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Civil Engineering and Geosciences

The Role of Bacteriohopanepolyols as Biomarkers for Soil Bacterial Communities and Soil Derived Organic Matter

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Declaration

The content of this thesis is solely my responsibility and the original work herein is my own, except where specified otherwise in the text. Neither the thesis nor any of the original work comprising it has been submitted to this or any other institution for consideration for a higher degree.

Signed…….

Date...……..

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Abstract

Bacteriohopanepolyols (BHPs) are a group of membrane lipids produced by a wide range of bacteria which have a high degree of structural specificity relative to bacterial source. This is the widest study to date into the BHP content of soils from around the world and investigates their use as biomarkers in modern soils and modern and ancient sediments using reversed phase HPLC-MS^n. A total of 46 different BHP structures were observed during the study including the tentative identification of 11 novel BHP structures.

Analysis of the BHP content in a series of soils from North East England, South West England, Canada, Sweden, Vietnam and Southern Spain has shown that soil BHP distributions are dominated by up to 5 different BHPs: bacteriohopane-32,33,34,35-tetrol (BHT), 35-aminobacteriohopane – 32,33,34-triol (aminotriol), 30-(5'-adenosyl)hopane (adenosylhopane), bacteriohopanetetrol carbopseudopentose ether (BHT cyclitol ether) and, less frequently, adenosylhopane type-1, although generally more than 20 different BHPs are identified in each soil sample.

Soil bacterial population dynamics were investigated using existing knowledge of the relationship between BHPs and their specific bacterial sources and has demonstrated, for example, a decrease in the concentration of cyanobacteria related BHPs with soil depth in pasture (Palace Leas) and woodland (Hack Hall farm) environments confirming the validity of these BHP – bacteria relationships to infer BHP producing bacterial activity. Principle Component Analysis of the different bacterial types, identified by source BHPs, in the different soils can clearly identify variations between the soils with, for example, the influence of methanotroph and cyanobacteria derived BHPs having significant and opposite effects on the separation of the soils. This potentially enables BHPs to be used to identify source locations or environments.

Investigation of seasonal variations at two sites in NE England (Palace Leas and Hack Hall Farm) has shown that soil BHP concentrations increase during the summer months and decline during winter with no net accumulation indicating that the aerobic conditions observed in near surface soils result in the rapid diagenesis or metabolism of BHPs.

An investigation of estuary sediments from the Congo Fan, River Rhone and 7 arctic rivers has shown that this rapid degradation of these highly functionalized structures does not occur

in anaerobic marine sediments with the aerobic methane oxidizing bacteria biomarker, 35-aminobacteriohopane-30,31,32,33,34-pentol (aminopentol) and other complex BHPs being identified in Congo Fan sediments up to 1 million years old (100 metres below sea floor), thus enabling BHPs to be used as biomarkers in palaeo-environments.

The estuary and fan sediments also contained a suite of BHPs that are ubiquitous in soil but absent in many lacustrine and open marine environments where their concentration decreases proportionally with distance from land. These BHPs; adenosylhopane, adenosylhopane type-1 and their C-2 methylated homologues can therefore be used as markers for the transport of soil derived organic material (SOM) from terrestrial to marine environments.

These results have opened up a wide range of opportunities for BHPs to be used as bacterial proxies in a variety of environments and ages, accessing a wide range of different proxies for different bacterial populations and processes from a single analysis.

Publication History

As a direct result of this research the following papers have been published:

Cooke, M. P., Talbot, H. M., Wagner, T., 2008a. Tracking soil organic carbon transport to continental margin sediments using soil-specific hopanoid biomarkers: a case study from the Congo fan (ODP site 1075). Organic Geochemistry 39, 965-971.

Cooke, M.P., Talbot, H.M., Farrimond, P., 2008b. Bacterial populations recorded in bacteriohopanepolyol distributions in soils from Northern England. Organic Geochemistry 39, 1347-1358

Cooke, M.P., van Dongen, B.E., Talbot, H.M., Semiletov, I., Shakhova, N., Guo, L., Gustafsson, O., 2009. Bacteriohopanepolyol biomarker composition of organic matter exported to the Arctic Ocean by 7 of the major Arctic rivers. Organic Geochemistry 40, 1151-1159.

Redshaw, C. H., Cooke, M. P., Talbot, H. M., McGrath, S., Rowland, S. J., 2008. Low biodegradability of fluoxetine HCl, diazepam and their human metabolites in sewage sludge-amended soil. Journal of Soils and Sediments 8, 217-230.

Xu, Y. P., Cooke, M.P., Talbot, H.M., Simpson, M.J., 2009. Bacteriohopanepolyol signatures of bacterial populations in Western Canadian soils. Organic Geochemistry 40, 79-86.

The following papers were published as a result of research inspired by this thesis

Handley L., Talbot, H.M., Cooke, M.P., Anderson, K.E., Wagner, T., 2010. Bacteriohopanepolyols as tracers for continental and marine organic matter supply and phases of enhanced nitrogen cycling on the late Quaternary Congo deep sea fan. Organic Geochemistry 41, 910-914.

Rethemeyer, J., Schubotz, F., Talbot, H.M., Cooke, M.P., Hinrichs, K-U., Mollenhauer, G., 2010. Distribution of polar membrane lipids in permafrost soils and sediments of a small high Arctic catchment. Organic Geochemistry, 41, 1130-1145.

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4. Hack Hall Farm Case Study

Abbreviations used in text.

Abbreviation	Description
Ac, Acet	Acetyl, CH_3COOH
APCI-LC/MS^n	atmospheric pressure chemical ionisation liquid chromatography / multi-stage ion trap mass spectrometry
ave	average
BIT index	ratio of branched/isoprenoid tetraethers
BHPs	Bacteriohopanepolyols
°C	degrees celcius
CLPP	community level physiological profiling
cm	centimetres
cmy^{-1}	centimetres per year
C_{mic}:C_{org}	Ratio of microbial C to organic C
CR	Chicken Road Landfill site
Da	Daltons
DNA	Deoxyribonucleic acid
HPLC-MS^n	High Performance Liquid Chromatography – Mass Spectrometry
g	gram
GC-MS	Gas Chromatography – Mass Spectrometry
GRARs	Great Russian Arctic Rivers
h	hour
HDPE	high density polyethylene
km	kilometres
$kg\ ha^{-1}$	kilograms per hectare
m	metres
Ma	Million years ago
Me	Methyl
MeOH	methanol
ml	millilitres
m/z	mass per unit charge
mM	millimolar
min	minute
$\mu g_{BHP}\ g^{-1}$dry soil	micrograms of BHP per gram of dry soil
µg/g dry sediment	Micro grams BHP per gram dry sediment
$\mu g_{BHP}\ g^{-1}{}_{TOC}$	micrograms of BHP per gram of Total Organic Carbon
NMR	Nuclear Magnetic Resonance
N-S	North to South
NE England	North East England
OC	Octavia Court Landfill site
ODP	Ocean Drilling Program
OM	Organic matter
PLFAs	Phospholipid fatty acids
PCR	Polymerase chain reaction
PLFAs	Phospholipid fatty acids
PCA	Principle component analysis

Abbreviation	Description
PNSB	Purple Non-Sulphur Bacteria
PC	Principle Component in PCA
PCDDs	polychlorinated dibenzo-*p*-dioxins
PAHs	polycyclic aromatic hydrocarbons
pmoA	methane mono-oxygenase
Q	glutamine
QW motifs	Chain of amino acids in squalene hopene cyclase beginning with glutamine (Q) and ending in tryptophan (W)
qCO_2	rate of carbon dioxide respiration per unit biomass
rbf	round bottom flask
RNA	Ribonucleic acid
rpm	revolutions per minute
RSD	relative standard deviation
S	Seghill landfill site
Shc	squalene hopene cyclise
SOM	Soil Organic Matter
SOC	Soil Organic Carbon
TLE	total lipid extract
TOC	Total Organic Carbon
TOM	terrestrially derived organic matter
[TOC/TN]	total organic carbon/total nitrogen
v/v	volume/volume
V_{max}	maximum rate of glucose induced respiration
W	Ttryptophan
WA	West allotment landfill site

1. Introduction

The aim of this thesis is to assess the potential of bacteriohopanepolyols (BHPs) to act as biomarkers for bacterial activity of specific environmental conditions in soils and sediments.

The role of a biomarker is to act as a proxy for a biological process that cannot be measured directly in the environment. The biomarker must be specific and have a known biological fate that can be related directly to a biological process or an environmental condition. For example, alkenones, long chain ketones produced by prymnesiophycean algae, are used as a marker for sea surface temperature where an increase in unsaturation of the alkenone relates directly to a decrease in sea surface temperature (Brassell et al., 1986). The recalcitrance of the alkenones makes it possible to study the sea surface temperature in the past, a process that could not be undertaken previously.

A further set of biomarkers are long chain *n*-alkanes which are derived from the surface waxes of terrestrial higher plant leaves and can be leached from leaves by rain or eroded with soils and subsequently transported by rivers to the coastal marine environment (Eglinton and Hamilton, 1963) or transported as dust by prevailing winds to marine environments (e.g. Gagosian et al., 1981; Poynter et al., 1989) where they are preserved.

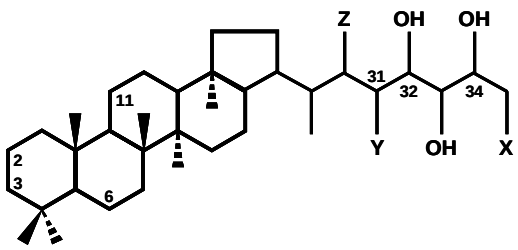
In soils less than 1% of the 10^9 bacteria per gram of soil are able to be cultured (Davis et al., 2005) and therefore other techniques have to be used to measure the characteriatics of the soils microbial community. Phospholipid fatty acids (PLFAs) are used to measure rapid changes in soil microbial populations (Zelles, 1999). PLFAs have a labile structure that results in a rapid turnover in environmental conditions outside dead cells, with up to 50% of PLFAs being degraded or transformed in the first 8 days after cellular death (Tollefson and McKercher 1983). O'Donnell et al., (2001) compared over 50 different investigations and found that the measurement of rapid changes in soil microbial populations using PLFA measurement was at least as good as microbial methods such as polymerase chain reaction (PCR) and community level physiological profiling (CLPP) and in many cases were able to detect changes that these methods could not. However PLFAs are not recalcitrant in soils and therefore can only provide information about the current soil bacterial population.

Alternatively, molecular techniques that use specific markers for bacterial DNA and RNA can be used to measure the soil microbial activity (Nannipieri et al., 2000, Saleh-Lakha et al., 2005). These methods again use a labile marker that can only give information on the current microbial activity. A final method to measure soil microbial activity is respiration analysis that measures the bacterial response to a variety of substrates (Degens and Harris, 1997, Schipper et al., 2001). Once again iot is a measure of the current activity.

In these cases a specific biological source is used to measure environmental conditions or processes and provide useful insights into current or ancient biological processes in Earth history. There are hundreds of different biomarkers in the organic geochemist's toolbox (Peters et al., 2005) and the ability to identify a biomarker and relate it to specific environmental conditions is the key task of an organic geochemist.

This research will investigate the usefulness of BHPs as biomarkers in modern and ancient environments. BHPs are pentacyclic triterpenoids, with a 5-carbon side chain (Fig. 1.1), biosynthesised by many prokaryotes as membrane components (Ourisson and Rohmer, 1982; Rohmer et al., 1984; Ourisson et al., 1987). Most BHPs have four, five or six functional groups on the side chain (Tetra-, Penta- and Hexa-functionalised BHPs) with the functional groups being dominated by up to 6 hydroxyl groups, although the tetra-functionalised form is the most common. These hydroxyl groups can be substituted, at the terminal C-35 carbon (Fig. 1.1) by other functional groups, predominantly amine or composite groups. The composite groups typically comprise sugar or nucleoside derivatives (Fig. 1.2). In addition to the wide variety of function groups variations also occur in the triterpenoid ring system with methylation being observed at either C-2, C-3 or C-12 (Fig 1.2) (Bisseret et al., 1985, Cvejic et al., 2000b, Costantino et al., 2000 respectively) and unsaturation being observed at C-6 or C-11 (Ourisson and Rohmer 1992). More than 100 different BHP structures have been identified to date. BHPs are also the precursors of the more widely recognised and ubiquitous geohopanoids, e.g. hopanoic acids, hopenes, hopanes and hopanols (Ourisson and Albrecht, 1992).

A wide range of different bacteria have been shown to produce BHPs (e.g. Rohmer at al., 1984; Farrimond et al., 1998 and see section 1.3) with the alpha- beta- and gammaproteobacteria and cyanobacteria being the most prolific sources although not all species tested in each group produce hopanoids. The size of the BHP producing



Tetrafunctionalised
Z=H, Y=H, X=OH or NH_2 or composite group

Pentafunctionalised
Z=H, Y=OH, X=OH or NH_2 or cyclitol ether
Z=OH, Y=H, X=OH

Hexafunctionalised
Z=OH, Y=OH, X=NH_2

Side chain variants include a methyl group at C-31, a ketone group at C-32, -$OCONH_2$ at C-34, and structures with the side chain condensed into a cyclic ether form.
Variants in the ring system include methylation at either C-2 or C3, and a double bond at C-6 and/or C-11.

Fig.1.1. Generalised structure of BHPs.

bacteria population varies between 50% of all bacteria (Rohmer et al., 1984 – based on extraction of BHPs from pure bacterial cultures) and 5% (Pearson et al., 2007 – based on genomic analysis). A wide variety of these bacteria have been shown to only produce a few specific BHPs (e.g. only methanotrophs produce aminopentol, **1c**, Neunlist and Rohmer, 1985b; Fig. 1.2) whereas some BHPs are produced by many bacteria (e.g. BHT, 1a, is the most commonly observed BHP in bacteria, Renoux and Rohmer, 1985; Rohmer 1988; Neunlist et al., 1988; Rohmer, 1993 and others).

This research represents the largest study ever carried out into the occurrence of (BHPs) in soils and sediments to identify the variety, concentration and recalcitrance as intact structures of BHPs in the terrestrial environment and to understand the capability of BHPs to describe the activity of BHP producing bacteria at each location. It has involved the production of a BHP profile of each sample which describes the concentration and occurrence of a wide range of BHP structures using solvent extraction techniques and subsequent analysis of the total lipid extract (TLE) using high performance liquid chromatography – mass spectrometry (HPLC-MS^n).

Soils were collected and analysed from a wide range of global locations; North East England, The Lake District (North West England), South East England and South Wales, Canada (Alberta), Sweden, Southern Spain and North Vietnam. In addition to their location the soils were selected for their specific environmental setting; fertilised pasture (Palace Leas, NE England), woodland (Hack Hall Farm, NE England), landfill cover soils (West Allotment, Chicken Road, Octavia Court and Seghill, NE England), peat (Priest Pot, The Lake District), forest fire damaged soils (Seville, Southern Spain), rice paddy fields (North Vietnam). Sediments were collected from the estuaries of 7 Arctic rivers (Russia and Alaska), the Rhone delta and from the Congo

fan (West Africa). This wide range of locations and environments has enabled the comparison of BHP profiles on the global scale.

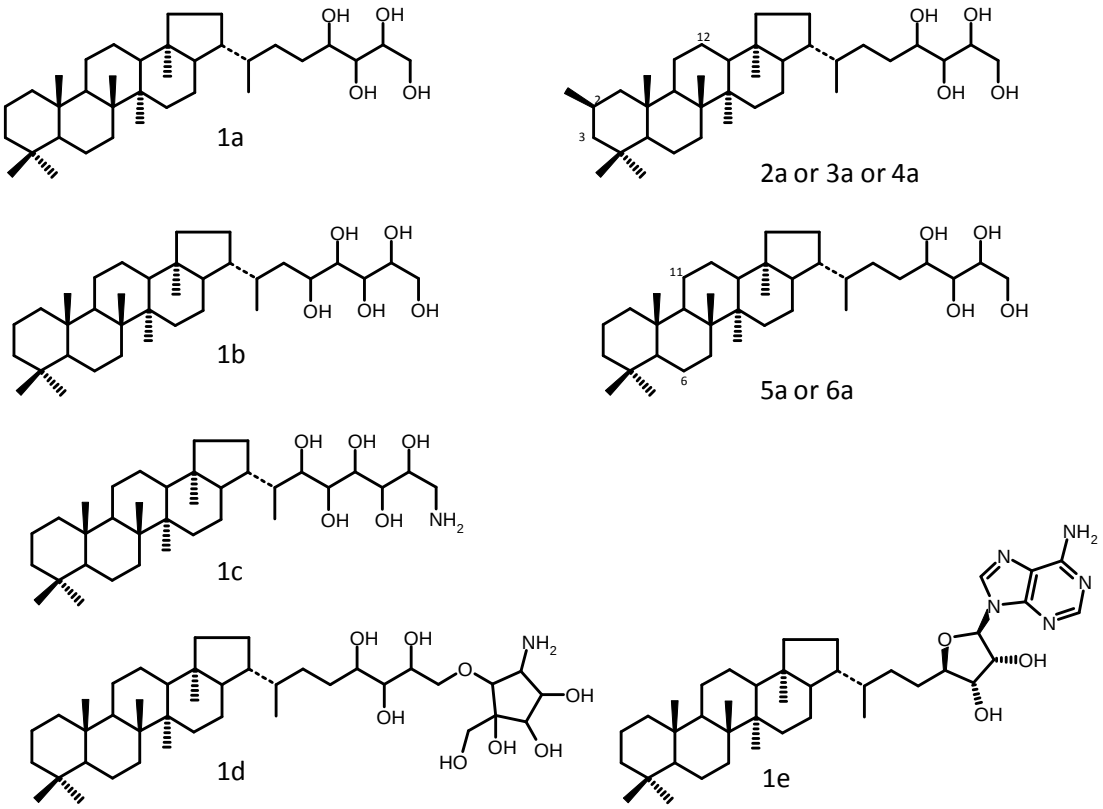


Fig. 1.2 Examples of variety of BHP structures

1a = bacteriohopane-32,33,34,35-tetrol (BHT), (Langworthy 1976).

1b = bacteriohopane-31,31,33,34,35-pentol (BHpentol), (Zhao et al., 1996).

1c = 35-amino-bacteriohopane-30,31,32,33,34-pentol (aminopentol), (Neunlist and Rohmer, 1985b).

1d = bacteriohopanetetrol carbopseudopentose ether (BHT cyclitol ether) (, (Cvejic et al., 2000a).

2a = 2-methyl bacteriohopane-32,33,34,35-tetrol (2me BHT), Bisseret et al., 1985). Methylation can also occur at C-3 (**3a**) (Cvejic et al., 2000b), or at C-12 (**4a**) (Costantino et al., 2000)

5a = Δ^6 bacteriohopane-32,33,34,35-tetrol (unsaturated BHT) (Ourisson and Rohmer 1992). Unsaturation can also occur at Δ^{11} (6a), (Talbot et al., 2007b)

1e = 30-(5'-adenosyl)hopane (adenosylhopane) (Neunlist et al., 1988)

It is the variety of BHPs, the range of bacteria and the specificity of certain bacteria to produce specific BHPs that potentially enables BHPs to be used as biomarkers for bacterial populations. However before being able to use BHPs as biomarkers it is important to fully understand their formation, role and fate in bacteria and the environment.

1.1 Formation of Bacteriohopanepolyols in Prokaryotes

All bacteria are surrounded by a membrane comprised of a predominantly phospholipid bilayer (Singer and Nicolson, 1972). The membrane is essential to the life of the cell and is not a rigid or strong structure due to the fluidity of the aliphatic tails on the lipids (Fig 1.3).

