin et al., 2010). The detrital remains of phytoplankton and other organisms grazing on the algae contribute to the BDOC (e.g., Søndergaard & Middelboe, 1995). Algal DOC (more significant in Harding) is generally more biodegradable than terrigenous derived DOC (Kawamura et al., 1987; Søndergaard & Middelboe, 1995; Kragh & Søndergaard, 2004; more significant in Bolganup). The distribution of treated Harding water is prone to microbiological re-growth (including algae, EPS and at times *Naegleria* spp.) and has historically had a high chlorine demand (Water Corporation, 2005b).

Harding Reservoir also experiences greater sunlight intensity and higher temperatures than Bolganup. Microbial growth and activity increases with temperature (White et al., 1991; Geldreich et al., 1996; Smith et al., 2000; Keinanen et al., 2002; Ndiongue et al., 2005; Rao, 2010) whilst photodegradation can increase the lability (Bano et al., 1998; Anesio et al., 2005) and biodegradability of organic matter

In summary, differences in sediment flux (higher for Bolganup) and algal biomass (higher for Harding) may be a major factor in the influence the aquatic microbial community structure of these source waters and as reflected by subtle differences in their PLFA profiles.

5.4.3.3.1 Microbial Sub-groups

Figure 5.18 shows the relative contributions of Gram -ve, Gram +ve and fungal (or eukaryotic) microbial subgroups. The different community compositions of the source waters was reflected by higher proportions of Gram -ve bacterial PLFAs (34 %, B *Cf*. 22 %, H) and lower proportions of fungal/eukarotic sourced PLFAs in B-PLFA compared to (2 %, B *Cf*. 6 %, H) H-PLFA. Both showed similar 21–24 % of Gram +ve bacterial PLFAs.

PLFAs indicative of Gram +ve (e.g., *i*16:0; Zelles, 1997) and Gram -ve (18:1ω11*c*, 18:1ω9*t*; Zelles, 1997) bacteria were detected in both samples, but at higher concentrations in H-PLFA (Table 5.9). 18:1ω11*c* has been associated with Gram -ve *Proteobacteria* (Zelles, 1997), which are commonly found in aquatic microbiota (Axmanova et al., 2006).

The slightly higher proportion of microeukaryotic/eukaryotic/fungal PLFA markers in the Harding water may relate to the Spinifex grasses that dominate this catchment. The Spinifex leachate supported a relatively high proportion of fungal PLFAs compared to the

other leachates (Chapter 3, Section 3.4.1.1). The thick waxy cuticle of Spinifex blades (of the Harding catchment) are high in polyphenols (Webster & Benfield, 1986) which can be utilised by fungi (Mutabaruka et al., 2007).

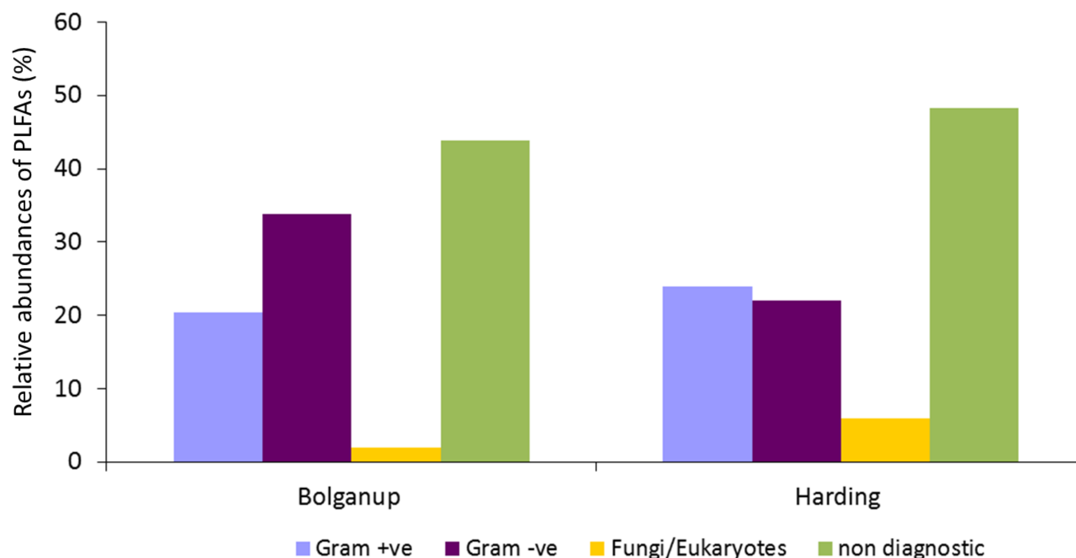


Figure 5.18 The relative abundances of PLFAs diagnostic of microbial sub-groups, of Harding and Bolganup waters. Gram-positive PLFAs (∑ [*i*14:0, *i*15:0, *a*15:0, *i*16:0, *i*17:0, a17:0]), Gram-negative PLFAs (∑ [12:0(3OH), 14:0(3OH), 16:1ω9*c*, 16:1ω7*c*, cy17:0, 18:1ω9*t*/18:1ω11*c*, cy19:0]) and Fungal PLFAs ∑ [18:2 (9,12) + 18:1 ω9*c*]). Others are non-diagnostic.

5.4.3.3.2 Statistical Insights

The PLFA abundance data shown in Table 5.9 was statistically analysed using the same MDS analysis described in Chapter 3. Figure 5.19 shows the Case study PLFA clusters (denoted "X with appropriate prefix; HD1, HD2, Bo1 or Bo2) superimposed on the PLFA fractions of the leachates described in Chapter 3. Further details of the statistical analysis, results and additional diagrams are given in Appendix A. ANOVA of PLFA distribution and Total concentration was also run (using IBM SPSS 21 software), no statistical significance between the groups was shows.. The details of the ANOVA analysis are presented in Appendix B.

MDS analysis of the PLFA data showed that each case study sample clustered into a uniquely discrete grouping separate from the other samples (i.e., distribution system biofilms; vegetation leachate biomass) subjected to PLFA analysis in this PhD project.

So whilst the differences between test groups (i.e., the reservoir waters Bo1,Bo2, HD1, HD2 as distinct from the other test groups of (i) pipeline biofilms and (ii) vegetation leachates) were statistically significant ('significance' described in detail in Appendix A), the

differences within the test group were not so distinct (Bo1,Bo2, HD1, HD2 all fell into individual groupings of statistical similarity). This reinforces the argument that the sensitivity of the PLFA analysis was too low to facilitate reliable statistical delineation. More samples would allow more definitive conclusions to be made. This should be addressed by any future work which should also examine the control of phytoplankton, BDOC, sediments and seasonal variability on microbial-PLFA structure and dynamics.