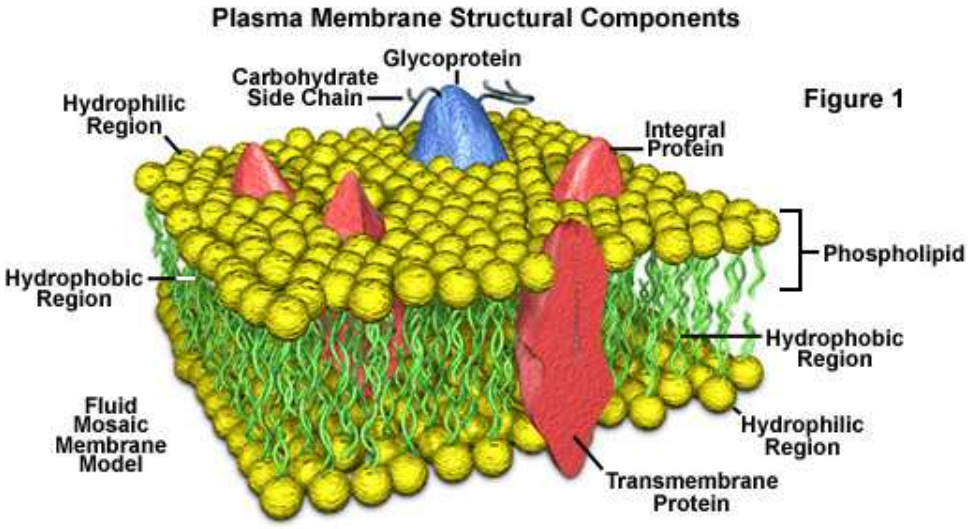


Fig. 1.3.The cell membrane. From http://imcurious.wikispaces.com/Midterm+Exam+2010+Review+P2

To maintain membrane integrity it can be strengthened by numerous methods. The simplest method is the unsaturation of the hydrophobic tail groups. This adds a rigid bond to the tail increasing the fluidity of the lipid by reducing the van der Waals forces in the bilayer. This maintains the fluidity of the membrane a relatively low temperatures. The reverse is also true with increased saturation increasing stability at higher temperatures. The second method is the addition of molecules to the membrane with a polar head group and a rigid hydrophobic tail. These act as anchors or barriers to free movement, in the membrane reducing the fluidity and increasing the stability of the membrane. The greater the concentration of rigid molecules in the membrane the greater the stability of the membrane. However an appropriate balance must be struck between the phospholipids and the stabilisers to enable the membrane to fulfil its other functions. In eukaryotes these rigid molecules are sterols, in many prokaryotes this function is believed to be carried out by bacteriohopanepolyols (e.g. Poralla et al., 1980; Kannenberg and Poralla, 1999; Saenz in press).

In BHPs the C_{30} triterpenoid isoprenoids with a distinctive aliphatic 5-ring system act as the hydrophobic end of the molecule and the polyfunctionalised polar C_5 side chain fulfils the same role of the polar head group in phospholipids in providing the orientation of the hopanoid within the membrane. The ring system is rigid and planar providing the stability to the membrane.

Bacteriohopanepolyols are formed from squalene (e.g. Woodward and Bloch 1953; Wendt et al., 1997; Kannenberg and Poralla 1999; Wendt et al., 2000; Rajamani and Gao 2003; Reinert et al., 2004; Poralla 2004; Hoshino et al., 2004) in a reaction catalysed by squalene hopene cyclise (*Shc*).

The cyclisation of squalene to hopene is the most complex single enzyme catalysed reaction known (Fig 1.4) and has been widely studied in *Bacillus acidocaldarius*, a thermophillic bacterium easily cultured at 60^{o}C, where its structure has been identified (e.g. Wendt et al., 1997 and 2000).

The reaction pathway is highly exothermic releasing 40-50 kcal per mol (Poralla 2004) which is well in excess of the usual protein stabilisation energy of 5-15 kcal per mol. It would be expected therefore that the reaction could not proceed as the protein would simply denature. Poralla et al (1994) identified a series of 7 to 8 repeating aromatic rich, alpha helical, QW motifs beginning with glutamine (Q) and ending in tryptophan (W) that appear to tighten the protein structure due to aromatic interactions that link the helices in the protein together, greatly increasing the protein stability. This prevents the protein from denaturing and enables the reaction to continue. The high energy is thought to partially melt the internal structure of the protein giving a more fluid centre enabling the free movement of the large substrate molecule through the active centre.

The final stage of the reaction is the deprotonation of the carbocation produced in the reaction by water molecules which produces either diploptene (80%), simple deprotonation, or diplopterol (20%) from hydration of the carbocation (Rajamani and Gao 2003).

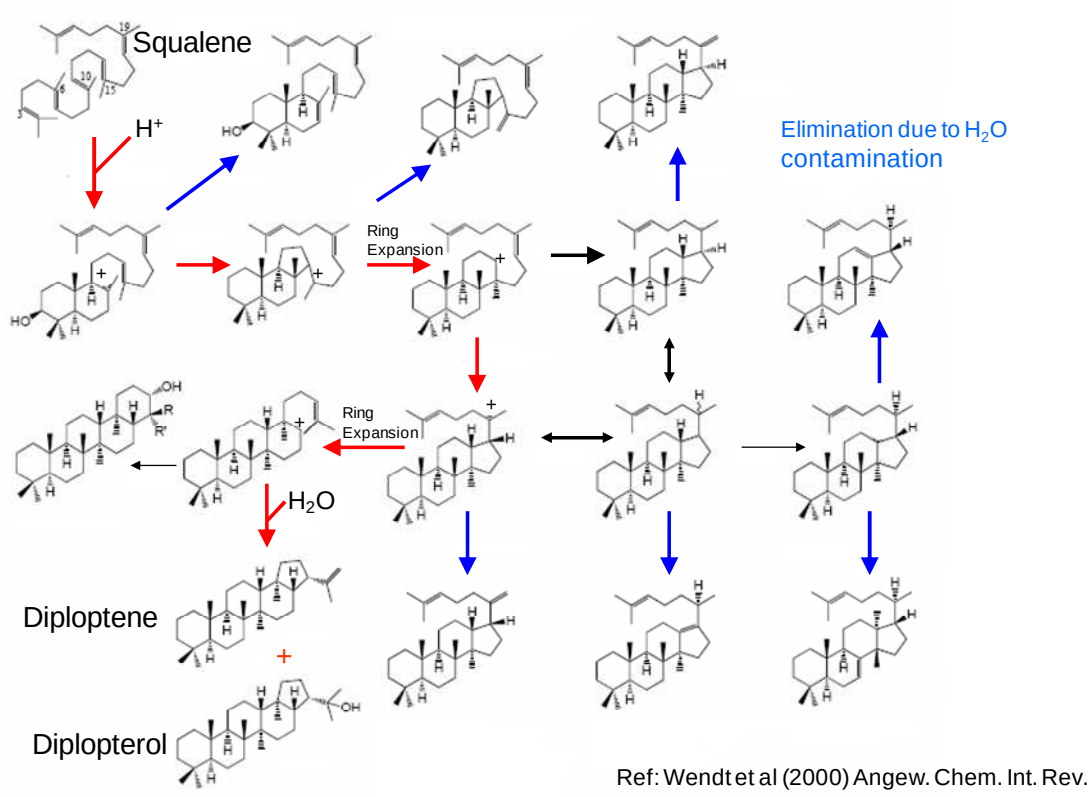


Fig. 1.4 Cyclisation of squalene to form diplotene and diploterol. Adapted from Wendt et al., 2000.

During the investigation into the structure and function of squalene – hopene cyclase more than 25,000 associated terpenoids have been identified (Reinert et al., 2004). This has lead Poralla (2004) to suggest that more than one cyclase is involved in the reaction series. However three factors lead me to believe that this is not the case. The first is the high degree of complexity involved in a highly energetic reaction. This must make the pathway liable to failure due to the production of non standard terpenoids which abruptly stop the pathway and lead to the elimination of the defective molecule from the enzyme where they will be quenched (Wendt et al., 2000). Secondly the reaction is readily quenched by water and any ingress of water into the active centre of the enzyme is liable to produce further non standard products. Finally Douka et al., (2001) identified significant quantities of 12 novel polycyclic terpenoids from the bacterium *Zymomonas mobilis* that appear to have no structural function in the bacterium due to their very low levels in other bacterial species. The presence of these molecules is attributed to poor efficiency of the squalene cyclase enzyme and its ability to use a wide range of initial substrates that subsequently fail to produce hopene (Douka et al., 2001).

The resulting diploptene, or diplopterol, are subsequently ejected into the core of the membrane before addition of the polar head group (Wendt et al., 1999). The enzyme responsible for the addition of the polar head group to diploptene must also reside in the membrane where it "picks up" the molecule from the membrane core and attaches it to the head group presumably obtained from the cytoplasm. Diplopterol automatically orientates within the membrane with the hydroxyl group providing the orientation direction.

The mechanism of polar head group attachment methodology is unknown but work by Flesch et al (1988) and Rohmer (1989 and 1993) using ^{13}C labelling identified D-ribose as the origin of the side chain. This would suggest that Bacteriohopanetetrol, BHT, is the precursor to all other BHPs where additional functional moieties are added to give the different hopanoid structures.

A second mechanism has been proposed where an adenine nucleoside is added to the triterpenoid ring system to give adenosylhopane (**1e**). The subsequent removal of the adenine group opens up the associated 5 carbon ring system to give the side chain (Neunlist et al., 1988). The stereochemistry of the adenine nucleoside is such that this mechanism would fit the resulting side chain orientation. Recent work by Bradley et al., (in press) appears to confirm this hypothesis. They created mutant strains of *Methylobacterium* deficient in the gene *hpnG* which accumulate adenosylhopane (1e) and do not produce BHPs with other side chains. This indicates that adenosylhopane (1e) is a key step in the formation of the BHP side chain.

1.2 Role of BHPs in Bacteria

The BHP formation mechanism is important because it will explain the origin and function of the additional moieties such as cyclitol ether, amino and other functional groups that are found in BHPs (e.g. Rohmer 1993). If the function of the BHPs were simply to act as membrane rigidifiers then the orientation would simply be provided by the hydroxyl ion in diplopterol. The addition of D-ribose increases the length of the molecule giving it a better spatial fit within the membrane bilayer (Rohmer 1989). Poralla et al (1980) investigated the properties of BHT glucosamine (**1g**) in lipid monolayers and found that it had very similar properties to cholesterol in that it has the effect of condensing a lipid monolayer and therefore adding stability to lipid

membranes. This appeared to answer the long standing question “if prokaryotes don’t produce sterols to stabilise their membranes what do they produce?”

Rohmer et al (1979) tested the hypothesis that BHPs are restricted to the membranes of prokaryotes by growing the eukaryotic protozoan *Tetrahymena pyriformis* on steroid containing and steroid free media. In the presence of steroids *Tetrahymena* incorporates the steroids into its membrane. However in the absence of steroids *Tetrahymena* biosynthesizes diplopterol and tetrahymanol in levels equivalent to that of the steroids when they are available to the protazoan. These molecules are restricted to the membranes and the phospholipid composition is adjusted to maintain membrane fluidity. Rohmer et al (1979) used this information to assume that BHPs are restricted to prokaryotic membranes. Latterly the role of BHPs was investigated using a mutant strain of *Rhodopseudomonas palustris* that is unable to synthesise BHPs (Welander et al., 2009). It was found that under normal growth conditions the bacterium was able to grow and function normally but under acidic or alkaline conditions growth was severely inhibited and increased membrane permeability compared to a wild strain of *R. Palustris was observed*, which is consistent with BHPs providing a membrane stabilising role. This was further confirmed by the identification of increased levels of BHT cyclitol ether in a detergent resistant strain of *Crocosphaera watsonii* implying that in this case BHT cyclitol ethers are important in stabilising membrane lipid rafts (Saenz, in press).

Kleemann et al (1994) investigated the nitrogenase nodules of a *Frankia* species. These were found to be rich in BHPs, approximately 80% of the extracted vesicle lipids. Nitrogenase nodules are sensitive to molecular oxygen and it is believed that the high percentage of BHPs act to prevent any ingress of oxygen into the enzyme cluster thus maintaining the function of the nitrogenase. As oxygen tension was varied the lipid composition changed. At low oxygen concentration the lipids decreased and at high oxygen concentration they increased with the ratio of 2 phenyl monoester hopanoid isomers changing, indicating that the different BHPs confer different oxygen penetration characteristics. This is due to the van der Waal’s interactions between the hopanoid and the membrane lipids. These interactions are primarily influenced by the spatial orientation of the molecules. Therefore one of the two isomers (Kleemann was unable to identify which) has a greater condensing effect on the membrane than the other thus conferring greater oxygen protection to the nitrogen nodule.

Further work was carried out on the composition of BHPs in bacteria under stressed conditions. *Streptomyces coelicolor* was found to produce BHPs during the transition from substrate to aerial hyphae (Poralla et al 2000). When grown on a liquid medium no BHPs are produced but when sporulating on a solid medium BHPs are produced. Work on mutant strains indicates that the production of aerial mycelium switches on the production of BHPs, predominantly BHT, hopane acetate and aminotriol. The production of the BHPs will condense the membranes preventing desiccation of the cytoplasm or excessive molecular transport across the membrane. Joyeux et al., (2004) investigated the effect of temperature on the membrane lipids in *Frateuria aurantia*. They found that as temperature increased the % of BHPs with a hydroxyl group at C_{31} increased, as did the percentage of saturated fatty acids. These modifications were believed to regulate membrane fluidity at higher temperatures.

The additional functional groups would not be necessary for this membrane condensation function. This is especially so in the case of the addition of amino groups such as seen in the commonly occurring 35-amino-bacteriohopane-32,33,34-pentol (**1f**) where the addition of the amino group reduced the polarity of the side chain and would reduce membrane condensation. It therefore seems logical that BHPs fulfil some other more complex role in bacterial membranes in addition to that of membrane condensers. BHPs, specifically BHT (**1a**), BHT cyclitol ether (**1d**) and bacteriohopanetetrol glucosamine (BHT glucosamine) (**1g**), appear to modulate the activity of soybean 15-lipoxygenase in *Zymomonas mobilis* (Moreau et al., 1997) with BHT (**1a**) inhibiting activity and the other 2 BHPs stimulating activity.

Neunlist and Rohmer (1985a) isolated an adenosylhopane (**1e**) from *Rhodopseudomonas acidophila*. They postulated that this functional group may have an additional function as well as that of membrane stabilisation as adenosine can hydrogen bond with thymine it could act as a physical link between prokaryotic DNA and membranes, an interaction that is often seen but not fully understood.

This evidence indicates that the role of BHPs is greater than that of membrane condensation and other roles are possible.

1.3 Variety and Source of Bacteriohopanepolyols in Bacteria

In 1971 Bird et al. first isolated and identified diploptene in *Methylococcus capsulatus*, this was the first instance of a triterpenoid being identified in any prokaryotic organism. Subsequently a C_{35} terpene with four hydroxyl groups on a C_5 side chain, now described as bacteriohopanetetrol (**1a**), was first isolated and identified from *Acetobacter xylinum* by Förster et al in 1973. They described the triterpenoid ring system as bacteriohopane and used NMR analysis to confirm its pentacyclic triterpenoid structure. They also tentatively proposed the biosynthetic pathway by the addition of a polyfunctionalised C_5 chain to the triterpenoid ring system.

In 1976 Langworthy et al isolated a tetracomposite structure, BHT glucosamine (**1g**), from the extreme thermoacidophile *Bacillus Acidocaldarius* using the Bligh and Dyer extraction technique (Bligh and Dyer 1959).

Until 2004 (Sinninghe Damsté et al) no evidence of any BHPs with a strictly anaerobic bacterial origin was available. Until this time it was assumed that all BHPs found in anaerobic sediments were molecular fossils that had been deposited under aerobic conditions and subsequently buried and transformed into their diagenetic products, geohopanes (Ourisson et al 1979). This understanding has been used for many years in the oil industry, for example, to plot the diagenetic history of sediments and to assess oil maturity. It was assumed that all the BHPs and geohopanoids, were the same age as the sediments. Sinninghe Damsté (2004) identified anammox bacteria (*Candidatus* "*Brocadia anammoxidans*"), planctomycetes capable of anaerobic ammonia oxidation, that produce BHPs, in anaerobic conditions. This creates a problem with the assumption that the age of the BHPs is comparable to that of the sediment. It is now possible that there is a continuing input of BHPs into the sediment from bacteria living in the sediment. This may result in the sediment appearing less mature than it really is, or conversely increasing the believed recalcitrance of the BHPs in the environment. This work was further investigated by Härtner et al (2005) who studied *Geobacter* species, strict anaerobes, that grow in subsurface and sediment environments. These were found to produce a wide range of BHPs as well as diploptene and hop-21-ene. It is these compounds that are used to estimate paleo-sedimentary conditions. Blumenberg et al. (2006) found high concentrations BHT (**1a**), aminotriol (**1f**) and some 35-amino-bacteriohopane-31,32,33,34-tetrol

(aminotetrol) (**1h**) in some species of anarobic sulphate reducing bacteria *Desulfovibrio spp.*

Michel Rohmer (1993) described 23 different side chain structures in addition to possible methylation and unsaturation of the triterpenoid ring system and many as yet unidentified BHPs, indicating the wide range of BHP structures.

Neunlist and Rohmer (1985b) reported the identification of aminotriol (**1f**), aminotetrol (**1h**) and aminopentol (**1c**) derivatives of bacteriohopanepolyols from the methanotrophic bacteria *Methylococcus caspsulatus* and *Methylococcus methanica*.

Peiseler and Rohmer (1991) found a further hopanoid with an unsaturated ring system,Δ^6 BHT cyclitol ether (**5d**) (Fig. 1.2), in *Burkholderia* species. Costantino et al (2000) isolated bacteriohopanepolyols from the marine sponge Plakortis simplex. The origin of these BHPs is almost certainly from symbiotic and dietary bacteria but the identification of the novel hopanoid 12-methylbacteriohopanetetrol (Fig.1.2) led Costantino to postulate that this molecule may actually be transformed by the sponge, a capability that sponges have been shown to have with fatty acids and sterols.

Whilst it may appear that much of this research has no single direction with random potential BHP producers being tested for BHPs when available this is not the case and there have been concerted efforts to link specific bacteria to specific BHP structures to enable the BHPs to be used confidently as biomarkers.

Cyanobacteria have been reported to be the only significant source of BHPs methylated at position C-2 (Summons et al., 1999) although C-2 methylation is regularly found for C_{30} hopanoids in *Methylobacterium* spp. (Bisseret et al., 1985; Knani et al., 1994), *Beijerinkia* sp. (Vilcheze et al., 1994) and *Bradyrhizobium* spp. (Bravo et al., 2001) and to some extent for BHT in *Methylobacterium organophilum* (Renoux and Rohmer, 1985). More recently an adenosylhopane methylated at C-2 (**2e**) has been identified as a minor component from the nitrogen fixing bacterium *Bradyrhizobium japonicum* (Talbot et al., 2007a). Other BHPs more typically associated with cyanobacteria, which are present in all soils, include BHT (**1a**) and 2-methylBHT **(2a)** which are known to occur together in various cyanobacteria including *Nostoc* spp. (Bisseret et al., 1985; Zhao et al., 1996) and *Prochlorothrix hollandica* (Simonin et al., 1996).

However, Rashby et al. (2007) identified the presence of 2Me BHT (**2a**) and 2Me aminotriol (**2f**) in the anoxygenic phototroph *Rhodopseudomonas palustris* indicating that not all 2 methyl BHPs are produced by bacteria. This was investigated further by

Doughty et al., (2009) who identified that 2-methylhopanoids were produced by the cyanobacterium *Nostoc punctiforme* when in the form of an akinete, a resting phase. Therefore 2-methylhopanoids are not simply related to oxygenic photosynthesis in cyanobacteria and may be present in other bacteria within the soil. However the current understanding of 2-Me BHPs does indicate that cyanobacteria are the dominant source.

In recent years it is this use of targeted genomic detection of biosynthestic pathways (e.g. Fischer et al., 2005; Pearson et al., 2009a and 2009b) has superceded the use of laboratory grown bacteria, especially since the identification of primers for *Shc*. These techniques are an improvement as they do not require the use of bacteria that can be grown under laboratory conditions and tend to reflect the natural expression of BHPs by bacteria and not that seen in artificially designed environments.