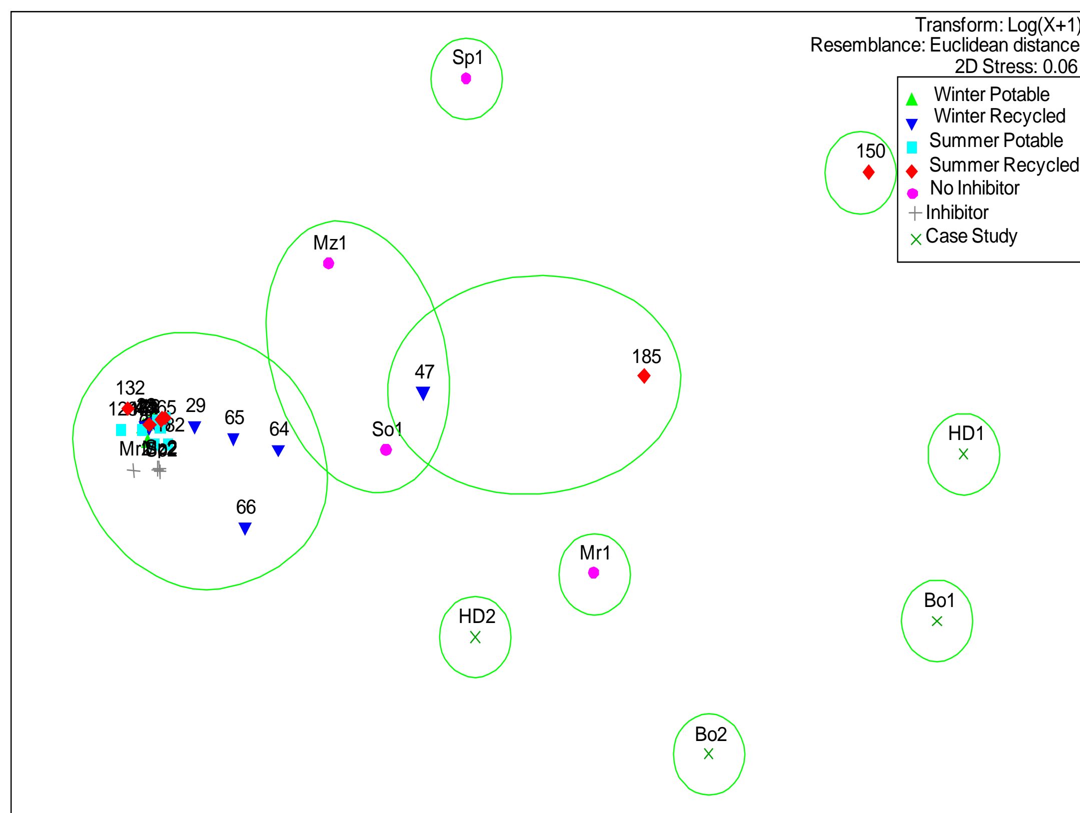


Figure 5.19 MDS analysis of PLFA profiles in whole sample suite. The input of the cluster analyses were Bray-Curtis similarity matrices of Log (X+1) transformed PLFA profiles (% of total PLFA content). Resemblance based on Euclidean distance. 2D stress = 0.06. The 2D arrangement represents relative differences in similarity. HD1 = Harding concentrated BD1 = Bolganup concentrated. HD2 Harding non concentrated BD2 = Bolganup non concentrated. Green lines correspond to Euclidean distance of 11.68.

5.4.3.4 CSIA of PLFAs

The $\delta^{13}C$ values of four of the most abundant PLFAs were able to be measured from the concentrated reservoir waters (Table 5.10). This represents a very small subset of the PLFAs detected by GCMS, and not all are indicative of particular microbial sources, reflecting the sensitivity challenges confronting the CSIA analyses of PLFAs in source waters. Each of the PLFA for which $\delta^{13}C$ could be measured were ^{13}C depleted relative to their

parent HPO-fractions (Table 5.3), consistent with previous analyses which show PLFA to be lighter than the bulk of their parent samples (De Niro & Epstein, 1977; Monson & Hayes, 1982; Blair et al., 1985; Hayes, 1993; Abraham et al., 1998; Boschker et al., 1999; Teece et al., 1999; Jones et al., 2003).

Table 5.10 $\delta^{13}C$ of specific PLFA products from GC-irMS analysis of B-PLFAs and H-PLFAs. $^{(n)}$ = Number of replicates on which analyses was based. Bulk $\delta^{13}C$ of B-HPO and H-HPO fractions are also shown. The $\delta^{13}C$ variance (Δ) of the PLFAs relative to 16:0 and the corresponding HPO fractions are also shown.

PLFA	B-PLFA			H-PLFA		
	$\delta^{13}C$ PLFAs (‰)	Δ (‰) B-HPO	Δ (‰) 16:0	$\delta^{13}C$ PLFAs (‰)	Δ (‰) H-HPO	Δ (‰) 16:0
*i*16:0	-35.9 ± 0.05$^{(3)}$	-7.8	+1.5	-27.2 ±0.21$^{(2)}$	-3.2	+4.2
16:0	-37.4 ±0.12$^{(2)}$	-9.3	-	-31.4 ±0.04$^{(3)}$	-7.4	-
18:1ω9t/18:1ω11c	-36.0 ±0.07$^{(2)}$	-7.9	+1.4	n/d	-	-
18:0	-31.8 ±0.07$^{(2)}$	-3.7	+5.6	-26.3 ±0.55$^{(2)}$	-2.3	+5.1
Average PLFA $\delta^{13}C$	-35.3	-7.2	-	-28.3	-4.3	-
Bulk $\delta^{13}C$ of HPO NOM	-28.1	-	+9.3	-24.0	-	+7.4

The PLFAs were more ^{13}C depleted in Bolganup than Harding, consistent with a greater contribution of C3 substrates which would be plentiful in a heavily forested catchment. The Bolganup PLFAs were also generally ^{13}C depleted relative to their HPO fraction by a greater amount than the Harding PLFAs. This would be consistent with an increase in anaerobic conditions (Teece et al., 1999) or increased utilisation of C3 derived organic matter (e.g., Cranwell, 1978 & 1981). The $\delta^{13}C$ values of B-PLFA, however, do not support the presence of metal reducers which are typically characterised by extensive ^{13}C depletion.

PLFA 18:0 was consistently the least ^{13}C depleted compound in B-PLFA and H-PLFA, with $\delta^{13}C$ values only 3.7 ‰ (B) and 2.3 ‰ (H) less than their corresponding bulk values. Furthermore these values were 5.6 ‰ (B) and 5.1 ‰ (H) higher than the respective 16:0 $\delta^{13}C$ values. The consistent trend evident in the two reservoir systems may reflect common biochemical processes.

In addition to source controls, the $\delta^{13}C$ of lipid membranes can be influenced by growth rate, competition, type and bioavailability of substrate, light and temperature effects (Jahnke et al., 1999; Wick et al., 2003; Londry et al., 2004; Zhang et al., 2005; Staal et al., 2007) as discussed below.

Regarding substrate, sulfate and iron complexes are mobilised in Bolganup sediment during stratification (Garbin et al., 2010) and can be used by sulphate/iron reducing bacteria. Sulphate reducing bacteria can produce PLFA showing a broad range of $\delta^{13}C$ values, depending on metabolism and structure (Londry et al., 2004). Zhang et al. (2003) investigated $\delta^{13}C$ of PLFAs isolated from the commonly identified Fe(III)-reducing *Geobacter* spp. (strict anaerobes; Lovley, 1991) which have been detected in fresh water sediments (Coates et al., 1996; Snoeyenbos-West et al., 2000). Zhang et al. (2003) reported that 14:0 was depleted by -3.8 ‰, *i*15:0 was depleted by -8.2 ‰ and 16:1ω7*c*/ω7*t* (co-eluting isomers) was depleted by -9.4 ‰ relative to bulk $\delta^{13}C$ values. With the exception on 16:0 (a generic, non-diagnostic PLFA representing the overall bulk and therefore not useful at reflecting subtle differences), the magnitude of these depletions was similar to the trends observed in the Bolganup PLFAs (i.e., -3.7 ‰ for 18:0, -7.8 ‰ for *i*16:0 and 7.9 ‰ for 18:1ω9t/18:1ω11c). This was not observed for Harding, and may indicate the presence of sulfate reducing bacteria.

None of the PLFAs (16:0, *i*16:0, 18:0, 18:1) for which $\delta^{13}C$ values were measured are diagnostic of a singularly specific bacterial type, but collectively their $\delta^{13}C$ values (-28‰ to -37 ‰) are generally typical of either heterotrophic, anaerobic, oxygen deprived aerobic bacteria (i.e., also consistent with elevated Cyclo-PLFA concentrations) or sulfate reducing bacteria, all of which can flourish during periods of stratification (Molongoski & Klug, 1976, 1980; Dahm et al., 2005).

Harding reservoir (*Cf*. Bolganup) has a large surface area, higher incident temperatures and UV radiation and a known history of algal and cyanobacterial blooms. Microbial growth rates (Jahnke et al., 1999; Boshker et al., 2005; Ekblad et al., 2005) and substrate utilisation are both dependant on temperature (Wiebe et al., 1992; Yumoto et al., 2004). The PLFAs of Harding, where the water temperature is higher, were on average 6.7 ‰ more ^{13}C enriched than B-PLFAs. This greater enrichment may be due to increased biomass growth rates at the higher temperature and subsequent high demand for CO_2 (as during a bloom period there would be competition for $^{12}CO_2$ and algae would consequently take in more $^{13}CO_2$; Grice et al., 1996b).

There was no visible bloom in Harding at the time of water sampling, and consequently no singularly dominant source input. The molecular and the $\delta^{13}C$ signatures of H-PLFA reflect a mixed source, including C3, C4 and algal inputs. These sources contribute to the complex

molecular composition of DOC which can range from very 'labile' (algal) to more refractory (C3/C4) organic substrates supporting a diverse microbial community.

The effectiveness of water management practices which aim to minimise both the organic loading and microbial activity of source waters typically results in very low concentrations of microbial biomass and hence also detectable PLFA compounds. This represents a major challenge to the isotopic characterisation of PLFAs in pristine waters. The sensitivity of the analysis was improved by processing a relatively large water volume (i.e., 100 L), but still most PLFAs were below the $\delta^{13}C$ detection threshold. This suggests even larger volumes of pristine waters would be required for comprehensive PLFA characterisation.