1.4 Occurrence Bacteriohopanepolyols in the Environment

The identification of BHPs in environmental samples is fundamental to their use as biomarkers. Until the development of HPLC-MS^n analysis which enables the identification of intact BHPs (Talbot et al., 2001; 2003a; 2003b), BHPs were analysed using GC-MS. Most BHPs are not amenable to analysis by GC-MS and periodic acid cleavage was used to remove the functional groups leaving C-32, C-31 and C-30 hopanols that indicate the presence of tetra-, penta- and hexa-functionalised BHPs, respectively. This resulted in a very restricted use of BHPs as biomarkers before 2001.

Recent and ancient sediments are the most widely studied environments for the presence of BHPs. Innes et al (1997) investigated the hopanoid signatures of the sediment from a fresh water lake, Priest Pot. They found relatively high hopanoid concentrations in the lake sediment, between 500 and 3500 µg/g dry sediment, which they attributed to high productivity in the water column. They mapped the concentrations of the total BHPs, geohopanoids, and BHT (**1a**) (Fig. 1.5).

For significant lengths of the core the ratio of geohopanoids to BHPs is relatively consistent with geohopanoids accounting for 30 -50% of the BHPs. This implies that the initial diagenesis could occur rapidly and then slows, probably beginning in the water column. The exception to this is the sediment between 5 and 7 cm where the BHP concentration dips, explained as a drop in bacterial productivity.

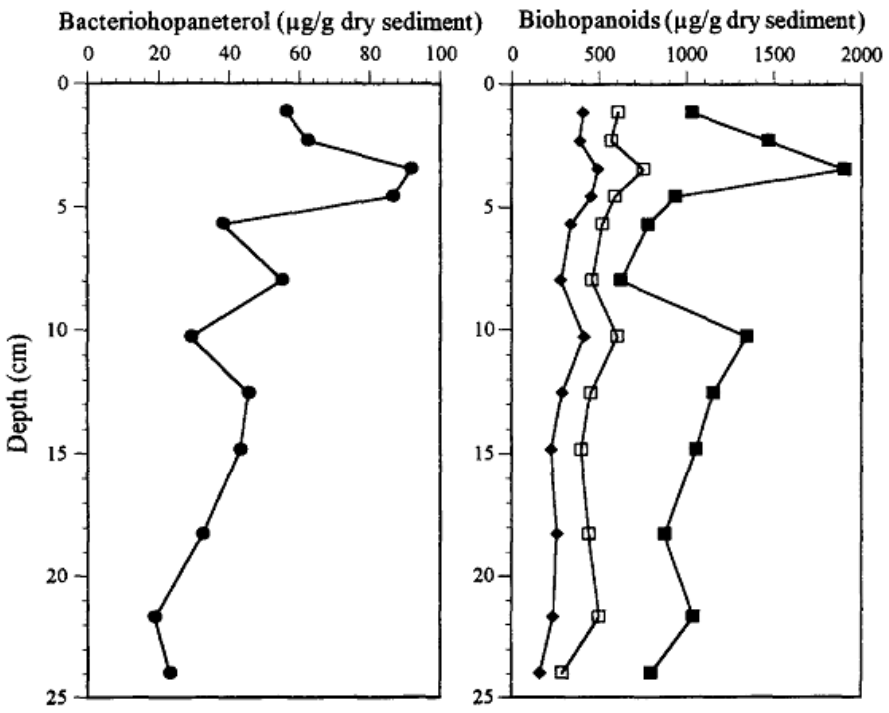


Fig. 1.5 Geohopanoid, Biohopanoid and BHT distribution down sediment cores from Priest Pot Innes et al (1997). Geohopanoids are the lower value.

The BHT (**1a**) concentration is very low throughout the core indicating that it only accounts for a small fraction of the total BHPs and that other BHPs are more significant. The gradual reduction in BHPs down the column is believed to be due to the incorporation of the BHPs into macromolecular material such as humin or kerogen.

Innes et al., (1998) also investigated Lake Pollen, Norway, a lake that was formerly linked to the sea. The hopanoid profile of the sediment (Fig. 1.6) shows a significant increase in tetra-functionalised BHPs immediately post the change from fjord to freshwater conditions with a drop in the penta- and hexa-functionalised BHPs. They believe that this increase in tetra-functionalised compounds is due to a rise to dominance in a single bacterial source immediately after the change from salt to freshwater. The changes in relative compositions may enable hopanoid signatures to be used as markers of environmental conditions (Farrimond et al., 2000)

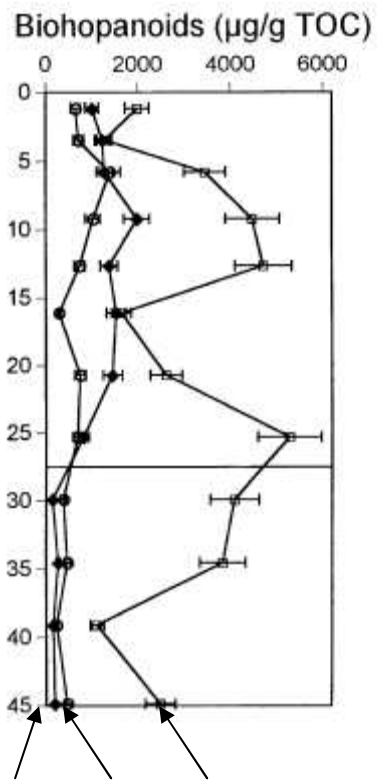


Fig. 1.6 Biohopanoid profile, Lake Pollen. Innes et al (1998)

Farrimond et al (1998) returned to Priest Pot in an attempt to identify the bacterial origin of the BHPs in the lake sediments. They used rRNA to identify the dominant bacteria in the sediment and found that the sediment was dominated by non-hopanoid producing bacteria indicating that the source of the BHPs was in the water column. This fitted with the understanding at the time that no obligate anaerobes were known to produce BHPs. This belief has subsequently been disproved by Sinninghe Damsté (2004) and Härtner (2005) however this does not disprove the belief that the majority of BHPs are produced in the water column.

The first work on the analysis of BHPs in soil was carried out by Ries-Kautt and Albrecht (1989) who investigated a wide variety of French soils with a wide range of biological activity. Low levels of BHPs were found in all the samples and in acidic peat increased with depth, indicating accumulation down the soil column due to leaching in this soil type. The upper layer of the peat also contained high levels of BHT which was assumed to imply high bacterial activity.

Jaffé et al (1996) studied the lipid composition in seasonally flooded Amazonian rain forest soils. They identified that diagenesis occurs rapidly during the annual flooding

period, less that 3 months. They raised concerns that the origin of soil lipids was complicated by the flooding making the source of the lipids difficult to identify. This problem is of use as soil derived BHPs can be tracked into sediments and therefore give an estimation of soil input from terrestrial environments (see Chapter 6).

Winkler et al (2001) identified that BHPs accumulated in forest soil during leaf litter decomposition and humification. The accumulation increased moving down the O horizon, surface zone, reaching a peak in the eluvial zone, the zone of maximum leaching before dropping away. There is a partial recovery in the illuvial zone, where accumulation of recalcitrant molecules would be expected. The increase in the eluvial zone implies that the BHPs are relatively resistant to leaching either as a result of binding to clay particles which carry a net negative charge or by accumulation into humin. Binding to clay particles would have to be via metal ions that are bound to the clay minerals.

Shunthirasingham and Simpson (2006) investigated BHP inputs into soils from Western Canada. They investigated the differences in free and bound BHPs in 5 different types of soil. 3 of the soils, 2 grassland and 1 forest contained a higher proportion of free BHPs than bound BHPs. The other 2 soils, a grassland and a transition soil contained more bound than free BHPs. This variation in the ratio free to bound BHPs in different soils is best explained by the variation in equilibrium between the various states described in Fig. 1.9 and would be influenced by factors such as organic content of the soil, soil pH, rainfall and therefore leaching rate, sorption to clay minerals in soil and depth of aerobic zone in soil.

Recent work, using HPLC-MS^n, on the occurrence of BHPs in sediments has identified more than 15 different BHPs in lacustirne sediments from around the world (Talbot and Farrimond, 2007 and work on the Congo Fan sediments, ODP1074, identified intact BHPs to a depth of 100 m 1Ma), (Cooke et al., 2008a, Handley et al., 2010).

Work on soils using the same techniques has identified more than 22 different intact BHPs in soils from NE England (Cooke et al., 2008b), SE England and South Wales (Redshaw et al., 2008) and Canada (Xu et al., 2009). This work demonstrates a significant improvement on the previous studies using GC-MS and has allowed the use of BHPs to indicate bacterial processes and populations.

1.5 Diagenesis of Bacteriohopanepolyols

Cvejic et al (2000a) investigated type I methanotrophs including *Methylocaldum* and *Methylococcus* species. 3-methyl aminopentol was identified and for the first time a Δ^{11}3- methyl aminopentol (**7c**) was identified in the soil bacterium *Methylocaldum szegediense* (Fig. 1.7).

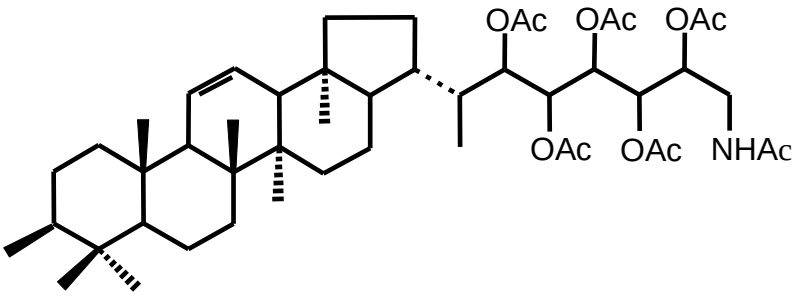


Fig. 1.7. Δ^{11} 3- methyl aminopentol (**7c**)

She proposes that this unsaturation of the ring system may the first step in the biodegradation of the BHP to the geohopanoid. However, as the BHPs were extracted from a bacterial culture it is my belief that the unsaturation is part of the standard structure of the hopanoid and not a degradation product. If the unsaturation were a result of biodegradation then unsaturation would not be common in laboratory cultures but is readily observed (Talbot et al., 2007b).

Biodegradation within a cell culture implies rapid onset of degradation but research by Innes et al (1997 and 1998) in BHPs in recent lake sediments indicates that the initial degradation steps are the removal of functionalised groups from the side chains and not action upon the ring system.

In 2001 Costantino et al isolated 32, 35-anhydrobacteriohopanetetrol (anhydroBHT) (**1j**) (Fig.1.8) from a sponge and proposed a biosynthetic pathway from adenosylhopane instead of the simple condensation reaction from bacteriohopanetetrol.

AnhydroBHT (**1j**) was subsequently isolated from a wide range a sediments by Bednarczyk et al (2005) and confirmed to be a diagenetic product of more complex BHPs (Cooke et al., 2008a.; Schaeffer et al., 2008)

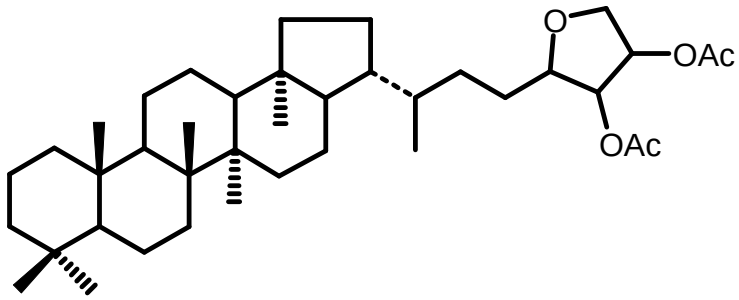


Fig. 1.8 Anhydrobacteriohopanetetrol (**1j**).

In environmental situations BHPs exist in 3 forms; free, bacterial and bound to kerogen and humin. Humin is formed when recalcitrant organic molecules such as lignin and BHPs combine with each other to form high molecular weight compounds that make up a significant proportion of soil organic matter (Killops and Killops, 1993). This material is highly stable to chemical and physical weathering effectively locking these molecules into the soil. In sedimentary deposits kerogen is a similar recalcitrant compound that is formed by the loss of hydrophilic functional groups from humin and fulvic acid material during burial to produce insoluble kerogen (Killops and Killops, 1993). Lichtfouse (1998) isolated the lipids in humin by pyrolysis and found it to be rich in linear, isoprenoid, steroid and hopanoid lipids from humin associated with a maize crop soil. He discussed 3 possible sequestration methods for the incorporation of lipids into humin, humification. The first is physical sequestration where small aliphatic molecules become incorporated into the humin due to van der Waal interactions. Resistant straight chain biopolymers are selectively preserved in soil because of their recalcitrant aliphatic skeleton. These form a significant part of the humin due to their longevity within the soil. BHPs are bound into the humin by chemical sequestration due to the formation of ester and ether bonds with other polar lipids such as isoprenoids. This results in humin being rich in BHPs and may help to explain their extreme recalcitrance in soils and sediments. The problem with this hopanoid sequestration is that it can mask short term changes in the bacterial hopanoid signature. Kerogen, effectively mature humin, was investigated by Mycke et al (1987); they succeeded in extracting BHPs from Messel oil shale kerogen, an Eocene deposit (50 Ma). This indicates the extreme age that BHPs can survive to when bound in kerogen. The rate of incorporation of BHPs into kerogen was investigated by Farrimond et al (2003) in freshwater lake and saltwater fjord

sediments. The results indicated that BHPs are incorporated into kerogen over a timescale of 0 to 350 years. They also identified that the binding was by strong ether bonds and weaker disulphide linkages, which form where the environment is rich in reduced sulphur species and low in iron.

Ohkouchi et al (1997) identified high levels of hopanoids in Cretaceous black shale (90.4 Ma) which they believe indicates a significant input of bacterial matter into the organic composition of the black shale. The results however show the concentration of hopanoids are relatively low, less than 0.01 µg/g sample, when compared with other recent and quaternary sources where the range of BHPs in soils is typically between 1 and 800 µg/g dry soil (Cooke et al., 2008b; Redshaw et al., 2008; Xu et al., 2009; this thesis).

Geohopanoids are formed by diagenesis of BHPs. This results in the cleavage of the side chain between adjacent hydroxyl groups giving hopanoic acids, hopanols, hopanes and aldehyde and ketone hopanoids. In addition to this cleavage there is a change in the stereochemistry of the hopanoids. In bacteria 17β- and 21β-stereochemistry dominates but as a result of diagenesis 17β-, 21α- and 17α-, 21β-forms arise as the steric hindrance is lower and therefore more stable (Rohmer et al 1992). Tritz et al (1999) investigated the biodegradation of hopanes and identified that under aerobic conditions a wide range of geohopanoids were formed. It is likely that BHPs can therefore exist in various forms within in the soil and that an equilibrium will exist dependant on soil conditions, depth within the soil core and age of the soil organic matter. Fig. 1.9 summarises these states. Degradation 1 (Fig 1.9) is the the cyclisation of BHPs or the removal of purine groups from adensosylhopane type BHPs to produce ahnydroBHT whereas Degradation 2 is the removal of functional groups, the shortening of the side chain and the change in stero chemistry found in geohopanoids. The relationship between these processes is unclear although it is likely that degradation 1 occurs before degradation 2.

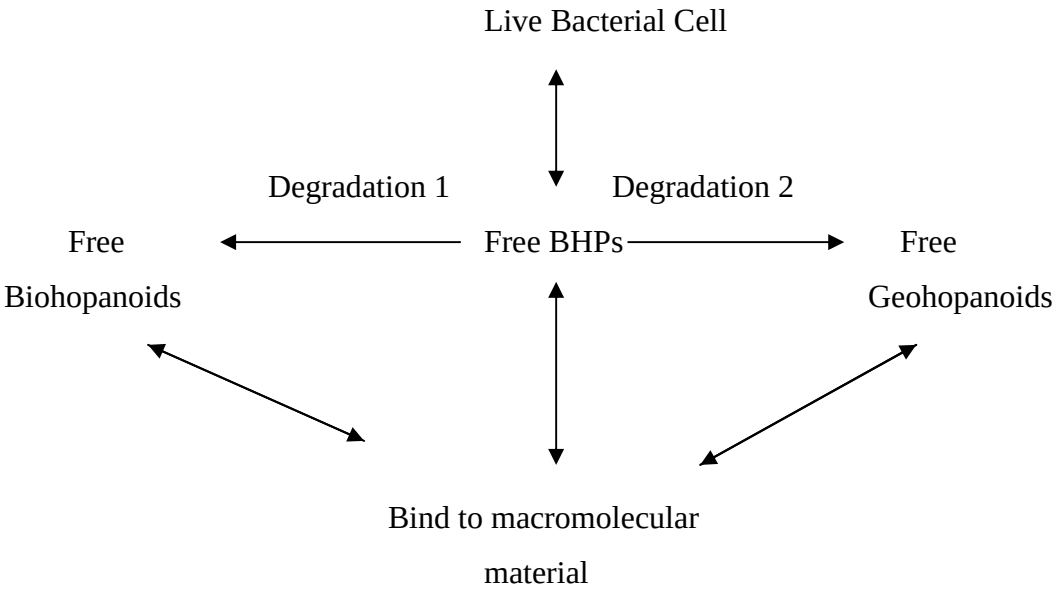


Fig. 1.9. Fate of BHPs in soil

Peat environments were studied by Quirk et al (1984) and they identified adundant αβ-hopanoids in the samples. αβ-hopanoids had previously been associated with thermally mature sediments (Seifert and Moldowan 1980) and were assumed to be late diagenetic products of hopanoid burial. Here the sediments are recent and it is believed that the acidic conditions in the peat drive forward the microbiologically mediated formation of this stereoisomer from the natural ββ-form that would be expected to be present in immature sediments and soils. This information can be used to track environmental change in soils and recent sediments where the presence of αβ hopanoids to be used to infer an input of peat derived material into the soil or a subsequent drying of a peat to form a soil.

Dehmer (1995) investigated the organic geochemistry of a series of tropical to temperate recent peat deposits. All the deposits contained αβ hopanoids which correlated with the research carried out by Quirke (1984) but a single sample contained virtually no 17α, 21β-(H)-22R-homohopane. This sample is a *Mariscus* peat that is unusually formed in neutral pH conditions. This confirms the belief that the formation of αβ hopanoids in peat is driven by the acidic conditions. Two further samples, *Alnus* and sedge/diatom peats are formed under neutral or weakly acidic conditions and contained very low levels of 17α, 21β-(H)-22R-homohopane. The acidic peats contained high levels of BHT indicating high levels of microbial activity which also conformed to the hypothesis that the formation of αβ hopanoids is

bacterially driven under acidic conditions. The high levels of BHT could be due bacteria producing more BHT to compensate for the acidity of the peat (Welander et al., 2009a, also see Chapter 3). Dehmer correlated the results with those obtained from a Borneo peat bog in 1993 where again high levels of 17α, 21β-(H)-22R-homohopane were observed.

1.6 Hypotheses

Prior to this thesis the current knowledge on the occurrence of BHPs in soils was very limited (see above, Ries-Kautt and Albrecht, 1989; Jaffé et al 1996; Winkler et al, 2001). This thesis was undertaken to fill that knowledge gap by conducting the largest study of the BHPs in soil.

For BHPs to be useful as biomarkers then a direct link must be able to be drawn between specific bacteria or environments and specific BHPs or groups of BHPs. To assess the potential roles of BHPs as biomarkers the following hypotheses will be tested using a series of goals.

Hypothesis 1

The BHP fingerprint of a soil indicates the current bacterial population.

For BHPs to be useful as a biomarker for bacterial activity it must be possible to identify different bacterial populations directly from the BHP profile of the soil. Analysis by many researchers has shown that different bacteria produce different BHPs. The presence on aminopentol (1c) (Cvejic at al., 2000a) has been linked to methanotrophs and 2-methyl BHPs associated with cyanobacteria (Summons et al., 1999). The presence of these BHPs in the soil will therefore indicate the presence of the bacteria without the need to directly culture the bacteria or the use of culture dependent DNA marker techniques. However bacterial identification can only be tentative as previous investigations on the link between bacteria and BHPs were conducted on cultured bacteria grown under ideal conditions and not under environmental conditions such as those observed in soil, where the production of BHPs may vary from that observed under ideal conditions.

Hypothesis 2

Complex intact BHPs are preserved in ancient marine sediments.