Despite the sensitivity limitation presently encountered, some valuable insight was gained from the four most abundant PLFAs for which $\delta^{13}C$ values could be determined. This extremely limited isotopic data set still provided some new insights including differentiation of different microbial communities and carbon substrate utilisation. Some of the more interesting findings were:

- The bulk $\delta^{13}C$ signatures of HPO-NOM fractions isolated from each source water was reflected in the $\delta^{13}C$ signatures of the PLFAs extracted from the same waters.
- Bolganup PLFAs were more ^{13}C depleted than Harding PLFAs, which was attributed to greater anaerobic conditions (associated with stratification) or increased mineralisation of C3 material.
- The more ^{13}C enriched PLFAs from Harding (*Cf.* Bolganup) may be related to algal blooms which are significant in this water.

5.4.4 Summary of NOM Characterisation

Bolganup and Harding Reservoirs represent two source waters with contrasts in geographical location, climate and catchment vegetation. Consequently, the molecular compositions of the NOM of these waters are likely to be very different, which in turn will impact the structure and function of the aquatic microbial community of each water. Some of these differences can be distinguished by complimentary molecular and stable carbon isotopic analysis. Table 5.11 shows a summary of the main findings from the holistic molecular and stable isotopic characterisation of these two waters.

The analytical data from the HPO-fractions of the two waters were generally very similar, but several subtle differences were evident. For example, FTIR and ^{13}C-NMR showed greater aromaticity and higher O/N alkyl content for Bolganup (*Cf*. Harding) HPO, which is consistent with their respective SUVA values. The larger response of ^{13}C-NMR spectra for Bolganup (*Cf*. Harding) is also consistent with the high DOC loads generally associated with a forested catchment. Algal blooms which occur very occasionally and anoxic conditions resulting from stratification in Bolganup Reservoir contribute to a more complex biogeochemical system (Harris & Antennuci 2009; Garbin et al., 2010). Harding did show greater alkyl character, possibly related to algal input from the blooms which occur frequently in the Harding Dam despite best efforts of water practice management.

Table 5.11 Comparison of molecular and δ^{13}C characterisation of the Harding and Bolganup source waters. Basic/general analyses are shaded green, established spectroscopic methods analysis are shaded purple and novel analysis with the complimentary molecular/CSIA methods developed in this project are shaded blue.

Technique	Bolganup	Harding	Reference
Basic	Higher **UV, DOC**,	Higher **Nitrogen** Content	Table 5.1 & Table 5.3
NOM isolation	HPO 80 %	HPO 60 %	Table 5.2
Bulk δ^{13}C HPO	-28.1 ‰	-24.0 ‰	Table 5.3
FTIR & ^{13}C-NMR	Relatively aliphatic, Moderate carboxylic Greater **aromaticity**	Relatively aliphatic, Moderate carboxylic Greater **alkyl character** More **N-products**	Section 5.4.2.2 & Section 5.4.2.3
Flash-Py	More **lignin, tannin & alkyl phenol** material	More **carbohydrate** derived material	Figs 5.10 & 5.11
MSSVpy	Greater **phenolic** input More **aromatic**	More **N-products** More **carbohydrate** More **aliphatic**	Figs 5.12 & 5.13
SPME GCMS & irMS	No differentiation		Section 5.4.3.1 & 5.4.3.2
PLFA GCMS	Lower total PLFA conc; Gram -ve PLFAs dominant; **Cy, OH and MCB PLFAs** prominent; oxygen stress	Higher **total PLFA conc**; Greater **PLFA/microbial diversity**	Table 5.9 Fig. 5.17 & 5.18
δ^{13}C of PLFAs	More **^{13}C depleted** Greater **C3 input** **Metal reducing bacteria** Input from **anaerobic heterotrophs**	More **^{13}C enriched** Mixed input – more C4 or Algal contribution 'labile' sugar substrate	Table 5.10

Of the new methods, the molecular distributions of PLFA detected provided a snapshot of the reservoir water column microbiota. Subsequent δ^{13}C analysis of the more concentrated

PLFAs provided a small amount of additional data which could be useful for helping construct the carbon dynamics and substrate utilisation of the microbial communities observed. The $\delta^{13}C$ PLFA data of the HPO fractions identified differences in source inputs (i.e., B-HPO C3 , H-HPO mixed), suspected also from bulk ^{13}C analysis, and hinted at the influences of seasonal stratification on the microbiota, and periodical blooming of algae (autochthonous sources).

The application of SPME GCMS and SPME CSIA however yielded minimal information to assist the characterisation of the two NOM samples due to interfering analytical contamination. However, it did yield complementary information when trialled in control laboratory conditions (Chapter 2), which suggests potential merit if the contamination issues encountered here can be practically overcome. SPME does target the low molecular weight and microbiologically amenable fraction of aquatic NOM, complimenting the traditional analysis of resin isolated fractions which is prone to the loss of volatile organic compounds. The present case study did reveal the $\delta^{13}C$ of the thymol detected by SPME CSIA in Harding water was 3.4 ‰ lighter in ^{13}C than the parent H-HPO fraction (Table 5.2). A similar 3.2 ‰ depletion was also evident for thymol in the 150 day Marri leachate (*Cf*. Marri residue; Chapter 2, Table 2.17). The small difference (0.2 ‰) in depletion in thymol from Harding compared to the Marri may reflect the average isotopic signature of several different plant sources in the surface water.

Thus, the novel application of SPME CSIA may have the potential to provide information on constituents of aquatic NOM that are not easily characterised using conventional analysis. However, the method and associated protocols (e.g., water sampling and storage) need to be refined to minimise potential contamination.

Stable isotopic analysis of the SPME and PLFA fractions has the potential to yield unique analytical information, particularly with respect to the microbial communities present in the water column and the associated mineralisation of organic matter. However, further improvements in sensitivity will be necessary or larger sample volume obtained, if practical, to facilitate analysis of field samples. These objectives may be possible with further technique development in which case the extent to which stable isotopic analysis can complement of the molecular characterisation of NOM.

5.5 Conclusions

Outcomes from this case study in which the DOC of two source waters were characterised with a broad suite of established analytical methods as well as the complimentary molecular and isotope studies developed in this project include:

- The isolated HPO-NOM of Harding and Bolganup were of generally similar chemical character. UV, FTIR and ^{13}C-CPMAS NMR analysis of respective HPO fractions showed Harding was slightly more aliphatic and Bolganup slightly more aromatic in character. Greater volumes of water would be required to isolate sufficient amount of TPI, HPI and colloid fractions by XAD resin separation for similar analytical characterisation.
- SPME-CSIA was significantly compromised by contaminants of high relative concentration. These anthropogenic compounds overwhelmed the natural VOCs and SVOCs. Little useful data was obtained from the application of this novel method to the source waters. Greater concentrations of analytes from pristine aliquots of these samples will be required for meaningful analysis.
- PLFA-GCMS analysis recognised differences in the microbial biomass and community compositions, which likely reflects differences in the bioavailability of the NOM in each of Harding and Bolganup reservoirs.
- CSIA analysis showed the δ^{13}C values of a small sub-set of PLFAs were indicative of catchment sources of DOC and substrate available for microbial utilisation.
- Differences however were seen in the bulk δ^{13}C signatures of the NOM. Bolganup HPO had a more depleted δ^{13}C signature (-28.1 ‰), more characteristic of a C3 input than Harding (-24.0 ‰), which reflected a mixed input.
- Molecular and isotopic characterisation of PLFAs extracted from Harding and Bolganup waters provided new analytical data which complimented the traditional study of HPO fractions. Molecular characterisations (by Flash-Py, Fig.5.11, MSSVpy, Fig. 5.13 and SPME-GCMS, Fig. 5.14) consistently showed greater concentrations of phenols in the HPO fraction of Bolganup, compared to Harding-HPO. Phenols can derive from terrestrial sources, but many other sources also exist and there was little other direct evidence of specific plant biomarkers which might have been anticipated in Bolganup given the heavy forestation of its catchment. The δ^{13}C values of B-HPO were typical of a C3 source and the δ^{13}C value of PLFAs also reflected more significant C3 influence in Bolganup than Harding, whilst also

suggesting an influence of anaerobic conditions or metal-reducing anaerobic bacteria (many of which are Gram -ve).

- More interpretive information would be gained with an increase in PLFA concentration and CSIA resolution, which would also be achievable by processing larger volume water samples although the field and analytical practicalities of this may be questionable.
- A seasonal sampling regime comparing the molecular and $\delta^{13}C$ dynamics of PLFAs would provide more detail on substrate/consumer relationships as well as assist in studying the impacts of temperature (and growth-rate) variance and such episodes as stratification or algal blooming.

The molecular information obtained from SPME-GCMS analysis of concentrated surface waters was severely limited by an overwhelming anthropogenic signal, suspected to be from the plastic containers used during water sampling and storage. Such containers have been long used without apparent incident on such traditional analysis as metal speciation, traditional chlorine demand studies (Water Corporation, 2003) and even previous SPME water studies (Zander, 2008). However they may not be appropriate for trace analyses such as that presently performed by multiple SPME injections and RO concentrated waters. Given this issue it is advisable that plastic containers be avoided for future analyses—metal or glass containers would be less prone to contamination.

Chapter 6

Conclusions and Suggestions for Future Work

6.1 Conclusions

The practical utility of several analytical procedures to support the $\delta^{13}C$ measurement of water borne organics or related microbiota were tested on several laboratory prepared and real water samples including:

- Water leachates of contrasting C3 and C4 plants.
- Water sampled from two functioning Western Australian surface water reservoirs.
- A suite of biofilms isolated from various Australian distribution networks.
- Membrane biofoulant of a full-scale drinking water treatment plant.

The analytical methods investigated were: SPME analysis of volatile organics; isolation of PLFA (methyl ester) fractions; and innovative off-line pyrolysis in a H_2 rich atmosphere followed by traditional column chromatography preparation of saturate and aromatic hydrocarbon fractions. The organic and biologically concentrated leachates were first used to optimise several operational parameters associated with these analytical methods, which were then applied to the appropriate samples to assess their practical potential.

The quality, and thus extent, of isotopic data these analyses were able to provide were each seriously limited by sensitivity challenges. The low DOC concentrations of drinking water systems and the microbial communities they support hindered application and a thorough assessment of the value of stable isotope characterisations of NOM. However, $\delta^{13}C$ measurement of the most concentrated organic analytes from the different samples characterised did show that stable carbon isotope data can complement the more extensive data from contemporary molecular analysis on the sources and aquatic fate of certain organic constituents. A brief synopses of the three different analytical methods investigated follows. They were then applied with other standard procedures typically used to holistically characterise the nature of the organics in drinking water systems.

6.1.1 $\delta^{13}C$ Analysis of the VOCs and SVs of NOM by SPME-CSIA

Sensitivity was a significant limitation to the SPME-CSIA analysis – despite the use of analyte concentration strategies such as four "SPME-probe absorption-injection" cycles – and will need to be improved to be a practically relevant analytical method. Eighteen of the most abundant VOCs/SVs detected in the C3 plant (Marri) leachate and up to eight in the C4 plant (Maize, Sorghum and Spinifex) leachates were detected with sufficient sensitivity and GC separation to aid their $\delta^{13}C$ measurement. However, this small sub-set of analytes corresponded to organic products of the leachates. Most of the VOC/SV products for which $\delta^{13}C$ were measurable maintained the $\delta^{13}C$ signature of their respective plant sources with $\delta^{13}C$ of the C3 leachates (-28 ‰ to -40 ‰) more depleted than the C4 leachates (-15 ‰ to -22 ‰).

The $\delta^{13}C$ of particular structural classes (e.g., sugars, lignin) were also reflective of biosynthetic processes. Carbohydrate-derived products were ^{13}C enriched (0.5–2.1 ‰) relative to the bulk, e.g., 4-isopropyl-2,5-dimethoxybenzylacetate (C3 Marri leachate) showed a 2–2.5 ‰ enrichment and 1,2,3,4 tetrahydro-1,5-dimethyl-napthalene (C4 Spinifex leachate) was ~3 ‰ heavier – providing an insight into biodegradation or synthetic processes.

The majority of the VOCs/SVs of the leachates were from products derived from secondary plant metabolism. Fatty acids and terpenoids showed the largest ^{13}C depletion, attributed to the kinetic isotope effect of the pyruvate dehydrogenase reaction (Melzer & Schmidt, 1987; Gleixner et al., 1993). Secondary products such as lignin, aromatic compounds and flavonoids were also generally ^{13}C depleted due to the kinetic isotope effect of the shikimic acid pathway (Gleixner et al., 1993; Schmidt et al., 1995). The CSIA data from the leachates did show a pattern of consistent ^{13}C depletion with increasing MW, which was attributed to a biosynthetic effect or a mix of source inputs in biologically active samples.

Different $\delta^{13}C$ values of the monoterpene cymene also showed the potential for SPME-CSIA in following specific biosynthetic effects. The $\delta^{13}C$ values of cymene and the Spinifex (an extreme triodioid variant NAD-ME plant) from which it was derived were similar (-15.0 and 14.3 ‰, for cymene and Spinifex, respectively), but different to the $\delta^{13}C$ value of cymene (-20.7 ‰) from Sorghum (a classical NADP-ME plant cymene) where it was depleted by 3.2 ‰ relative to the bulk $\delta^{13}C$ value (-17.5 ‰) of the Sorghum plant. The different $\delta^{13}C$ values

of this analyte were ascribed to differing isotopic discrimination between NADP-ME subtypes (i.e., Sorghum) and NAD-ME species (i.e., Spinifex) as well as differing affinity of the enzyme RuBisCo (which is greater in Spinifex than Sorghum)—proposed to contribute to the greater ^{13}C depletion seen in Sorghum cymene. Although the details of how this occurs are not entirely understood at present, it may relate to the isotopic fractionation associated with the 'triodioid' variation of NAD-ME pathway as evidenced from the different $\delta^{13}C$ value of this monoterpene is two separate plant sources.

6.1.2 Molecular Distribution and Stable Isotope Signatures of PLFA Biomarkers from Biofilms to Assist in Source and Substrate Assignment of Biologically Available NOM

The sensitivity of the $\delta^{13}C$ PLFA analyses was also observed to be disappointingly low, with $\delta^{13}C$ values only measureable from a limited number of the most abundant PLFAs (typically <12) of each analyses. The PLFA concentrations of the biofilms were much lower than the leachates and proved to be insufficient for statistical validation of the $\delta^{13}C$ data.

PLFA $\delta^{13}C$ signatures of the plant leachates were shown to complement the GCMS measurement of their molecular distributions by providing specific information on carbon substrate utilisation and microbial dynamics, specifically regarding preferential microbial assimilation towards certain organic substrates.

The characteristic $\delta^{13}C$ values of the PLFAs were generally indicative of the respective C3 (more ^{13}C depleted) or C4 (more ^{13}C enriched) plants leached. The most diverse microbial communities were observed from the Marri and Spinifex plants—thought to provide plentiful supply of both 'labile' and refractory aqueous organic substances able to support different sub-communities.

The $\delta^{13}C$ of PLFAs indicative of Gram-negative bacteria showed ^{13}C depleted values (relative to the bulk value or the non-source specific 16:0 PLFA), consistent with preferential utilisation of the abundant terpenoid fraction (typically ^{13}C light) identified by SPME-GCMS. Gram-positive bacteria were more ^{13}C enriched, suggesting utilisation of more refractory and ^{13}C rich material such as lignin or lignocelluloses.

The molecular profiles (i.e., relative abundances) of the PLFAs in the distribution system biofilms were shown to be sensitive to a range of parameters including geographical site,

climatic season, water quality, water temperature, free chlorine residuals and substrate availability. The consistently high DOC and nutrient loads of recycled water (particularly those which had received little treatment) supported higher microbial activity, with greater representation of Gram-positive bacteria and fungi or algae with the ubiquitous presence of Gram-negative bacteria. The relatively high PLFA concentrations of these samples also provided the most $\delta^{13}C$ data; nevertheless these data-sets were still quite small and did not allow an extensive statistical evaluation of the biological controls on these distribution systems.

Interestingly, a ^{13}C enrichment of *anteiso*-branched PLFAs (Gram-positive bacteria) was measured in two separate recycled water schemes. This data mirrored previous isotopic-PLFA trends identified in tobacco plants which was attributed to the utilisation of different amino acid precursors (valine and *iso*-leucine for *iso* and *anteiso*- branched fatty acids, respectively; Grice et al., 2008; Zhou et al., 2010).

The $\delta^{13}C$ PLFA data measured in this study demonstrate the potential of CSIA to track source water inputs, and possibly help establish the connectivity and relationships between organic substrates and the biota they support at different stages of the drinking water system. But at present sensitivity levels the $\delta^{13}C$ measurement of PLFAs – particularly from low biological systems such as source waters with well protected catchments, or distribution systems with efficiently maintained chlorine residuals – would seem to be restricted to waters or related materials (e.g., biofilms) with very high microbial concentrations (e.g., recycled or waste water systems).

6.1.3 HyPy as a Preparative Technique for CSIA of Biofoulant NOM

The offline nature of catalytic HyPy supported subsequent column chromatography and derivatised preparation of fractions more amenable to GC procedures. These analyses were applied to a biofoulant sample collected from a nano-filtration membrane since quite large amounts of sample (typically > 40 mg) are required to derive quantitatively significant fractions. The success of these pre-treatment procedures was demonstrated by measuring the $\delta^{13}C$ value of *n*-alkanes and TMS-derivatised substituted aromatics from lipid and sugar precursors, respectively. However, many other components in the aromatic fraction and derivatised polar fraction were still not sufficiently resolved for uncompromised $\delta^{13}C$ measurement.