The recalcitrance of intact BHPs in ancient sediments is poorly studied with intact BHT (1a) having been identified in 65 Ma sediments (van Dongen et al., 2006) and more recently in Congo Fan sediments up to 1 Ma (Cooke et al., 2008a)

Analysis of BHPs in sediments has shown that they decrease rapidly with depth (Watson et al., 2002; Talbot et al., 2003) as a result of rapid diagenesis (Rohmer et al., 1980; Quirk et al., 1984; Innes et al., 1997). Research into BHP producing bacteria has identified that they are predominantly produced in aerobic conditions (e.g. Rohmer et al., 1984; Zundel and Rohmer, 1985; Neunlist et al., 1985; Ourisson et al., 1987; Summons et al., 1999). However BHPs have been identified in obligate anaerobes (e.g. Ourison et al., 1984; Sinninghe Damste et al., 2004; Fischer et al., 2005; Blumenberg et al., 2006) confirming production in anaerobic conditions. Therefore not all BHP production can be linked to aerobic conditions and the presence of some BHPs in deep sediments cannot be directly linked to preservation after burial and may be produced in situ (Saito and Suzuki, 2007).

However specific BHPs are used as biomarkers for aerobic processes, e.g. aminopentol as a marker for aerobic methane oxidation which occurs in the water column and surface sediments (e.g. Neunlist and Rohmer, 1985a; b; Cvejic et al., 2000; Talbot et al., 2001) and aminotetrol (e.g. Neunlist and Rohmer, 1985a; Cvejic et al., 2000). The presence of aminopentol and aminotetrol at depth in the sediment must be as a result of preservation after burial.

These 2 hypotheses form the basis of this thesis and will be investigated and tested using a series of goals that are described in each chapter and relate to specific characteristics of each sampling environment.

2. Methods and Method Development

2.1 Sample Collection and Preparation

Soil samples were collected from Palace Leas (Chapter 3), Hack Hall Farm (Chapter 4) and a variety of landfill sites in Tyne and Wear (Chapter 5) using a 35 mm diameter soil corer. Cores (15 cm depth), were wrapped in aluminium foil, placed in a snap lock plastic sample bag and frozen at -20 °C. These samples were subsequently defrosted for 12 h at room temperature and all surface vegetation and associated rhizosphere material removed to ensure that only the soil was analysed. The cores were then separated into three sections: 0-5 cm, 5-10 cm and 10-15 cm using a clean pallet knife. The wide variety of soils and the absence of visible soil horizons in the Palace Leas, except plot 7. (Chapter 3), Hack Hall Farm (Chapter 4) and landfill soils (Chapter 5) made the separation into soil horizons impractical so an arbitrary and consistent division of the soil layers was used. The sections were then placed in glass sample jars, covered with perforated aluminium foil and freeze dried at -40 °C for approximately 24 h. The freeze dried soil was ground in a clean, dry mortar and pestle and any obvious vegetable and animal material was removed. The soil was passed through a 2 mm brass sieve to remove small stones then sealed in the glass sample jar for storage.

Samples from Vietnamese paddy fields were collected by Dr Florianne Clement (Newcastle University) from northern Vietnam in September 2005 (Chapter 7). Samples were collected using a trowel, placed in glass collection jars and frozen in a domestic freezer. The samples were then transported to the UK frozen. On arrival the still frozen samples were defrosted for 5 h, placed in glass sample jars, freeze dried and sieved as described above.

All other samples were received as freeze dried material which was sieved (as above) and visible vegetable matter removed prior to extraction.

2.2 Analysis of Total Organic Carbon (TOC)

TOC analysis was carried out on all samples, with replication every 8^{th} sample to assess the accuracy of the analysis. Freeze dried sample (0.1 g) was weighed into a porous crucible and HCl (1 ml, 4 mM) was added to remove any inorganic carbon

from the soil. After 24 h samples were analysed using a LECO-CS-244 analyser with AR077 iron chip and SL266 tungsten accelerators added to ensure complete combustion of the soil. A standard sample was analysed after every 10 samples to check accuracy.

2.3 Modified Bligh and Dyer Extraction

The samples were extracted using a methodology adapted from Summons et al. (1994) and is based on the Kates modification (Kates, 1986) of the original Bligh and Dyer extraction (Bligh and Dyer, 1959). Freeze dried material (1-3 g) was extracted in a 50 ml Teflon centrifuge tube using a monophasic mixture of water/methanol/chloroform (4 mL: 10 mL: 5 mL). The water was added first and the sample shaken vigorously by hand to ensure that the sample was fully dispersed before the other solvents were added. The mixture was initially sonicated at 40ºC for 1 h, followed by shaking for a further 2-4 h. The mixture was then centrifuged at 12,000 rpm for 15 min and the supernatant removed to a second centrifuge tube. The sample was then extracted again using the same solvent mixture and was left to shake overnight following sonication for 1 h (as above). The sample was again centrifuged and the supernatant transferred to a third tube. A third extraction was carried out for an additional 4 h and transferred to a fourth tube. To tubes 2, 3 and 4 chloroform (5 mL) and water (5 mL) were added and then centrifuged for 5 min to allow complete separation of the organic (chloroform) fraction from the methanol (MeOH)/water phase. The chloroform fraction from tube 2 was then transferred to a 100 mL round bottom flask (rbf). The chloroform fraction from tube 3 was transferred to tube 2, the 5 min centrifugation repeated and the chloroform fraction added to the first chloroform fraction in the rbf. The chloroform fraction from tube 4 was similarly transferred via tube 2 to the rbf. In all cases the interface layer between the chloroform fraction and the aqueous fraction was removed to prevent loss of BHPs to the discarded fraction. The 3 combined chloroform fractions (total lipid extract, TLE) were then rotary evaporated to near dryness, transferred to a weighed vial using a solution of warm chloroform/MeOH (2:1 v/v) then evaporated to dryness under a stream of nitrogen.

Each set of extractions comprised 15 soil samples and a method blank, to ensure no cross contamination issues.

Test analyses were conducted on random aqueous phase samples and in all cases no BHPs were recovered indicating that all the BHPs were collected in the chloroform and interface layers.

2.4 Standard Addition

To each extract 3 standards were added: 5α-androstane (2 µg mg^{-1} TLE), for quantitative assessment of hopanes and hopenes using GC-MS; 5α-androstan-3β-ol (2 µg mg^{-1} TLE), for quantitative assessment of hopanols produced by periodic acid treatment and identified by GC-MS and 5α-pregnane-3β,20β-diol (pregnanediol) (5 µg mg^{-1} TLE), for semi-quantative measurement of acetylated BHPs by HPLC-MS^n, i.e. by comparison with known concentration of pregnanediol The extract was redissolved in chloroform/MeOH (2:1 v/v), (ca. 1 mL), split into 2 aliquots and both blown down under a stream of nitrogen. One of the aliquots was used for HPLC-MS^n analysis and the second reserved for GC-MS analysis where required.

The pregnanediol standard, occurring as a diacetate post acetylation (Section 2.5), gives a characteristic spectrum (Fig. 2.1) with a base peak of *m/z* 345 (= [M+H-CH_3COOH]) and a peak at m/z 285. The m/z 285 peak indicates the loss of a second acetylated hydroxyl from the structure, -60 Da.

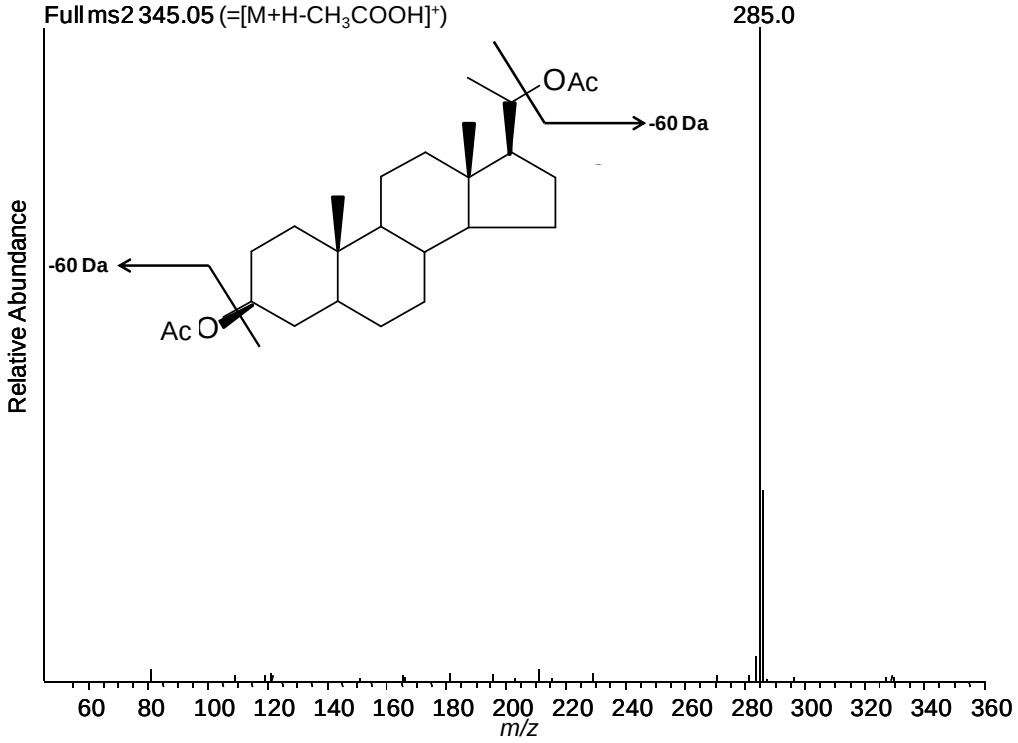


Fig. 2.1 APCI HPLC-MS^2 spectrum of the diacetate of pregnanediol standard.

2.5 Acetylation

Samples were derivatised prior to analysis according to Innes et al. (1997). Pyridine (2 ml) and acetic anhydride (2 ml) were added to the extracts and heated in a closed vial for 1 h at 50 °C before leaving overnight at room temperature. The acetylated samples were then the solvent removed via rotary evaporation, transferred to 3.5 ml glass vial using DCM, blown down under nitrogen and re-dissolved in methanol: isopropyl alcohol (60:40; 0.5 ml) and transferred to HPLC vials for analysis. This acetylation results in the addition of an acetyl group to each hydroxyl group in the structure and makes the BHP amenable to HPLC analysis.

2.6 Identification of BHPs by APCI-HPLC/MS^n

2.6.1 Methodology

Talbot et al. (2003b, c) used reversed phase atmospheric pressure chemical ionisation liquid chromatography / multi-stage ion trap mass spectrometry (APCI-LC/MS^n) to identify intact BHPs. The method used during this research is based on the methods developed by Talbot et al. in these two papers and subsequent development work carried out in the department (e.g. Talbot et al., 2007a, b, 2008; Cooke et al., 2008a, b). The final method used was that described in Cooke et al. (2008a, b) and detailed below.

Reversed-phase HPLC was accomplished using a Surveyor HPLC system (ThermoFinnigan, Hemel Hempstead, UK) fitted with a Phenomenex (Macclesfield, UK) Gemini C_{18} 5 µm HPLC column (150 mm x 3.0 mm I.D.) and a security guard column cartridge of the same material.

Separation was achieved at 30ºC with a flow-rate of 0.5 mL min^{-1} and the following gradient profile: 90% A and 10% B (0 min); 59% A, 1% B and 40% C (at 25 min) then isocratic to 70 min (where A = MeOH, B = water and C = propan-2-ol; all HPLC grade, purchased from Fisher [Loughborough, UK]) returning to the starting conditions in 5 min and stabilising for 15 min.

LC-MS^n analyses were performed using a ThermoFinnigan LCQ ion trap mass spectrometer equipped with an APCI source operated in positive ion mode. LC-MS

settings were as follows: capillary temperature 155°C, APCI vaporiser temperature 400°C, corona discharge current 8 μA, sheath gas flow 40 and auxiliary gas 10 (arbitrary units). The instrument was tuned as described previously (Talbot et al., 2003c). Detection was achieved at an isolation width of *m/z* 5.0 and fragmentation with normalised collisional dissociation energy of 35% and an activation Q value (parameter determining the *m/z* range of the observed fragment ions) of 0.15. LC-MS^n analysis was carried out in data-dependent mode with three scan events: SCAN 1 – full mass spectrum, range *m/z* 300-1300; SCAN 2: data-dependent MS^2 spectrum of the most intense ion from SCAN 1; SCAN 3: data-dependent MS^3 spectrum of the most intense ion from SCAN 2. Structures are identified based on comparison with previously identified published spectra where possible (Talbot et al., 2003b, c; 2007a, b; 2008) or by comparison of APCI MS^2 and MS^3 spectra with those of known compounds.

The dynamic exclusion function was used during the analysis. This function is designed to limit the number of times that ions of a specific *m/z* value are isolated and fragmented in a preset period of time. To maximise the possibility of obtaining MS^2 spectra of minor compounds which may be partially co-eluting with more abundant compounds the number of times any particular *m/z* value was repeated was set to 2 rather than 3 (as used previously; Talbot et al., 2003a,b, 2007a,b, 2008) during this project.

The semi-quantitative estimate of BHP abundance is calculated from the characteristic base peak ion peak areas of individual BHPs in mass chromatograms (from SCAN 1) relative to the *m/z* 345 (=$[M+H-CH_3COOH]^+$) base peak area response of the acetylated 5α-pregnane-3β,20β-diol standard. Averaged relative response factors, based on a suite of five acetylated authentic BHP standards, were used to adjust the BHP peak areas relative to that of the standard where BHPs containing one or more nitrogen atoms give an averaged response approximately 12 times that of the standard and compounds with no nitrogen atoms give a response approximately 8 times that of the standard (e.g. Cooke et al., 2008a, b, 2009; Redshaw et al., 2008; Xu et al., 2008).

2.6.2 Reproducibility

The capability of the HPLC-MS^n to accurately reproduce the results was assessed initially by running six repeat analyses of 2 sample extracts of different BHP content. Statistical analysis of these results indicated that there was a precision of +/- 17% in the results. The samples were run subsequently at a later date to assess any variation temporal change in the function of the HPLC-MS^n. These results indicated no decrease in BHP content during the timeframe of the research and no change in the precision in the analysis.

During the project three types of duplicates were analysed to assess the consistency of the methods employed. For the Palace Leas samples (see Chapter 3) three cores were collected from each location and two cores were analysed to assess the variation in the results due to variations in the soil and soil bacterial content. The second set of duplicates were tested to assess the consistency of the extraction technique and HPLC-MS^n analysis, by testing one complete core twice from each sampling period (Chapter 3). The third set of duplicates was obtained by repeating the HPLC-MS^n analysis on a single extract from each batch to assess the reproducibility of the HPLC-MS^n results.

Comparison of the latter 2 sets of systematic duplicates indicated that the variation in BHP content and distribution due to variations in extraction and analysis were within the 17% relative standard deviation identified during the reproducibility results.

2.6.3 Statistical Analysis

Statistical analysis was carried out using Minitab 15, Minitab Ltd (USA), for Principle component analysis (PCA). Prior to PCA the data was normalized to remove excess influence of dominant BHPs and standardised to remove errors due to normalization (Rietjens, 1995). The standardization method used was to subtract the means and divide by the standard deviation for each value in the data set. These values were then used in the PCA calculations. BHPs that were identified only once were not included in the PCA as this has the effect of skewing the analysis.

2.7 Identification of BHPs

During this work all BHPs were analysed as acetylated derivatives and are identified by comparison of their APCI MS^2 spectra with known standards and previously identified structures (see section 2.11) using characteristic parent ions and subsequent fragmentation product ions and in some cases by consideration of relative retention times to other known structures. A full list of all references associated with these structures can be found in Table 2.2.

Using reversed phase chromatography the more polar BHPs are the first to elute. Relative polarity is based upon the number of functional groups, their polarity and the molecular weight of the structure. In general a structure that is pentafunctionalised will elute earlier than its equivalent tetra functionalised form and by the same measure a hexafunctionalised structure earlier than its pentafunctionalised form. The addition of a non-polar methyl group will generally increase the size of the structure without increasing the polarity and therefore a methylated structure will elute later than its non-methylated form. Unsaturation of the BHP reduces its overall mass of the structure and therefore has the effect of increasing the polarity relative to the saturated form. Unsaturation in the ring system would also cause distortion in the ring system potentially reduce the Van der Waal's interactions and similarly reduce the elution time. Therefore unsaturated structures elute earlier than their saturated equivalents.

The effect of these differences is illustrated in Fig 2.2 where the elution times of various BHPs with a terminal cyclitol ether group differ based upon the number of additional functional groups with the hexafunctionalised form (*m/z* = 1118, **1k**) eluting before the pentafunctionalised form (*m/z* =1060, **1l**) which in turn elutes before the tetrafunctionalised form (*m/z* = 1002, **1d**). The unsaturation at C-6 or C-11 (*m/z* =1000, **5d** and **6d**) reduces the elution time relative to the saturated tetrafunctionalised form (*m/z* =1002, **1d**) and the methylation at C-2 (*m/z* = 1016, **2d**) increases the retention time relative to the unmethylated form (*m/z* = 1002, **1d**). A fuller interpretation of these structures is given in section 2.7.3.

The BHPs have been grouped together in this report based upon structural similarities and not by bacterial origin or other possible groupings. These groupings have been used as the similarity between the identified spectra and relative elution times is the most natural grouping and makes the identification of related but novel structures easier.

During this investigation a total of 46 different BHP MS^2 spectra were observed and interpreted, including the tentative identification of 13 previously unknown structures. As APCI-HPLC-MS^n is unable to determine the stereochemistry of the bonds on the BHP ring system or side chain stereochemistries are assumed to be identical to those previously determined by other workers using NMR (see Table 2.2 for comprehensive list of related references); however, the possibilities of other stereochemistries cannot be excluded.

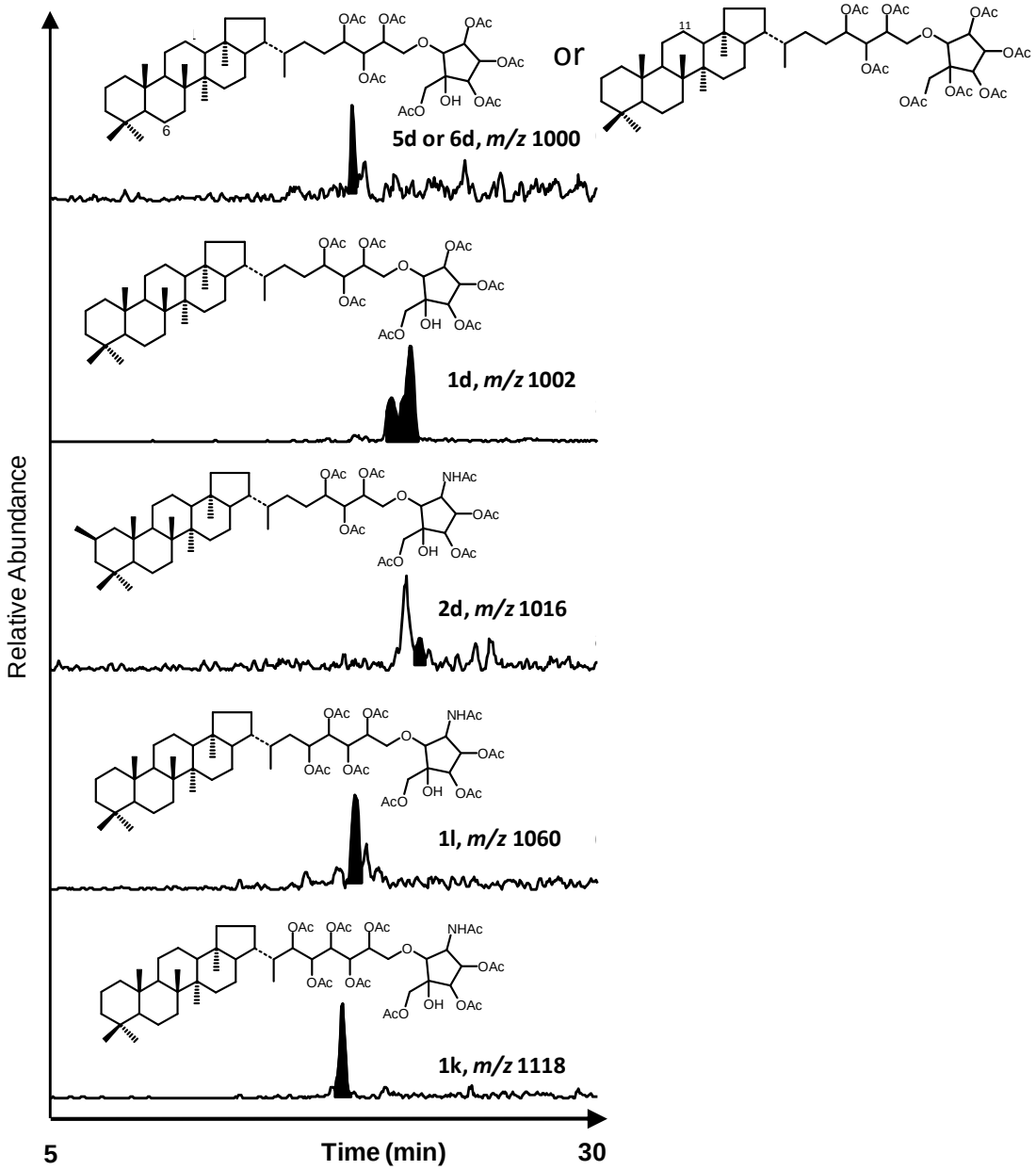


Fig. 2.2. Relative retention times for Bacteriohopanepolyols with terminal cyclitol ether group. Palace Leas plot 7 0-5 cm, September 2004. Note that BHT cyclitol ether (m/z 1002, **1d**) has 2 known isomers that produce the characteristic double peak seen here.