The prominent even-number *n*-alkanes and MMAs in the aliphatic fraction, and the sugar products in the derivitised polar fraction are indicative of their microbial source.

Other studies have similarly reported significant abundances of microbial molecular markers (Croué et al*.* 2003a) and a *Sphingomonas* dominant community (EPS related) determined by comprehensive molecular biological analysis (including PCR-DGGE and FISH analysis; Bereschenko et al., 2008, 2010). The $\delta^{13}C$ profile of the *n*-alkanes showed an even-over-odd preference, which reflects an inversed 'saw-tooth' profile to the odd-over-even preference often reported for microbial sources (e.g., Collister et al*.*, 1994; Huang et al*.*, 1995; Grice et al*.*, 1997 & 2001; Chikarashi & Naraoka, 2003; Grice & Brocks, 2011). This data reflects the different biosynthetic pathways of even and odd fatty acid precursors. The relatively low $\delta^{13}C$ values of the odd numbered *n*-alkanes (e.g., *n*-C_{19} = average of -30 ‰), formed by decarboxylation of even carbon numbered fatty acids is consistent with a ^{13}C depleted acetate fatty acid precursor, whereas the notably higher $\delta^{13}C$ value of even numbered *n*-alkanes (e.g., *n*-C_{26} = average of -28.5 ‰), formed by decarboxylation of a C_{27} fatty acid, is more typical of propionate fatty acid precursors from the enzymatic reduction of the relatively ^{13}C enriched carboxyl group of pyruvate (DeNiro & Epstein, 1977; Monson & Hayes, 1980, 1982).

The water soluble and water insoluble fractions of isolated membrane foulant were separated in order to obtain two simple fractions of the complex biofoulant matrix prior to further analyses (to assist in achieving the required degree of baseline separation). In addition, these separate water soluble and insoluble fractions had also been previously molecularly characterised (by FTIR, ^{13}C-NMR and Py-GCMS analysis; Croué et al. (2003a), allowing inference to be made from the isotopic date obtained in the present study. The water insoluble fractions of the foulant contained a relatively high proportion of ^{13}C depleted microbial lipids evident from the relatively low $\delta^{13}C$ (-30 ‰ to -31.5 ‰) of the *n*-C_{16}–*n*-C_{22} alkanes (the higher end of which also exhibited the inverse saw-tooth profile described above), a progressive ^{13}C enrichment with increasing MW (noticeable over *n*-C_{19}–*n*-C_{33}) and ^{13}C-enriched EPS reflected by the relatively heavy $\delta^{13}C$ of the major sugar product levoglucosan (-15.8 ‰). Conversely, the water soluble foulant showed quite ^{13}C enriched *n*-alkanes ($\delta^{13}C$ = -26.7 ‰ to -30.2 ‰) attributed to the dominance of carbohydrate rich and isotopically heavy EPS. However, it is also possible that an unknown

degree of isotopic fractionation may have occurred during the solubility separation step (identified as requiring further investigation).

Protease-based cleaning of the membrane biofoulant samples led to differences in the $\delta^{13}C$ profiles of the *n*-alkanes. LMW *n*-alkanes (<C_{21}) were 1 to 2 ‰ enriched in $\delta^{13}C$ whereas some of the higher MW *n*-alkanes showed lighter $\delta^{13}C$ values, possibly reflecting the removal of an unidentified fraction during cleaning.

The CSIA of HyPy fractions, demonstrated here on the membrane foulants, would be equally applicable to characterisation of the NOM in source waters or related samples (e.g., pipeline biofilms) providing sufficient organic material could be isolated from them.

6.1.4 Case Study of WA Source Waters: - Practical Application of CSIA to the NOM of Source Waters.

Comprehensive characterisation of the HPO fractions of Harding and Bolganup drinking water sources, both from WA and with contrasting catchments, using a suite of contemporary analytical methods (i.e., UV, FTIR and ^{13}C-CPMAS NMR analysis) provided only a small degree of structural distinction. Harding was slightly more aliphatic (attributed to greater algal influence) and Bolganup slightly more aromatic (consistent with high terrestrial forest loads). The higher DOC content of the forest catchment-sourced Bolganup water (DOC_B = 9.8; DOC_H = 3.6) was reflected by the larger signal response of some analyses, including ^{13}C-CPMAS NMR. Bolganup also showed a greater concentration of phenols, but pyrolysis of its HPO fraction showed few of the methoxy phenols typical of the wood/lignin of a heavily forested area. EA-irMS analysis of the HPO fractions gave $\delta^{13}C$ values of -28.1 ‰ for Bolganup and -24.0 ‰ for Harding, consistent with the isotopic variance between the major C3 (Bolganup) and C4 (Harding) plant sources, respectively.

Complementary $\delta^{13}C$ data reflected the presence of allochthonous inputs (e.g., B-HPO -28.1 ‰, typical of C3 source). Stratification in Bolganup Reservoir may contribute to periodic algal blooms, anoxic bottom waters and a complex biogeochemical system (Harris & Antennuci 2009; Garbin et al., 2010). Some of the Bolganup $\delta^{13}C$ PLFA data (e.g., 18:1ω9*t*/18:1ω11*c*; 36.0 9 ‰) was also typical of anaerobic conditions and possibly metal-reducing anaerobic bacteria (many of which are Gram-negative). This molecular and $\delta^{13}C$ PLFA data is typical of a complex microbial community able to exploit an array of organic substrates (including DOC from sediment flux).

The $\delta^{13}C$ PLFA data demonstrates how it can complement conventional water quality and molecular speciation measurements by providing further insight into NOM supported microbiota. However, further improvements in sensitivity will be necessary or larger volume water samples obtained and processed to facilitate meaningful $\delta^{13}C$ PLFA analysis of field samples.

SPME-CSIA yielded even smaller sets of $\delta^{13}C$ data on VOC/SVs. This was largely due to high concentrations of contaminants which overwhelmed the natural abundances of VOCs and SVs. Nevertheless the minimal $\delta^{13}C_{VOC/SV}$ data that was able to be reliably measured did suggest that it can provide information on constituents of aquatic NOM that are not easily characterised using conventional analysis. For example, the present analyses did reveal the $\delta^{13}C$ of thymol detected in both Harding water and the 150 day Marri leachate (Chapter 2) was similarly ^{13}C depleted relative to the bulk NOM (-3.2 ‰ to 3.4 ‰). The consistency of this data suggests an isotopic trend which may recognise a precursor of this analyte common to the field and laboratory samples.

This SPME-CSIA study indicates that to be practically useful to source water characterisation studies, improvements in the overall procedure, particularly a reduction of contamination and increase in analyte concentration (possibly by processing larger water volumes) will be required. The plastic containers widely used to collect and store waters for other types of analysis may not be appropriate for trace level investigations such as multiple SPME injection GC-irMS of RO concentrated waters.

6.2 Suggestions for Further Work

Drinking water sources have traditionally been selected for their pristine nature, and regulation by water utilities strives to protect them from unexpected organic impacts, including contamination by anthropogenic chemicals, which represent a potential health risk. This means NOM levels in source waters are typically low, and on-going regulation and treatment (including disinfection) maintain very low DOC levels and microbial activity in water supplied to consumers. Drinking water sources thus pose significant challenges to organic analysis, and acutely so to assessing the developed CSIA methods of this investigation.

One worthwhile strategy to improve the feasibility of $\delta^{13}C$ analysis of VOC/SVs and PLFA fractions of relatively pristine aquatic samples would be to concentrate samples, particularly the organic analytes of interest, but taking care to avoid concentration of contaminants. New procedures for isolating the organic material from freshwater samples or biofilm should strive to address the sensitivity challenges of CSIA, which presently represent the major obstacle to more detailed data from the practical application of this analysis.

CSIA applications related to SPME isolation of VOCs/SVs and microbial PLFA markers of aquatic (and terrestrial) environments are at an early stage of development, but the potential of these techniques to provide useful information about sources and dynamics (and related impacts of climatic season, rainfall frequency and magnitude, as well as catchment type) of NOM and the symbiotic biota of freshwaters warrants further research and development.

A systematic seasonal sampling regime over a few years, allowing comparisons of PLFAs and their $\delta^{13}C$ signatures to be made before and after such phenomena as stratification or algal blooms would provide more detail on substrate/consumer relationships as well as to assist in determining the effect of physical parameters (e.g., temperature, water flux) on the growth-rate and $\delta^{13}C$ signatures, thereby offering greater insight into microbial community dynamics.

Fundamental development of these relatively new analytical approaches will benefit from further studies of more concentrated representative materials, such as the leachates used in the present study. Less complicated model systems (e.g., algal cultures, and bacterial isolates and associated EPS from organisms of interest, such as of *Sphingomonas*) would provide useful background information to elucidate specific sources and diagenetic pathways to help deconvolute complex isotopic data.

APPENDICES

Appendix A -Multivariate Statistical Analysis

Method

Multivariate statistical analysis (MSA) was performed by Suman George (University of Western Australia) using PRIMER-e software (Plymouth Marine Laboratory, Plymouth, UK), data interpretation and reporting was provided by the author. A data set based on the complete PLFA sample suite (Tables 3.6, 3.10 and 5.9), was established and then analysed by non-metric multidimensional scaling (MDS) to produce a two dimensional representation of statistical PLFA similarity. Similarity matrices of PLFAs associated with each sample location were Log(X+1) transformed. This treatment of the data served to improve interpretability by evening out the contribution of very rare and very dominant PLFAs (Legendre & Legendre, 1998). Cluster analysis was also carried out to create a hierarchy of PFLA clusters in the form of a tree structure dendrogram. The theoretical aspects of these analyses are described in Clarke and Warwick (2001).

A two dimensional plot is created in MDS, within which the distance between samples indicates the similarity of these samples relative to other samples in the plot (Legendre & Legendre, 1998). The accuracy of the two-dimensional representation in MDS is indicated by the stress value. All stress indicators in the present study were <0.1m indicating good reliability (Legendre & Legendre, 1998). Cluster analysis served to confirm the observed groupings of the MDS.

To investigate the significance of differences between groupings, an Analysis of Similarity (ANOSIM) permutation procedure was run using the following parameters: Sample statistic (Global R): 0.343. Significance level of sample statistic: 0.1%. Number of permutations: 999. R-values of 1 indicates separation, 0 indicates no separation. A value of 0.25 was used in the present study as the lower limit of resolvable groupings (Legendre & Legendre, 1998).

Statistical Analysis

General

As described previously, MDS was used to identify patterns among multiple samples that were subjected to PLFA analysis. These samples were:

- 35 distribution system biofilm samples shown in Table 1. These samples were grouped into; winter potable, winter recycled, summer potable, summer recycled.
- 8 vegetation leachate biomass samples (Table2).

- 4 case study PLFA samples from two source waters (described in Chapter 5) which were divided into two groups, corresponding to concentrated and raw water (Table 3).

The intention of the statistical analysis is to enable comparison to be made between the 47 samples extracted from different aquatic environments in order to determine if PLFA analysis could be used to differentiate aquatic microbial community profiles at a statistically significant level.

Table 1: Distribution system biofilm samples

Site & Season (w = winter, s = summer)	**Water Treatment**	**Biofilm ID**
A w	Potable	7, 9
	Recycled	10, 11, 12
B w	Potable	25, 26
	Recycled	28, 29, 30
B s	Potable	128
	Recycled	130, 131, 132
C w	Potable	43, 45
	Recycled	47, 48
C s	Potable	145, 147
	Recycled	150
D w	Potable	62, 63
	Recycled	64, 65, 66
D s	Potable	181, 182, 183
	Recycled	185
E s	Potable	163, 164, 165
	Recycled	166, 168

Table 2: Vegetation leachate samples

Sample	**Control (no inhibitor added, not discussed in thesis)**	**Test Samples (inhibitor added)**
Marri	Mr1	Mr2
Maize	Mz1	Mz2
Sorghum	So1	So2
Spinifex	Sp1	Sp2

Table 3: Case Study samples

Sample	**Harding**	**Bolganup**
raw	HD1	Bo1
concentrated	HD2	Bo2

Application

The results of the MDS analysis is a two dimensional plot (Figure 1) where objects are individual samples and variables are PLFA concentration and distribution. The distance between samples indicates the similarity of these samples relative to other samples in the plot (Legendre & Legendre, 1998). The accuracy of the two-dimensional representation in MDS is indicated by the stress value. A stress value of <0.1 indicates a good ordination (Legendre & Legendre, 1998). All stress indicators in the present study were <0.1, therefore unlikely to provide misleading information. Cluster analysis (Figure 2) served to confirm the observed groupings (Legendre & Legendre, 1998) and also assist in visualising data. Following ordination, Analysis of Similarity (ANOSIM) was performed to confirm significance of similarity/dissimilarity between groupings.

MDS Analysis

Overview of Groupings

Figure 1 shows that ten groupings of distance '11.68' (grouped in green) were determined and within the largest of these were three further groupings of distance '2' (grouped in red). The largest grouping contained 36 of the samples, showing statistically, that the PLFAs of these 36 samples were relatively similar. 32 samples in this group were distribution system biofilms.

Within this largest grouping were BFs 29, 64, 65 and 66, all of which were winter, recycled biofilms, and in addition, there was a tightly clustered grouping of all the remaining distribution system biofilm samples together with the abiotic leachate biomass. The abiotic vegetation leachate samples contained only trace amounts of the undiagnostic PLFAs 16:0 and 18:0 and functioned as a control in this dataset. This could be from dead biomass.

The recycled biofilm pair BF47 (winter) and BF185 (summer) grouped together, although there was some overlap with BF47 and the 'control' Maize and 'control' Sorghum leachate biomass. All four case study samples, the 'control' Spinifex, 'control' Marri and BF150 (summer, recycled) fell into seven separate and distinct distance classes.

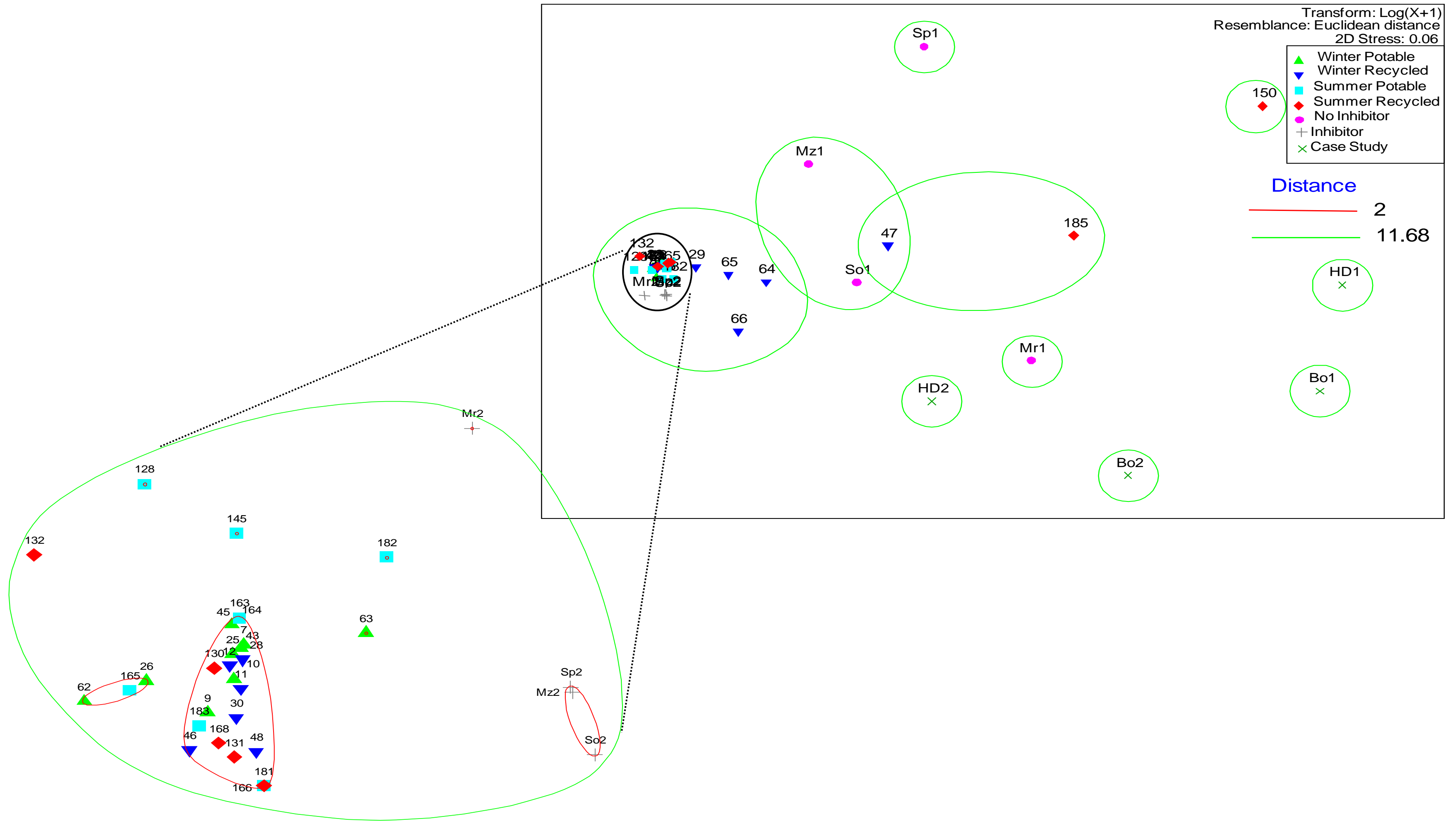


Figure 1 MDS analysis whole PLFA analytical suite. Resemblance based on Euclidean distance. 2D stress = 0.06. The 2D arrangement represents relative differences in similarity.