2.7.1 Polyhydroxylated BHPs

The polyhydroxylated BHPs are amongst the simplest and frequently observed BHPs in this study. They comprise a group of BHPs where all the functional groups on the side chain are simple hydroxyls.

BHT (**1a**) is the most commonly occurring BHP in the environment and has been identified in many different groups of bacteria (Table 2.2). It has a characteristic MS^2 spectrum (Fig. 2.3; see also Talbot et al., 2003a) produced via fragmentation of the base peak ion in the full mass spectrum of *m/z* = 655 (= $[M+H-CH_3COOH]^+$). BHT (**1a**) is identified by the serial loss of the three remaining acetylated hydroxyls (60 Da = CH_3COOH) from the side chain producing ions of *m/z* = 595, 535 and 475 respectively, with *m/z* = 475 equating to the loss of all 4 hydroxyls from BHT. Cleavage of the C ring is also observed resulting in a loss of *m/z* 192 which produces an ion of *m/z* = 463 (from *m/z* = 655). Subsequent loss of the 3 remaining hydroxyls from the side chain and the remaining D+E rings gives ions of *m/z* = 403, 343 and 283. The A+B ring fragment produced by the cleavage of the C ring is also observed (*m/z* = 191) and is a key fragment in the identification of BHPs.

Reading the spectrum from right to left (Fig. 2.3) there is a decrease in the relative abundance of the peaks which is characteristic of polyhydroxylated BHPs and many other BHPs.

Four structures related to BHT were identified during the course of this work including an unsaturated BHT (Fig. 2.4) and the C-2, C-3 (Fig. 2.5) and C-12 methylated forms of BHT (Fig. 2.7).

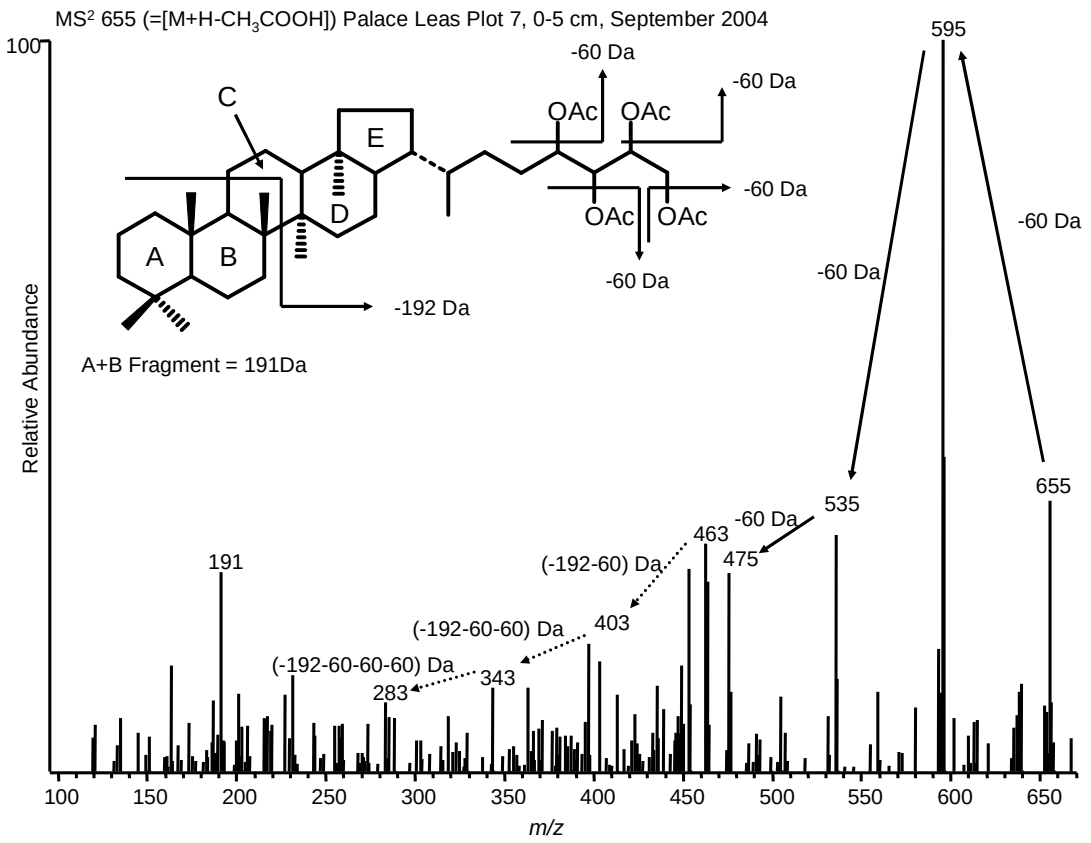


Fig. 2.3. APCI-HPLC-MS^2 Spectrum of Bacteriohopane-32,33,34,35-tetrol, as a tetra-acetate (**1a'**). Identified by comparison with Talbot et al., 2001; 2003a; b,

The unsaturated form of BHT (**5a** or **6a**) gives a very similar spectrum to that observed in BHT (Fig 2.3). However the unsaturation on the triterpenoid ring system (Fig. 2.4) results in a base peak that is 2 units less than that of the saturated BHT (**1a**), resulting in each of the subsequent fragments being 2 less than the saturated form (Fig. 2.3) giving fragment sequences of *m/z* = 653, 593, 533 and 473 when compared to the saturated sequence of *m/z* = 655, 595, 535 and 475. The fragmentation of the C-ring would be expected to produce an *m/z* = 189 fragment however the fragment m/z = 191 is formed instead (Talbot et al., 2007b). The presence of the relatively intense m/z = 449 fragment is indicative of unsaturation at C-6 (Talbot et al., 2007b). The presence of the m/z = 635 ion indicates the loss of an unacetylated hydroxyl ion from the structure giving a mass loss id – 18 Da (Fig. 2.4).

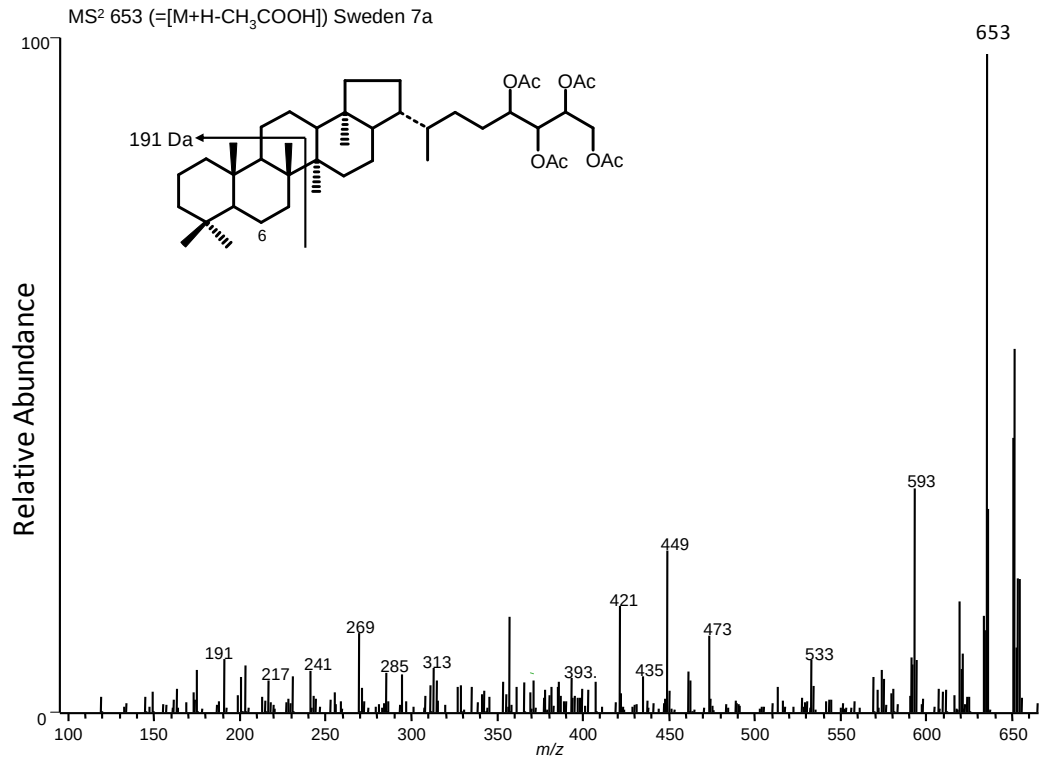


Fig. 2.4 APCI-HPLC-MS^2 Spectrum of Δ^6- Bacteriohopane-32,33,34,35-tetrol, as the tetra-acetate (**5a'**). Identified by comparison with Talbot et al., 2007b; 2008a.

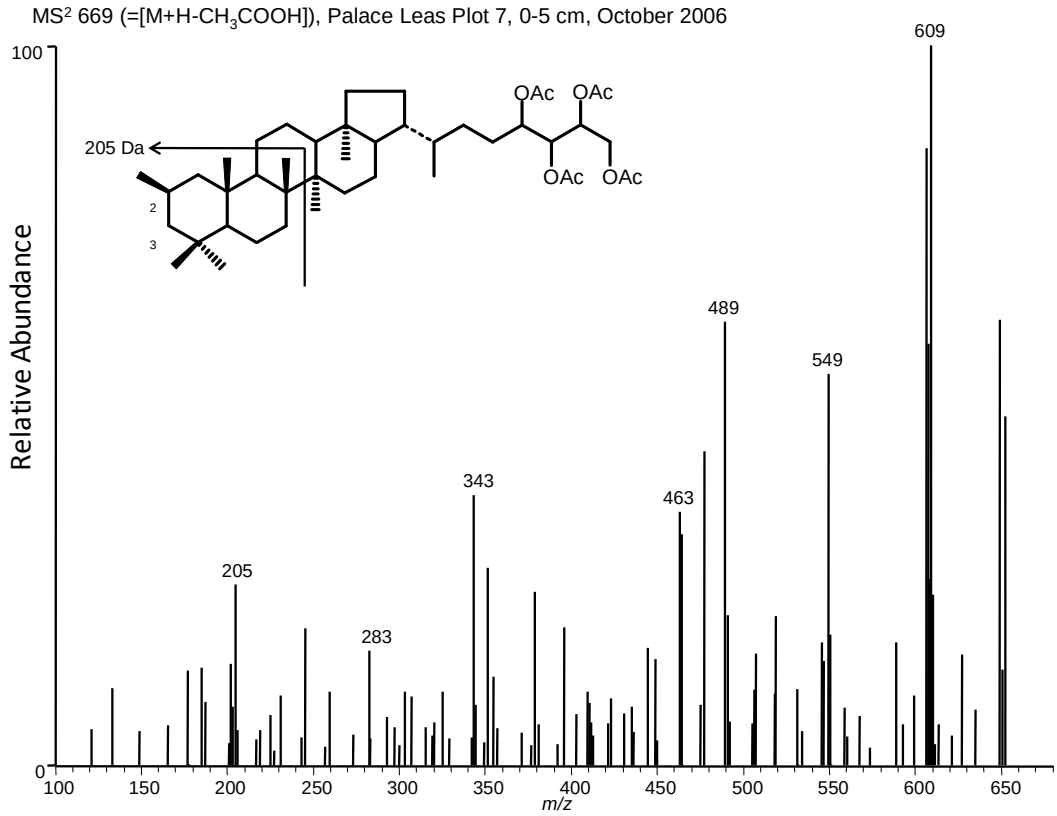


Fig. 2.5. APCI-HPLC-MS^2 Spectrum of 2-methyl Bacteriohopane-32,33,34,35-tetrol as tetra-acetate (**2a'**). Identified by comparison with Talbot et al., 2003b; 2007b.

Three methylated BHT structures were identified, each with a base peak 14 Da higher than the non-methylated tetrol at *m/z* 669 (**2a**, **3a**, **4a**). The position of the methylation on the triterpenoid ring system is indicated by the loss of the methylated A+B rings to give a fragment *m/z* = 205 (Fig. 2.5). As with BHT the spectrum shows the characteristic loss of an increasing number of acetylated hydroxyls in 2 suites; *m/z* = 609, 549 and 489 with the second set including the loss of the methylated A+B rings to give identical fragments to BHT; *m/z* = 463, 403, 343 and 283

It is not possible to distinguish between methylation at C-2 and C-3 based upon the spectrum fragmentation (Fig. 2.4). However, C-3 methylated BHT is known to elute later that C-2 methylated BHT (Fig. 2.6), (Cooke et al., 2008b). Confirmation of the position of methylation was carried out using GCMS analysis of BHP (Section 2.8).

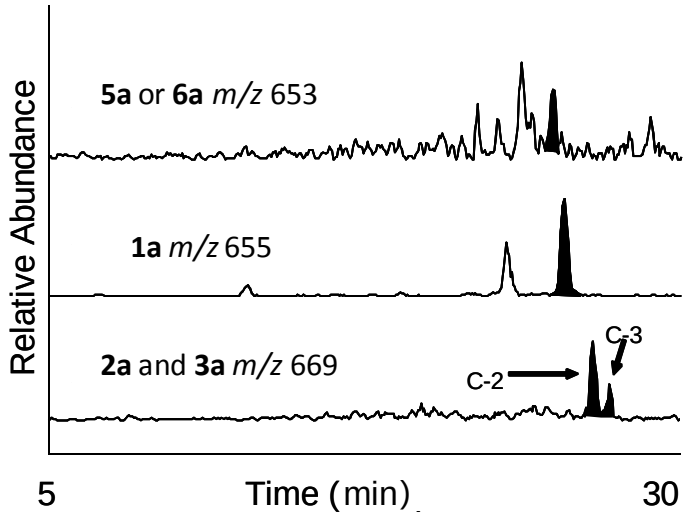


Fig. 2.6. Chromatograms indicating relative elution times for unsaturated BHT (**5a** or **6a**), BHT (**1a**), 2-methylBHT (**2a**) and 3-methyl BHT (**3a**). From Palace Leas Plot 2, January 2005, 0-5 cm.

A further methylated form of BHT was tentatively identified during this research as being 12-methyl BHT (**4a**) (Fig. 2.7), although it had previously been identified in the sponge *Plakortis simplex* by NMR (Costantino et al., 2000). Whilst the base peak ion of *m/z* = 669 indicates and additional methyl group relative to BHT (**1a**), the presence of strong *m/z* = 191 in the spectrum clearly indicates that the methylation could not be at position C-2 or C-3 (i.e. on the A or B rings). The fragmentation of the C ring results in the formation of a double bond between C-11 and C-12 (Talbot et al., 2007b). Normally this is formed by the breaking of a C-H bond on C-12. If C-12 were methylated then, as the bond energy for C-C bonds is less than C-H bonds, 348 kJ mol^{-1} compared to 412 kJ mol^{-1}, the C-C bond would break preferentially removing

the methyl at C-12 from the fragment. This can be clearly seen in Fig 2.7 where fragments are clearly shown to indicate the presence of methylation, 609, 549 and 489 which match the fragments seen in Fig 2.5. However the second sequence of fragments, 463, 403 and 283 (note that 343 is not clearly identified in this spectrum) which with the *m/z* = 191 fragment matches the fragments seen in BHT (**1a**) (Fig. 2.3). The most logical explanation for this sequence of fragments is the positioning of the methyl group at C-12. The methodology used in this research is unable to identify the stereochemistry for this methyl group and has been excluded from the molecular structure (Fig2.7)

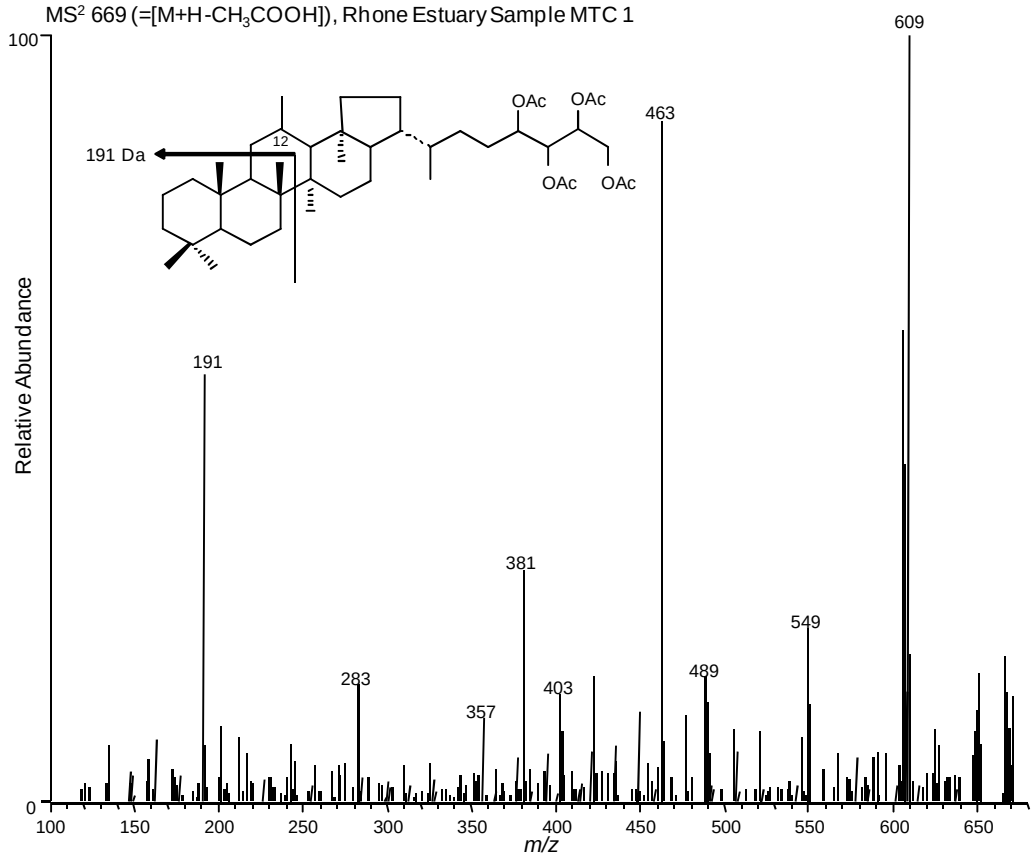


Fig. 2.7 APCI-HPLC-MS^2 Spectrum of 12-methyl Bacteriohopane-32,33,34,35-tetrol (**4a'**) as a tetra-acetate. Identified by comparison with BHT and 2me BHT

None of the other known purely hydroxylated BHPs including the bacteriohopanepentols (**1m**) (e.g. Talbot et al., 2008a) and bacteriohopanehexols (**1b**) (Talbot and Farrimond 2007) were observed during this work.