Distribution System PLFAs

For the distribution system pipeline PLFAs, the parameters used were relative abundance of PLFA concentration (m/z 74), PLFA and sample site. The majority of the biofilm samples showed a closely clustered grouping (labelled A), consistent with the community profiles discussed in Section 3.4.3.2.

The PLFA distributions of BFs 185, 150 and 47 grouped very differently to the rest of the biofilms, reflecting their very different community profiles and higher overall concentrations. These samples also had the highest PLFA concentrations. The tight clustering of all other biofilms may be due to the lower PLFA concentrations of these samples.

BFs 62, 63, 64, 65 and 66 showed considerable variance. These samples were taken during winter from potable (62 and 63) and recycled (64, 65 and 66) systems of site D. Variance was also shown between summer site D potable biofilms (181, 182) which may reflect the gradient of chlorine residual, as these were taken at different lengths along a pipeline (Storey & Kaucner, 2009). The winter recycled sample from site B (29) had a higher total PLFA concentration than the other BFs from site B, more comparable to the PLFA concentrations detected in winter site D BF samples (refer to Table 3.10). This offers a possible explanation as to why it grouped differently from the rest of the site B samples.

The effect of concentration could be further investigated by conducting a serial dilution test with the concentrated PLFA extracts of BFs 47, 150 and 185, however limited sample size prevented this additional analysis. This question of concentration is addressed by including the rich PLFA distributions of the vegetation leachates and of the PLFAs of biomass isolated from natural reservoirs (i.e., the case studies samples Chapter 5) in statistical comparison with the distribution system.

The statistical analysis suggests that high concentrations of PLFAs are required for reliable distinction on the basis of PLFA distribution. Within the group of three high PLFA concentrated samples, BF47 was the most different. Being the only winter sample this variance probably reflects the effect of season on quality or composition of the organic substrate supporting biofilm development.

Vegetation Leachate PLFAs

The vegetation leachate samples, which function as controls, are shown to group closely with potable drinking water samples, although the former are in a distinct cluster of their own. The vegetation leachate samples contained only trace concentrations of non-diagnostic PLFAs (described in Section 3.4.1.2). This was also the case for many of the potable samples, particularly from tertiary treated schemes (site A and B).

Case Study PLFAs

The case study samples comprise PLFAs extracted from natural reservoir systems (discussed in Chapter 5). Their presence in the dendogram shows that different microbial communities growing in different environmental conditions (vegetation leachate biomass, concentrated reservoir waters and distribution system biofilms) can be differentiated descriptively using PLFA analysis.

Cluster Analysis

The dendrogram shown in Figure 2 shows the clustering of specific groups. The further away samples are from each other, the less similar the two PLFA profiles are (Legendre & Legendre, 1998). As for Figure 1, this is a descriptive assessment. Interestingly, many of the samples which cluster together show some relationship towards their associated water temperature (discussed in Section 3.4.4.1). For example BFs 25, 7, 43, 28, 12, 10 and 11 cluster next to each other and all have associated water temperatures of <20 °C. Whereas 181, 166, 46, 183 and 168 (no water temperature data was available for BFs 30 and 9) were all associated with water temperatures mainly above or in two cases BFs 131 and 46) equal to 20 °C. This illustrates the importance of the 20°C recommendation of the ADWG (NHMRC, 2006) which was highlighted in Section 3.4.4.1.

The case study samples comprise PLFAs extracted from natural reservoir systems (discussed in Chapter 5). Their presence in the dendogram shows that different microbial communities growing in different environmental conditions (vegetation leachate biomass, concentrated reservoir waters and distribution system biofilms) can be differentiated descriptively using PLFA analysis.

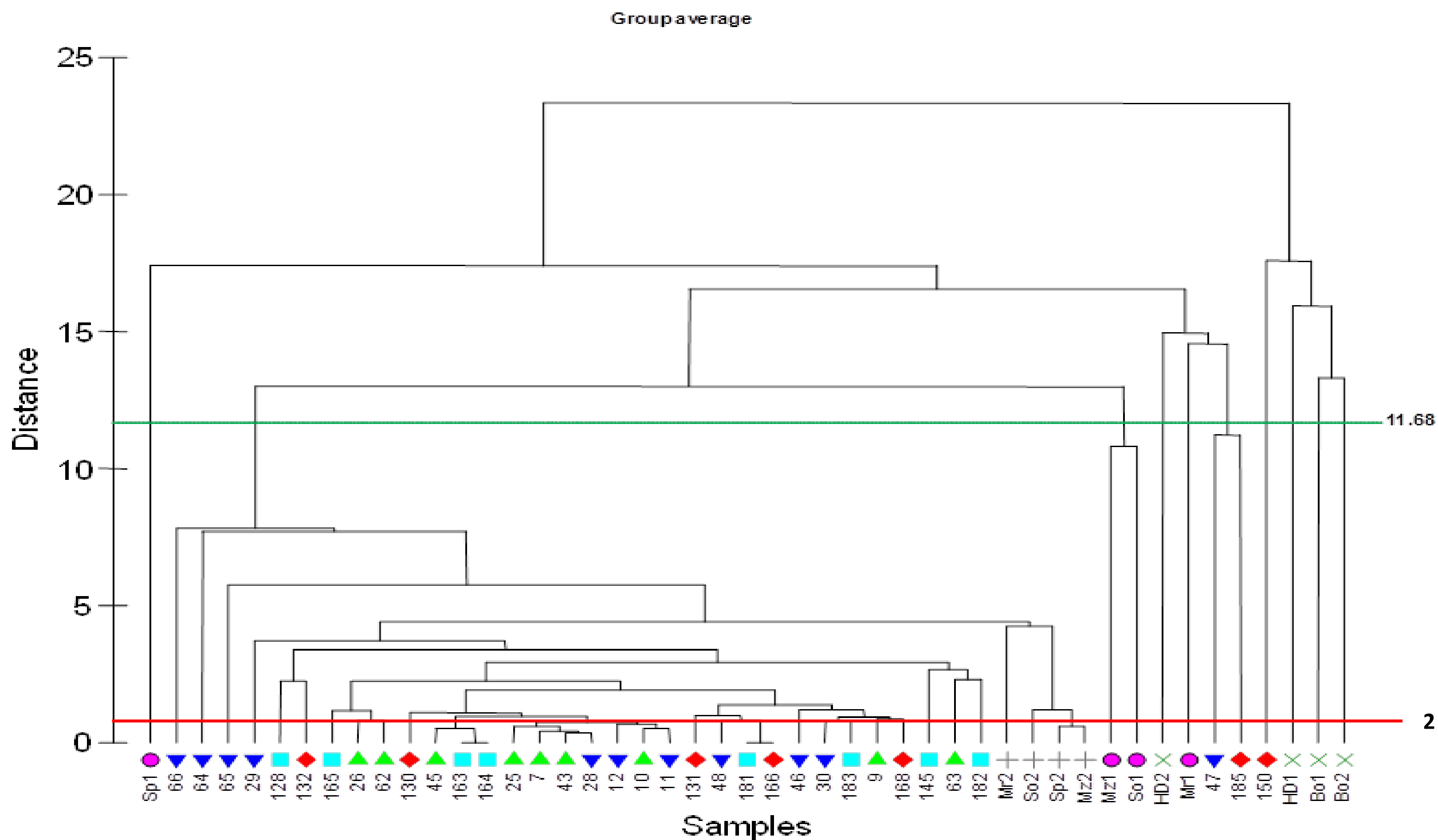


Figure 2 Hierarchical cluster analysis of PLFA profiles in whole PLFA analytical suite. Log (X+1) transformed PLFA profiles (% of total PLFA content). Resemblance based on Euclidean distance.