2.7.2 Bacteriohopanepolyols with sugar moiety at C-35

This group of BHPs contains a pentose at the C-35 position which is linked to the rest of the structure by an ether bond (Fig. 2.8). All of the other functional groups on the BHP are simple hydroxyls. The presence of the hydroxyls on the side chain and in the terminal sugar group results in the structure having characteristic base peak ions of $[M+H-CH_3COOH]^+$ (Talbot et al., 2008a) giving a base peak ion of *m/z* = 943 (**1n'**) for the most common saturated tetrafunctionalised form (Fig. 2.8). Other observed structures are an unsaturated form (**5n**) (Fig. 2.9) and a methylated form, either C-2 (**2n**) or C-3 (**3n**) (Fig. 2.10) all with a terminal group indicated by the same ion *m/z* = 331 (Figs. 2.8 – 2.10).

The loss of the terminal sugar moiety from the saturated tetrafunctionalised form (**1n**) results in a fragment identical to BHT (**1a**) (Fig. 2.3) with the same characteristic fragments, i.e. the sequential loss of hydroxyls resulting in a final fragment of *m/z* = 475 and splitting the C-ring to give the *m/z* = 191 A+B ring fragment. The loss of hydroxyls prior to the loss of the terminal sugar moiety results in the presence of fragments *m/z* = 883, 823 and 763 (absent from Fig 2.8). The loss of the terminal sugar group can result in the ether linking oxygen transferring to the sugar group giving a fragment of *m/z* = 347 (Fig 2.9).

The $\Delta^{6 \text{ or } 11}$ unsaturated form (**5n** or **6n**) (Fig. 2.9) and the C-2 (**2n**) or C-3 (**3n**) methylated form (Fig 2.10) both produce an *m/z* = 331 fragment due to the loss of the sugar and subsequently give characteristic fragmentations that are equivalent to those seen in the unsaturated and methylated forms of BHT (**5a** and **6a** respectively) (Figs. 2.4 and 2.5 respectively). These structures also produce fragments due to the loss of hydroxyls prior to the loss of the terminal sugar group, with the unsaturated fragments, *m/z* = 881, 821 and 761 due to the structure being 2 mass units less than the saturated form (Fig. 2.9) and 14 mass units higher due to the methylation, *m/z* = 897, 837 and 777 (Fig 2.10). The presence of the methylation at C-2 or C-3 results in the presence of an *m/z* = 205 fragment formed by the A+B rings, identical to that observed in the methylated BHT (**2a**) (Fig 2.5).

The absence of a strong *m/z* = 449 ion from the unsaturated form (Fig. 2.9) means that the position of the unsaturation cannot to identified more accurately than $\Delta^{6 \text{ or } 11}$.

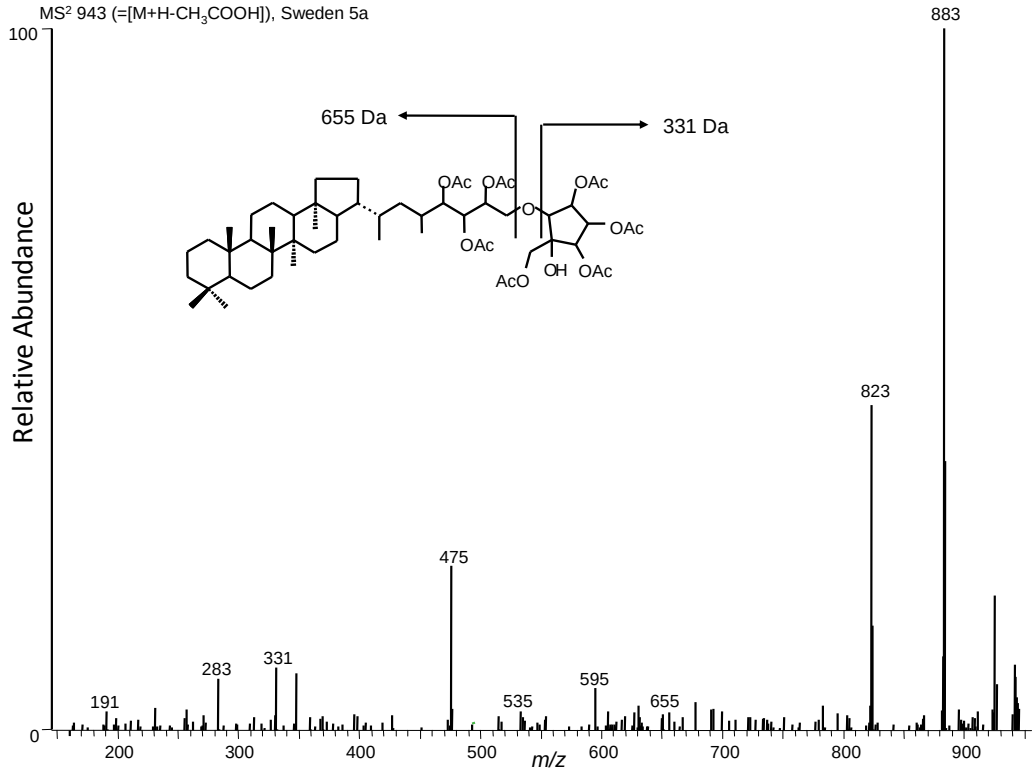


Fig. 2.8. APCI-HPLC-MS^2 Spectrum of BHT pentose as hepta-acetate (**1n'**). Identified by comparison with Talbot et al., 2008a

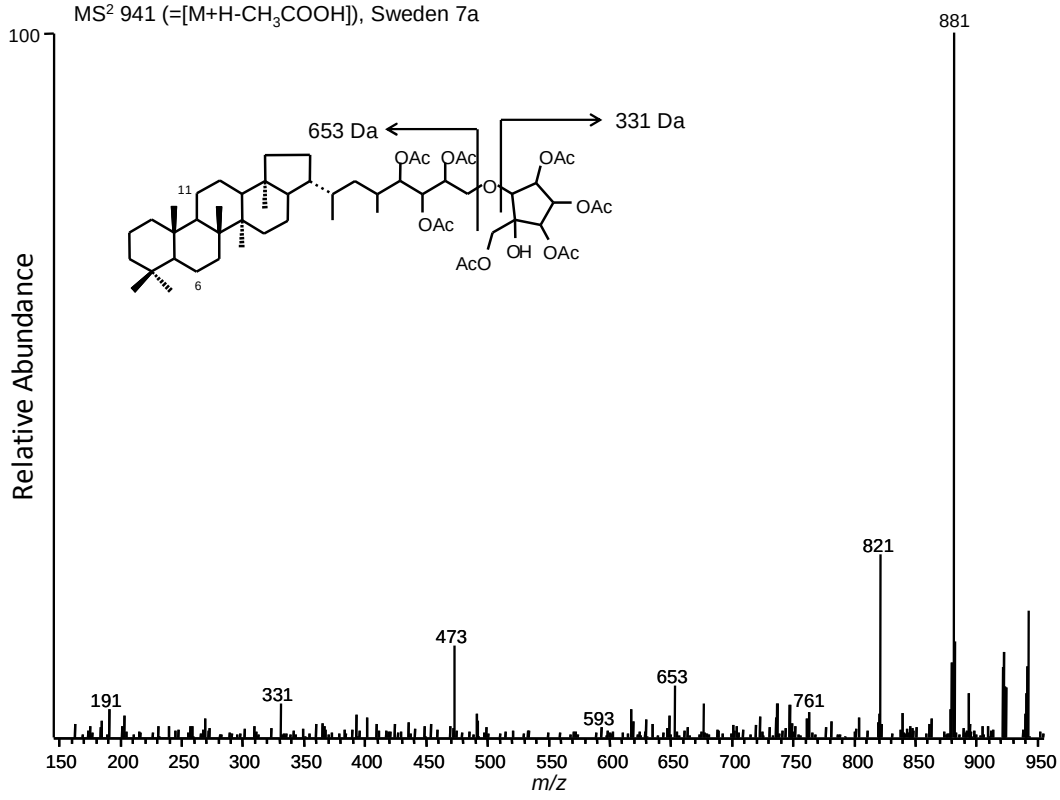


Fig. 2.9. APCI-HPLC-MS^2 Spectrum of mono-unsaturated $\Delta^{6 \text{ or } 11}$ BHT pentose as hepta-acetate (**5n'** or **6n'**). Identified by comparison with Talbot et al., 2008a.

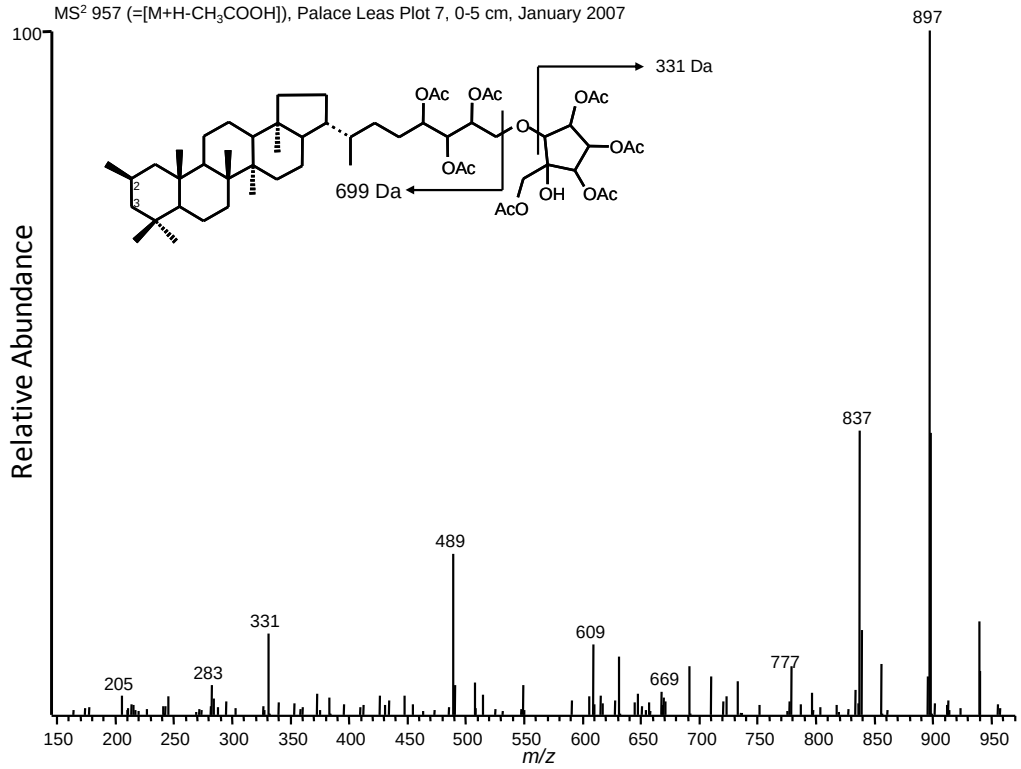


Fig. 2.10. APCI-HPLC-MS^2 Spectrum of 2 methyl BHT pentose as hepta-acetate (**2n'**). Identified by comparison with Talbot et al., 2008a.

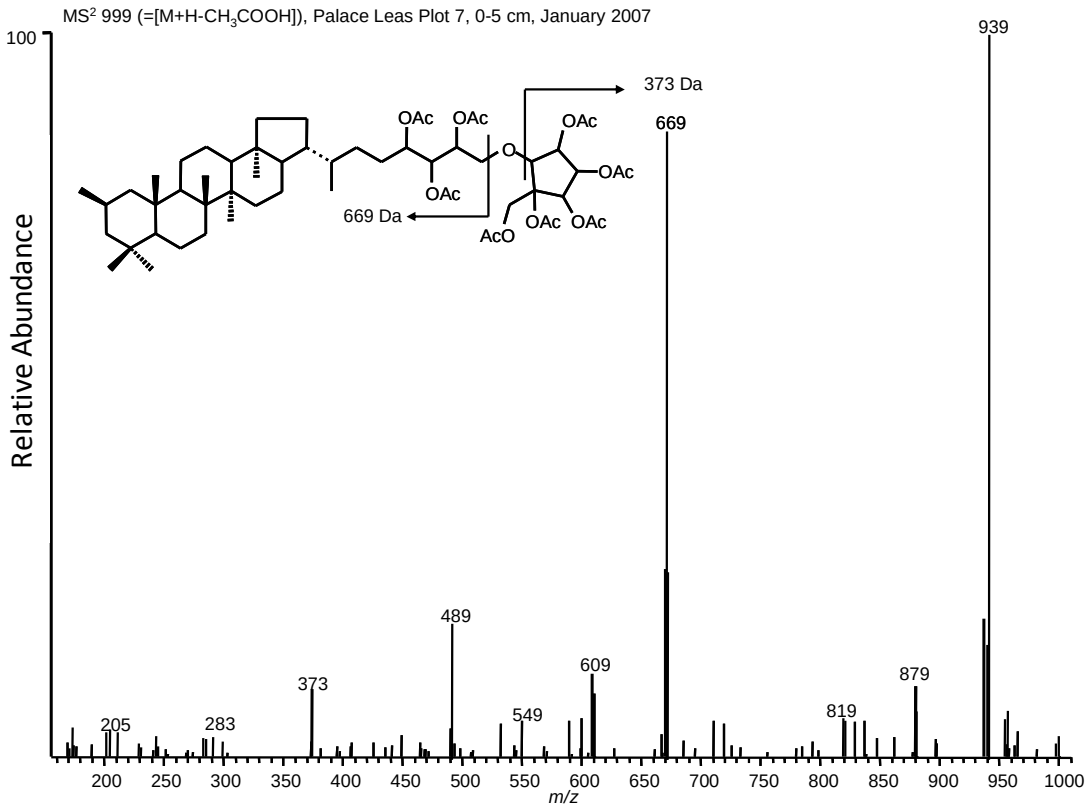


Fig. 2.11. APCI-HPLC-MS^2 Spectrum of 2 methyl BHT pentose as octa-acetate (**2n''**). Identified by comparison with Fig. 2.10, containing 7 acetylated hydroxyls.

It has been observed that acetylation of some BHPs, particularly those containing a tertiary hydroxyl in a terminal group sugar, can result in the observation of two different acetylated forms as the hydroxyls on the terminal sugar do not always undergo complete acetylation (e.g. Talbot et al., 2003c). Two observed MS^2 spectra indicating composite tetrafunctionalised BHPs, which differed by 42 Da (CH_2CO) from the form in Fig. 2.10, equivalent to one additional acetylation. The fragmentation of both structures follows an identical path with the structure in Fig.2.11 having a mass of +42 Da when compared to that in Fig. 2.10. For the fragments that show a loss of hydroxyls and not the loss of the terminal sugar (*m/z* = 939, 879 and 819 in Fig. 2.11 and *m/z* = 897, 837 and 777 in Fig. 2.10). The presence of the *m/z* = 373 ion (Fig. 2.11) when compared to the *m/z* =331 (Fig 2.10), confirms that the terminal sugar group has an additional acetylation when compared to the structure in Fig. 2.8. In these cases of multiple degrees of acetylation the quantification was calculated using the combined peak areas and a single semi-quantitative value obtained.

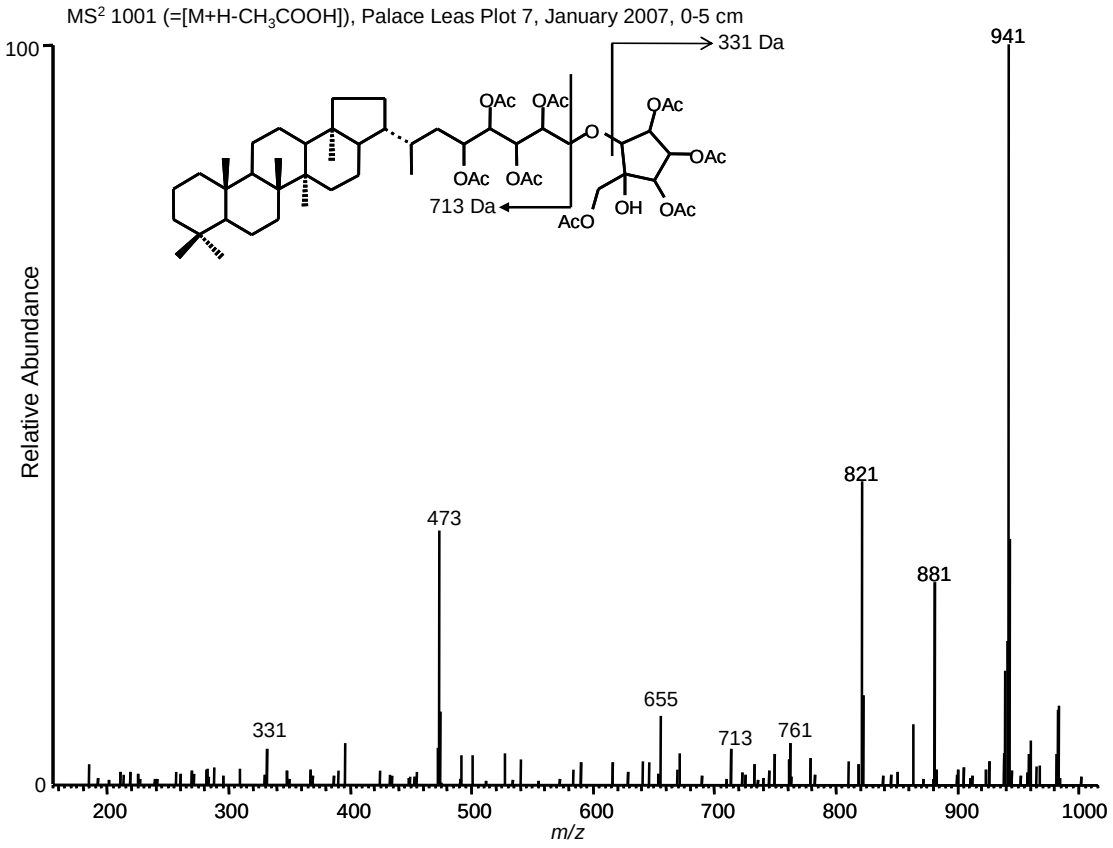


Fig. 2.12. APCI-HPLC-MS^2 Spectrum of Bacteriohopanepentol pentose as octa-acetate (**1o'**). Identified by comparison with Talbot and Farrimond 2007, Talbot et al., 2007b

Comparison of Fig. 2.12 with Fig. 2.8 indicates that there is an increase in the number of functional groups from 4 to 5. The increase can be identified by the presence of *m/z* = 473 and the serial loss of 4 hydroxyls. The loss of all the functional groups from the tetrafunctionalised form results in a fragment *m/z* = 475, the loss of a fifth functional group from the pentafunctionalised form will result in a fragment of *m/z* = 473 due to the formation of an additional double bond in the side chain structure, formed when the final hydroxyl is lost from the structure.

The loss of 4 hydroxyls from the side chain in addition to the loss resulting in the base peak *m/z* = 1001 is shown in the sequence of fragments *m/z* = 941, 881, 821 and 761. The presence of an *m/z* = 713 fragment indicates the removal of the terminal group leaving a fragment that contains 4 hydroxyls (c.f. BHT (**1a**), Fig. 2.3 where 3 remaining hydroxyls form a fragment of *m/z* = 655).

2.7.3 Bacteriohopanepolyols with terminal amino glycoside (glucosamine) or carbon pseudopentose group

This group of BHPs is amongst the most abundant and commonly seen during this study. As all of these structures contain one or more nitrogen atoms within the terminal group at C-35 they are all identified by a base peak ion of $[M+H]^+$ formed by the protonation of the acetylated amine group.

In addition to the tetra-functionalised form (**1d**) (Fig. 2.13) a wide range of unsaturation (**5d**), methylation (**2d** and **3d**) and increased functionalisation (penta- (**1l**) and hexafunctionalisation (**1k**)) as well as variation in the structure of the terminal C-35 group has been observed. In total 18 different BHP structures of this type were identified during this study with 7 novel structures being tentatively identified.

The tetrafunctionalised form produces a characteristic double peak on the chromatogram due to the presence of isomers in the structure (Fig 2.2). The tetrafunctionalised form (Fig. 2.13) produces a characteristic set for fragmentations beginning with the serial loss of hydroxyls from the side chain giving fragments *m/z* = 942, 882 and 822. The loss of the terminal group gives a characteristic fragments of *m/z* = 300 or 348, depending on the location of cleavage, and *m/z* = 655 (Fig. 2.13). Subsequent loss of hydroxyls after the removal of the terminal group will give

fragments of *m/z* = 595, 535 (absent in Fig 2.13) and 475, identical to those observed in BHT (**1a'**) (Fig. 2.3). The removal of the A+B rings, as seen in the previous BHPs will result in an *m/z* = 191 fragment and a 283 fragment, if all hydroxyls have previously been removed. The cyclitol ether functional group, *m/z* = 330, in BHT cyclitol ether (**1d**) (Fig. 2.13) gives a low intensity peak due to its relatively poor stability.

There is a second form of with a glucosamine terminal group (**1g**) as opposed to the cyclitol ether group (**1d**). The glucosamine terminal group in BHT glucosamine (**1g**, *m/z* = 330; Fig. 2.14) is much more stable and produces a characteristic high intensity peak due to the presence of the ether bond (Talbot et al., 2003c and 2007a). This more stable fragment can lose 2 hydroxyls from the fragment giving a sequence of fragments *m/z* = 330, 270 and 210, a sequence that is not seen in the BHT cyclitol ether (**1d**) due to the low stability of this fragment. In other respects the fragmentation of BHT cyclitol ether (**1d**) and BHT glucosamine (**1g**) are identical with serial losses of hydroxyls and fragmentation of the C-ring to give an A+B ring fragment.

The loss of the terminal functional group gives a set of fragments that are identical to those observed in the polyhydroxylated BHPs (**1a'**) (Section 2.7.1) and the BHPs with sugar moiety at C-35 (**1n'**) (Section 2.7.2), with changes due to unsaturation (**5g'**) and methylation (**2g'**) having the same effect on the sequence of fragments, i.e. unsaturation reducing the mass of the fragments by 2 Da (c.f. Figs. 2.13 and 2.15 with Figs. 2.8 and 2.9) and methylation increasing the mass of the fragments by 14 Da (c.f Figs 2.13 and 2.15 with Figs. 2.8 and 2.9).

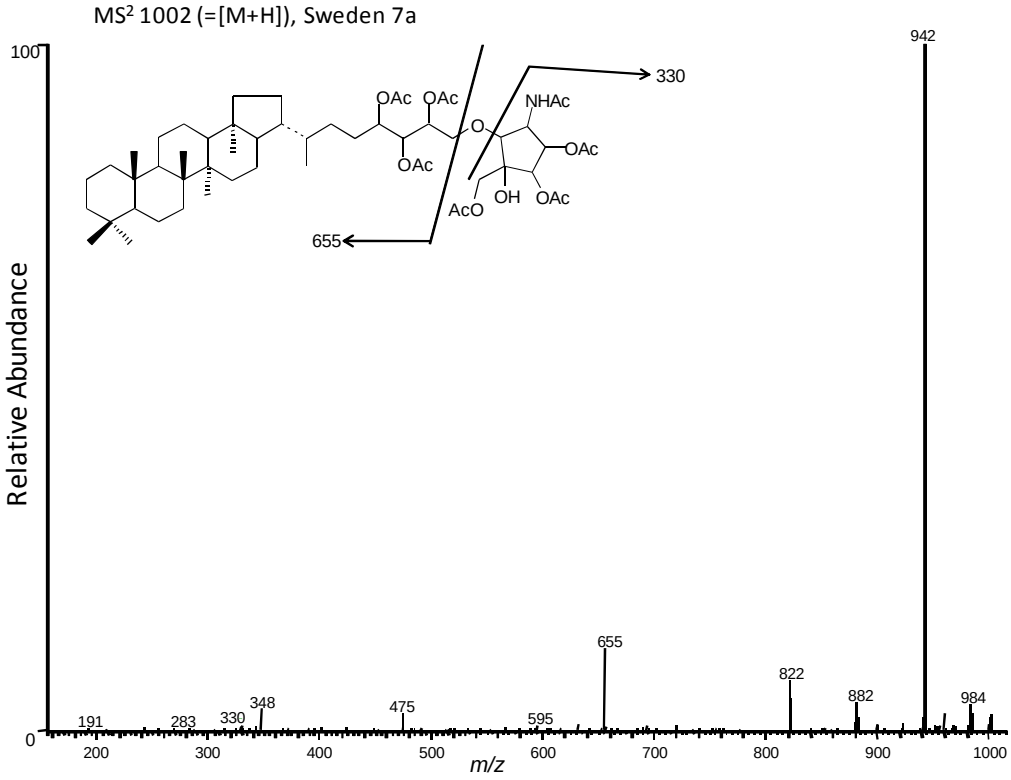


Fig. 2.13. APCI-HPLC-MS^2 Spectrum of Bacteriohopanetetrol cyclitol ether (**1d'**) as hepta-acetate. Identified by comparison with Talbot et al., 2003a.

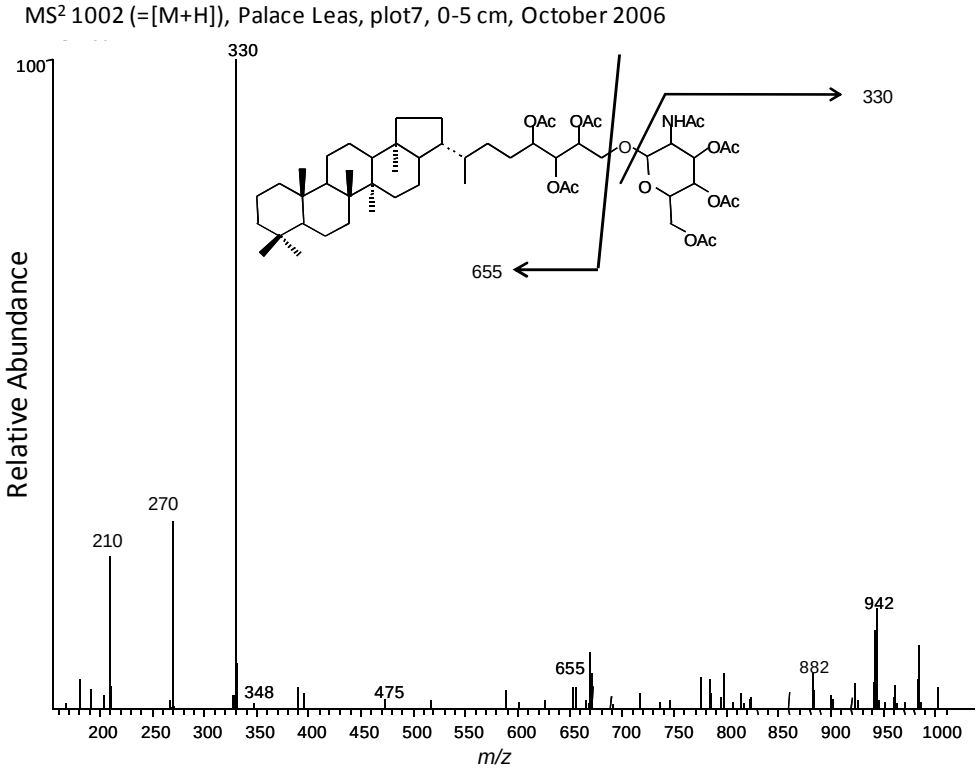


Fig. 2.14. APCI-HPLC-MS^2 Spectrum for Bacteriohopanetetrol glucosamine (**1g'**) as hepta-acetate. Identified by comparison with Talbot et al., 2003c; 2007a.

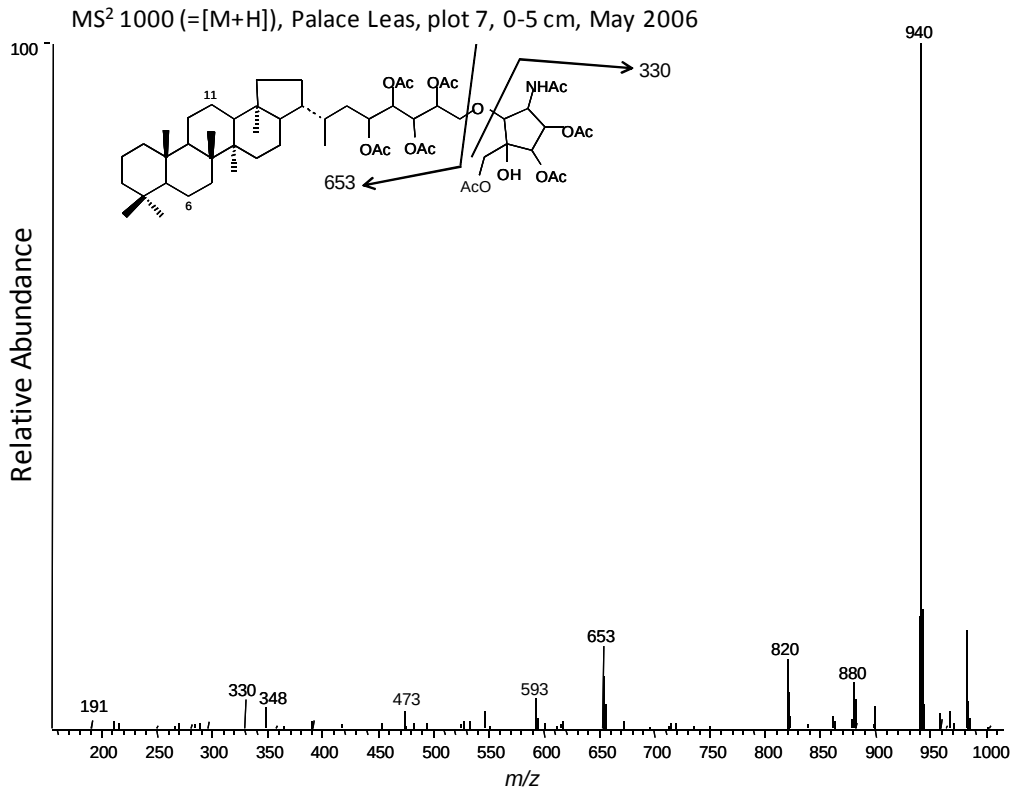


Fig. 2.15. APCI-HPLC-MS^2 Spectrum of mono-unsaturated $\Delta^{6 \text{ or } 11}$ unsaturated Bacteriohopanetetrol cyclitol ether (**5d'** or **6d'**) hepta-acetate. Identified by comparison with Talbot et al., 2007a; 2008a.

Three methylated forms of BHT cyclitol ether (Figs. 2.16 and 2.17) were found in the samples, with the methylation in Fig 2.15 believed to be at positions C-2 (**2d**) and C-3 (**3d**). Identification of these different structures was based upon the different elution times as illustrated above for the two different methylated forms of methyl BHT (Fig. 2.5). The methylation at C-3 was observed for the first time during this research.

In addition to these two forms a less common form was identified where there is an extra carbon on the terminal group, either as a methylation or as an additional CH_2 in the ring to give a 6-membered ring (**1p**). The differences are that C-2 or C-3 methyl BHT cyclitol ether (**2d** and **3d**) contains fragments *m/z* = 669 (c.f. C-2 methyl BHT (**2a**), Fig 2.5) and *m/z* = 330 indicating the terminal group which is identical to that observed in BHT cyclitol ether (**1d**) Fig. 2.13). However in *m/z* = 1016 with extra carbon on the terminal sugar (**1o**) these 2 fragments are replaced by *m/z* = 344 and 655, indicating the triterpenoid ring system does not contain extra methylation (c.f. BHT (**1a**), Fig 2.3) and the extra carbon is now on the terminal sugar group. The location of the methylation on the terminal sugar group could not be identified. All forms of methylation were also identified in an unsaturated form (**7d** and **5p**) (Figs

2.18 and 2.19). It must be noted that the ions *m/z* = 390 and 538 (Fig 2.18) are most likely derived from an unknown coeluting structure and not from the unsaturated methyl bacteriohopanetetrol cyclitol ether.

The presence of an unacetylated hydroxyl on the terminal group enables extra acetylation to occur as seen in Fig. 2.11, with the formation of the octa-acetate. This adds 42 Da to the mass of the terminal group fragment and give base peaks that are therefore +42 Da, e.g. BHT cyclitol ether (**1d'**) will have base peaks of *m/z* = 1002 and 1044. The methylated and unsaturated forms can also have this extra acetylation and will have base peaks that are correspondingly +42Da. The glucosamine terminal group has no unacetylated hydroxyls and therefore does not undergo the extra acetylation (Fig. 2.14).

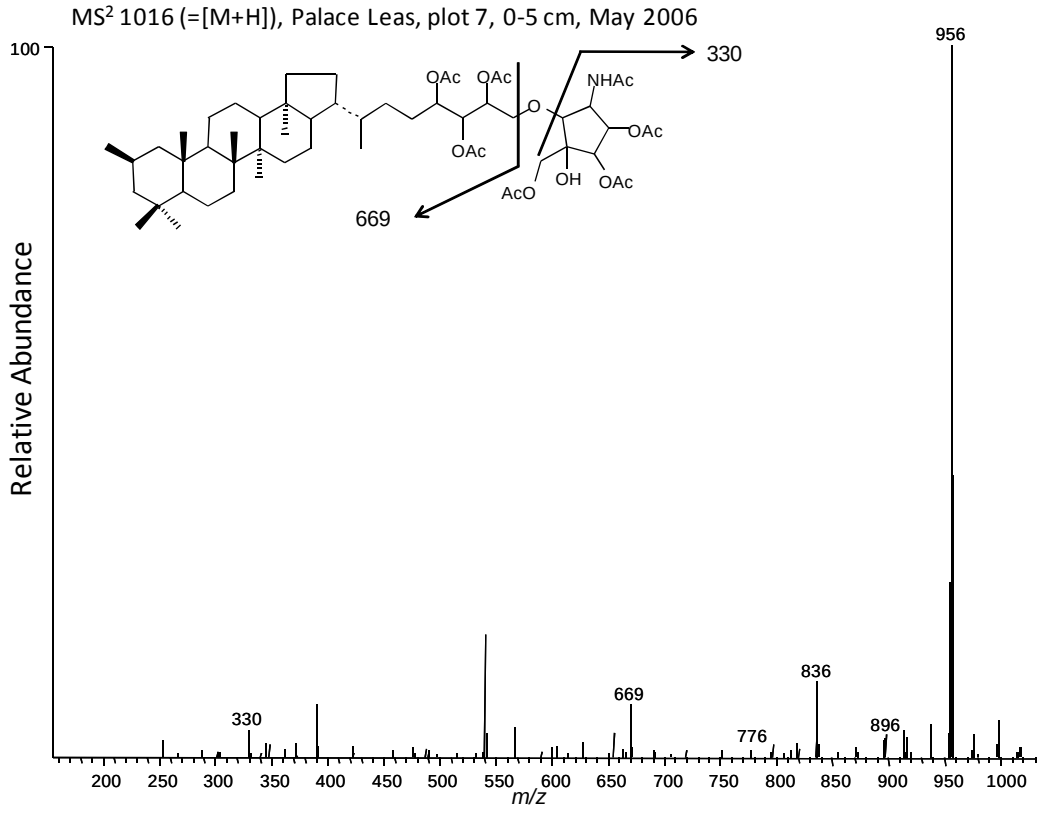


Fig. 2.16. APCI-HPLC-MS^2 Spectrum of 2-methyl BHT cyclitol ether (**2d'**) as hepta-acetate. Identified by comparison with Talbot and Farrimond 2007.

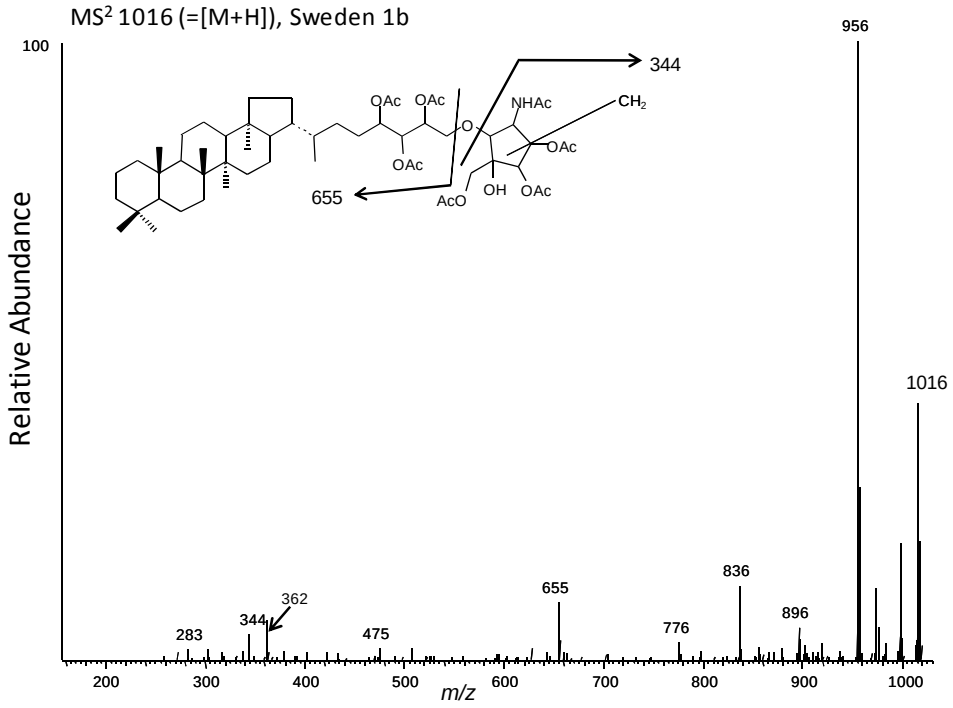


Fig. 2.17. APCI-HPLC-MS^2 Spectrum for bacteriohopanetetrol cyclitol ether hepta-acetate with extra carbon on terminal group (**1p'**). Tentatively identified for the first time during this research.

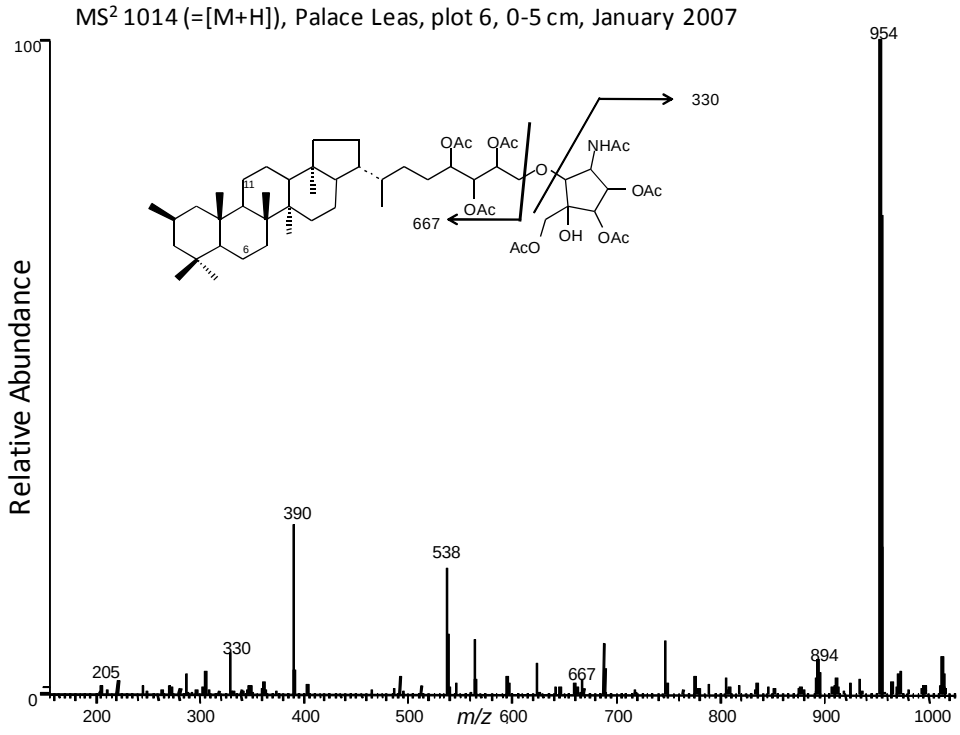


Fig 2.18. APCI-HPLC-MS^2 Spectrum for unsaturated methyl bacteriohopanetetrol cyclitol ether hepta-acetate (**7d'**). Tentatively identified for the first time during this research.