Green triangle = winter potable **Blue inverted triangle = winter recycled**

Red diamond = summer recycled **Turquoise square = summer potable**

Pink circle = vegetation leachate with no microbial inhibition (Mz1 = Maize, Mr1 = Marri, Sp1 = Spinifex, So1 = Sorghum)

Grey + = vegetation leachate with microbial inhibition (Mz2 = Maize, Mr2 = Marri, Sp2 = Spinifex, So2 = Sorghum)

Green X = case study PLFAs (refer to Chapter 5 for case study details)

Inferential Analysis

Figure 3 summarises the findings of the ANOSIM analysis performed on the entire PLFA data set.

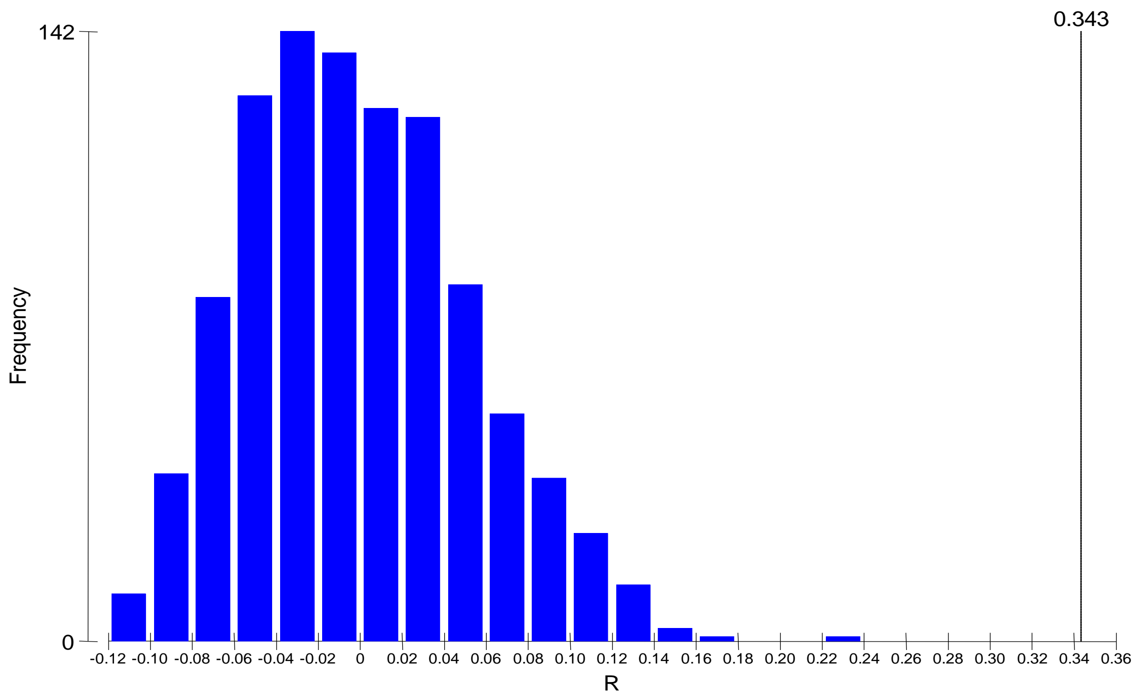


Figure 3 ANOSIM showing significance level of entire suite of PLFA analyses

The vast majority of differences between groupings were not statistically significant as their distribution frequency was highly concentrated on R-values well below the delineating R= 0.25. This suggests the PLFA concentrations were too low to facilitate statistical resolution. All samples showed a high level of variance, reflecting the different PLFA profiles associated with different types of vegetation biomass.

Permutation Test using ANOSIMS

Permutation testing was conducted to investigate significant differences between test groups. The ranks of distance between groups were compared with the ranks of distances within groups. The resultant R-test statistic and associated p significance levels (Table 4) measures whether complete separation is found (R=1) or no separation occurs at all (R=0). R values <0.25 are deemed as barely separable (Clark & Gorely, 2001). Significance levels (p) were calculated when R was >0.25. Levels of $p \le 0.05$ denote statistical significance (Fisher 1925).

Table 4 ANOSIMS Pairwise tests showing differences between distribution system groupings. Only pair tests with R >0.25 are shown below. Significance levels = *p < 0.05, **p < 0.01, **p < 0.001, $p \leq 0.05$ denotes statistical significance (Fisher 1925).

Groups Statistic	R
Winter Potable, Summer Recycled	0.264**
Winter Potable, Leachate Biomass	0.927**
Summer Potable, Leachate Biomass	0.814**
Winter Potable, Case Study	0.964**
Winter Recycled, Case Study	0.949***
Summer Potable, Case Study	0.954**
Summer Recycled, Case Study	0.479*
Leachate Biomass, Case Study	0.885*

Table 4 shows that of the distribution system PLFAs, only the winter potable and summer recycled groupings were statistically resolved. With the exception of one grouping, which was statistically different (p=<0.01), the sensitivity of the PLFA analysis was too low to facilitate reliable statistical delineation between the majority of the distribution system PLFAs.

Potable distribution system PLFA groups from summer and winter samples could be distinguished with statistical confidence from the vegetation leachate biomass tests. This reflects the very different PLFA profiles associated with the two different aquatic environments. The recycled distribution system PLFAs showed less of a distinction. Surprisingly, the summer recycled PLFAs and the vegetation leachate PLFAs were not significantly different. This shows that the technique is limited by sensitivity issues.

Case study reservoir PLFAs, were statistically different from winter potable and recycled distribution system groups, but only the summer potable group could be clearly distinguished. The summer recycled group did not show a statistical difference from the Case Study. The relative similarity in these groups could relate to the algal bloom DOC source discussed in Sections 3.4.5 and 5.4.3.3.

The leachate vegetation biomass groups could also be distinguished from the case study reservoir PLFAs with some statistical significance, however the differences were less distinct than observed for the distribution system groups. This finding is consistent with the relative similarity of these samples groups compared to a pipeline system. The vegetation leachate systems were designed to emulate a reservoir receiving a simplified allochthonous DOC input. However, the fact that these were PLFAs were statistically

different, reflects the inherent differences in the microbial communities between a simple 'model' and a complex real aquatic environment.

The addition of $\delta^{13}C$ data was proposed to increase the correlation capacity of PLFA data. However, statistical analysis of the concentration and $\delta^{13}C$ values (results not shown) showed a very similar level of correlation to that obtained for just the PLFA concentration data, probably as a result of the limited number of $\delta^{13}C$ values obtained.

Appendix B -ANOVA Statistical Analysis

Method

Analysis of Variance (ANOVA) was performed by the author using IBM® SPSS® Statistics 21 statistical software. Four separate ANOVA analyses were run on the PLFA data (from Table 3.6, Table 3.10 and Table 5.9) in order to investigate the statistical significance of differences in PLFA concentrations and distribution between and within sample groupings. Firstly one-way ANOVA was run on the PLFA data from vegetation biomass (Table 3.6). The *p* values for both total concentration and distribution were significantly >0.05 indicating that no significant differences were observed between the PLFAs extracted different plant species, qualifying the findings of the same sample group in Appendix A.

A two-way ANOVA analyses was run on the PLFA data from distribution system biofilm PLFA (Table 3.10). The parameters and results are shown below; values of interest are highlighted in yellow.

Tests of Between-Subjects Effects

Dependent PLFA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	45240684.752[a]	5	9048136.950	35088.092	.000
Intercept	20895311.094	1	20895311.094	81030.669	.000
Season	16825027.042	1	16825027.042	65246.370	.000
Treatment	20260137.682	2	10130068.841	39283.754	.000
Season * Treatment	18654182.987	2	9327091.493	36169.859	.000
Error	1547.215	6	257.869		
Total	53507704.020	12			
Corrected Total	45242231.967	11			

a. R Squared = 1.000 (Adjusted R Squared = 1.000)

These values show that both season and treatment and a combination of both all had a statistically significant effect on PLFA concentration. The two-way ANOVA offered a simple and clear analysis of the distribution system biofilm samples, obscured in some ways by the detail of the sample set provided in Appendix A.

The third set of the analyses was conducted on the PLFA data from the PLFAs extracted from the case study waters (Table 5.9). As was observed for the vegetation biomass samples, the *p* values obtained were both > 0.05 therefore any distinction between groupings was of not reliably statistically significant. Finally, all the data was run together in a one way ANOVA investigating PLFA groupings and total concentration, again *p* values were >0.05 therefore no statistically significant differences between the entire groupings were observed greater than the variation within the groups themselves.

Appendix C - Permissions

- **Figure 1.6 (Hayes, 2001)**

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- **Figure 1.5 (Brugnoli & Farquhar, 2000).**

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- **Figure 2.7 (Edwards et al., 2011).**

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- **Figure 3.2 (Madigan et al., 2003).**

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