The differences between the spectra in Figure 2.19 and that for unsaturated 2-methyl BHT cyclitol ether (**7d**) (Fig. 2.18) are identical to those seen between 2-methyl BHT cyclitol ether (**2d**) and BHT cyclitol ether with methylation on terminal sugar (**1p**) (Figures 2.16 and 2.17), i.e. presence of fragments *m/z* = 344 not 330 and *m/z* = 653 not 667. Therefore the location of the extra carbon is on the terminal group. This structure was only observed at one sample location.

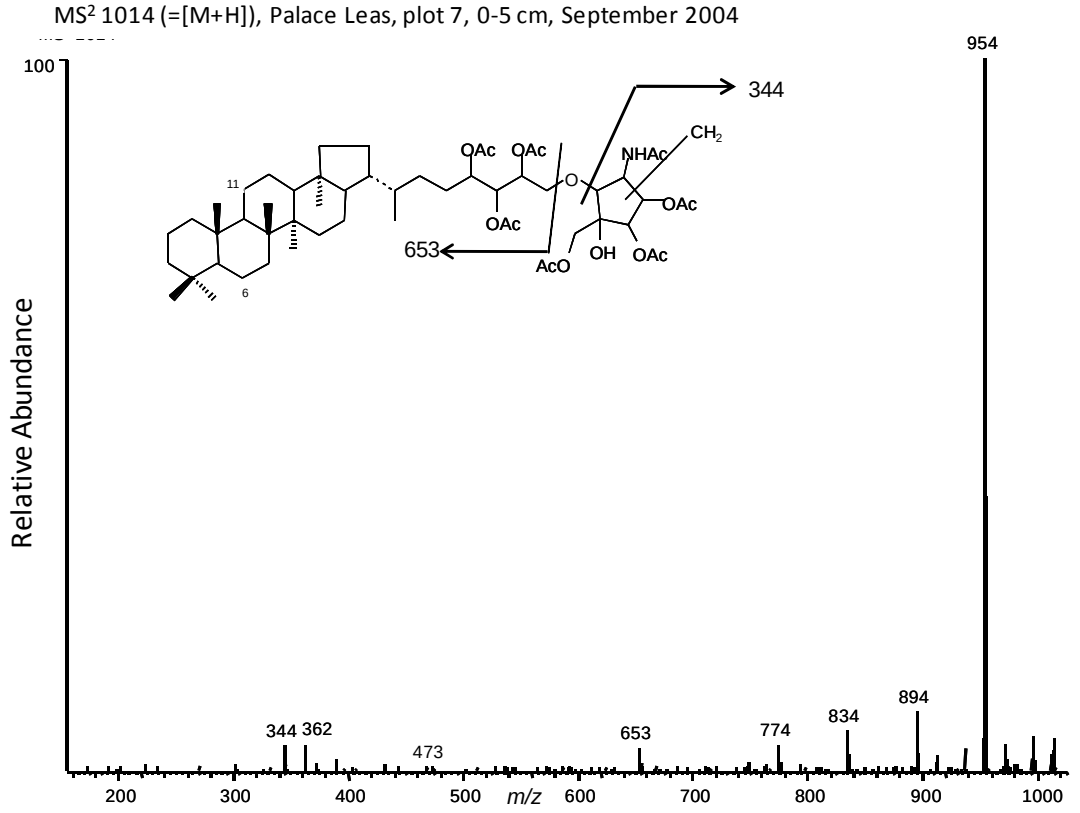


Fig. 2.19. APCI-HPLC-MS^2 Spectrum for unsaturated bacteriohopanetetrol cyclitol ether hepta-acetate with extra carbon on terminal group (**5p'**). Tentatively identified for the first time during this research.

A group of pentafunctionalised BHPs were identified (Figs. 2.20 to 2.23 inclusive), including unsaturated and methylated forms. Their identification is characterised by the base peak being +58 Da greater than the pentafunctionalised equivalent, the presence of m/z =713, indicating the loss of the terminal group (Fig 2.20) leaving a fragment containing 4 hydroxyls (c.f. Fig. 2.12). The subsequent removal of these 4 hydroxyls leaves a fragment of *m/z* = 473 (c.f. Figs. 2.12 and 2.20). The loss of the 4 hydroxyls gives 2 characteristic sequences of fragments, *m/z* = 1000, 940, 880 and 820 (prior to loss of terminal group) and *m/z* = 653, 593, 533 and 473 (after loss of the

terminal group). This structure can exist with an extra acetylation giving a base peak of *m/z* = 1102.

A pentafunctionalised form containing the glucosamine terminal group (**1q**) (Fig 2.21) was identified by the relatively high intensity of the terminal sugar fragment, *m/z* = 330, when compared to that of the pentafunctionalised cyclitol ether structure (**1l**) (Fig. 2.20).

The presence of the unsaturated and methylated pentafunctionalised forms was identified by the differences of -2 Da for unsaturated fragments (Fig. 2.22) and the addition of +14 Da for methylated fragments (Fig. 2.23) as seen in the other structures discussed (e.g. Figs. 2.13, 2.15 and 2.16).

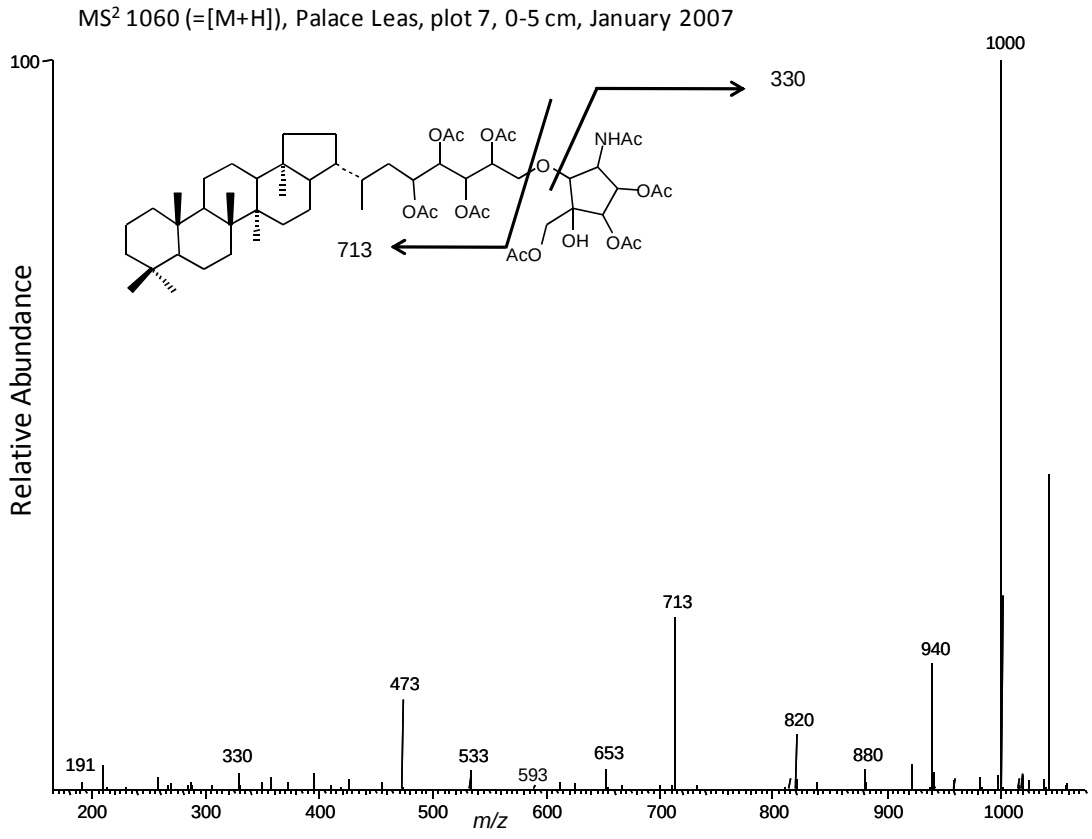


Fig. 2.20. APCI-HPLC-MS^2 Spectrum of Bacteriohopanepentol cyclitol ether octa-acetate (**1l'**). Identified by comparison with Talbot et al., 2007b.

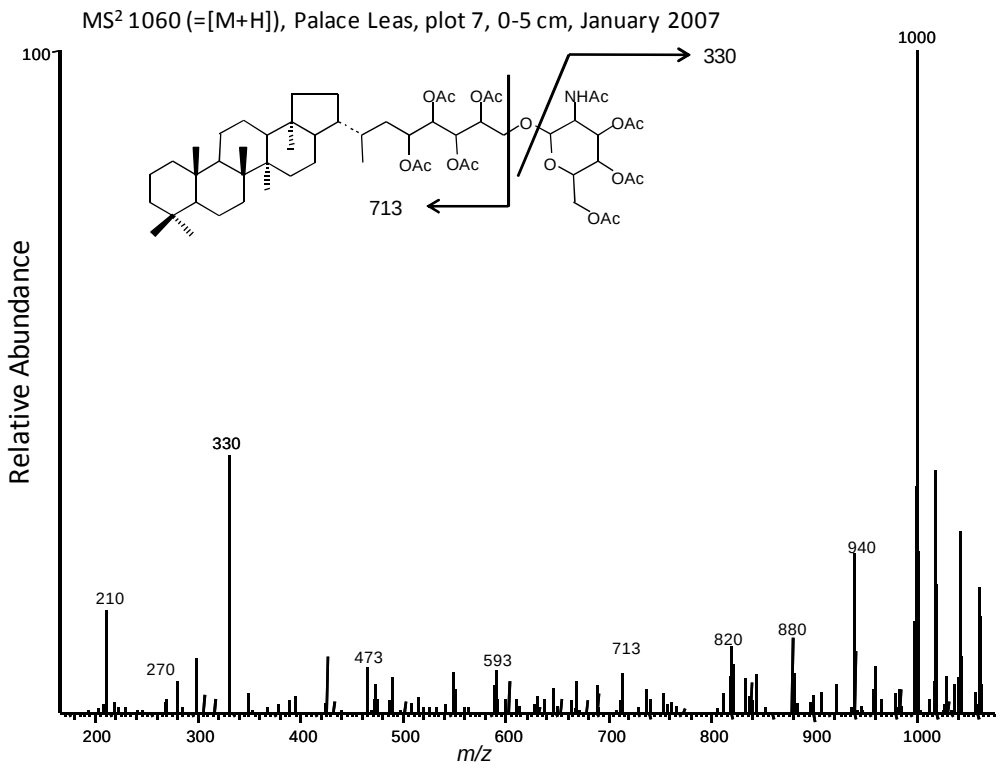


Fig. 2.21. APCI-HPLC-MS^2 Spectrum for Bacteriohopanepentol glucosamine octa-acetate (**1q'**). Identified by comparison with Talbot et al., 2007b.

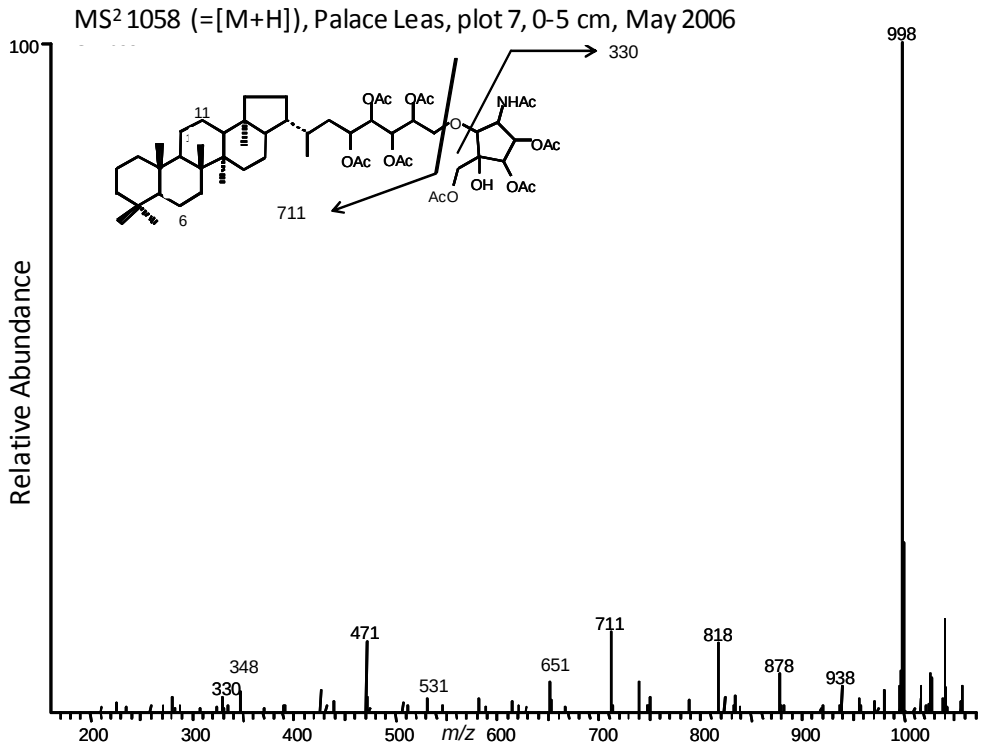


Fig. 2.22. APCI-HPLC-MS^2 Spectrum for unsaturated Bacteriohopanepentol cyclitol ether (**5l'**) octa-acetate. Identified by comparison with Talbot et al., 2007b.

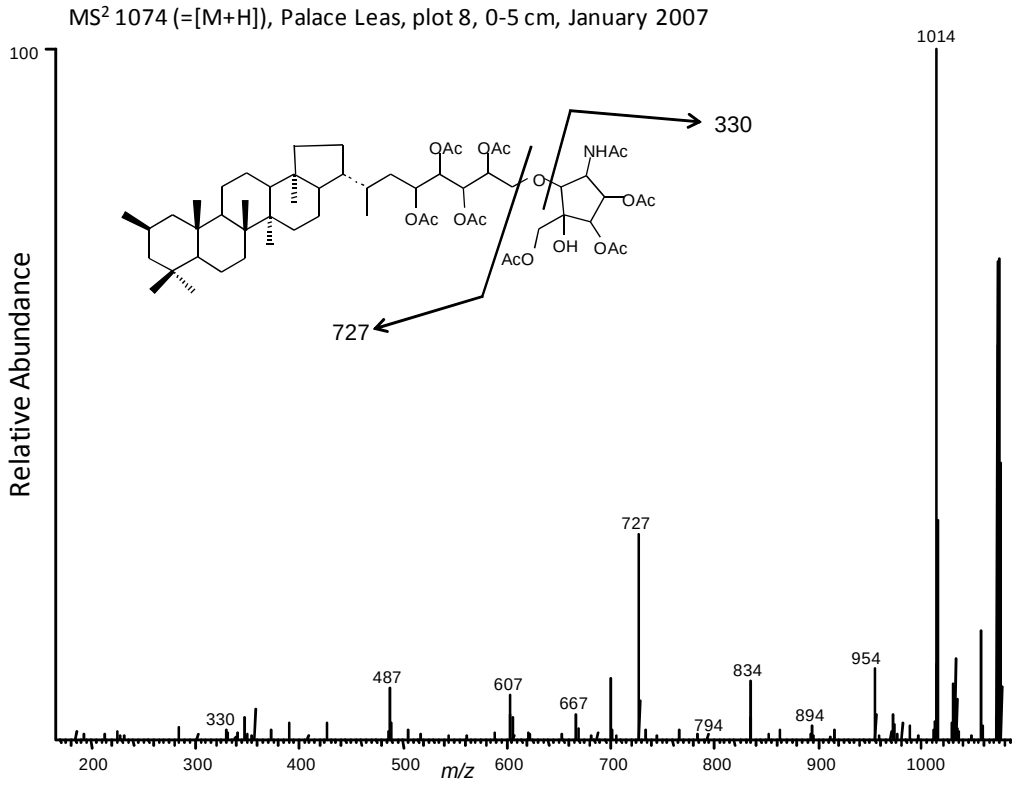


Fig. 2.23. APCI-HPLC-MS^2 Spectrum for 2-methylbacteriohopanepentol cyclitol ether octa-acetate (**2l'**). Tentatively identified for the first time during this research.

A group of hexafunctionalised structures with the cyclitol ether terminal group was identified. Bacteriohopanehexol cyclitol ether (Fig. 2.24, **1k'**, *m/z* = 1118) has a mass of +58 Da more than the pentafunctionalised form (**1l'**, Fig. 2.20) and forms a fragment *m/z* = 771 as a result of the removal of the terminal group indicating 5 hydroxyls on the side chain. The loss of these hydroxyls is confirmed by the loss of 5 sets of 60 Da as these hydroxyls are removed giving sequences of *m/z* = 1058, 998, 938, 878 and 818 (prior to removal of terminal group) and *m/z* = 711, 651, 591, 551 and 471 (after removal of terminal group). The fragment *m/z* = 471 indicates the ring structure after the removal of 6 functional groups (c.f. Figs. 2.13 and 2.20). The terminal group is identified by the presence of the *m/z* = 330 fragment.

Two other forms were identified, the C-2 or C-3 methylated form (**2k** or **3k**) (Fig. 2.25) and BHhexol cyclitol ether with an extra carbon on the terminal group (**1r**) (Fig. 2.26). The C-2 or C-3 methylated form (**2k** or **3k**) has a mass of +14 Da and the corresponding increase in mass of the fragments (c.f. Fig. 2.23). The BHhexol cyclitol ether with an extra carbon on the terminal group (**1r'**) produces a terminal group *m/z*

= 344 as seen in Fig 2.19 and a set of fragments identical to the unmethylated hexafunctionalised form (**1k'**) (Fig. 2.24).

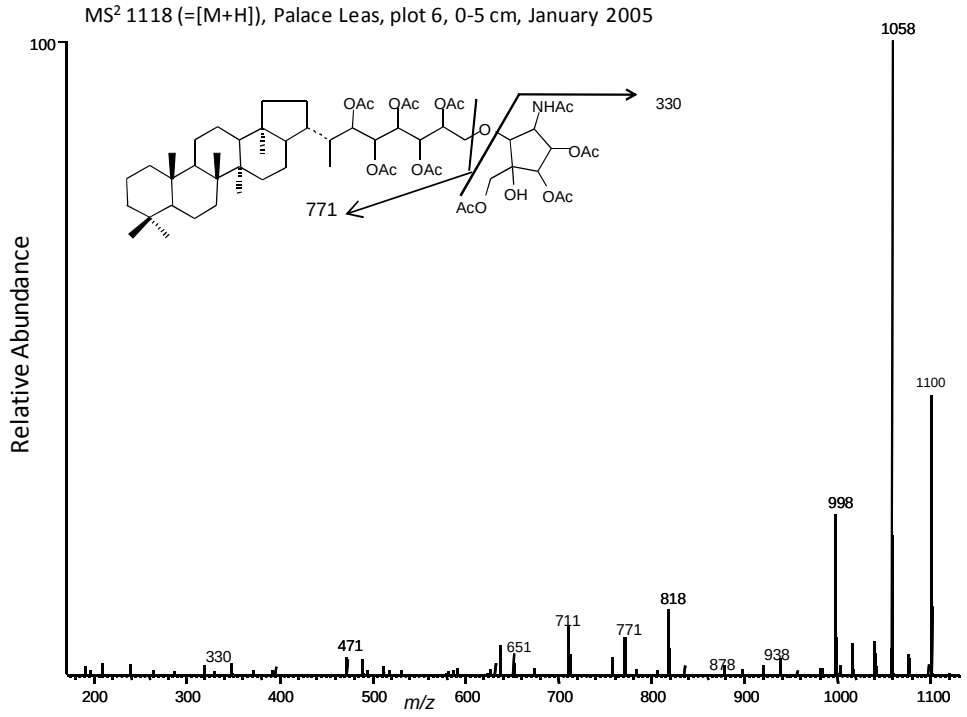


Fig. 2.24. APCI-HPLC-MS^2 Spectrum for Bacteriohopanehexol cyclitol ether nona-acetate (**1k'**). Identified by comparison with Talbot and Farrimond, 2007.

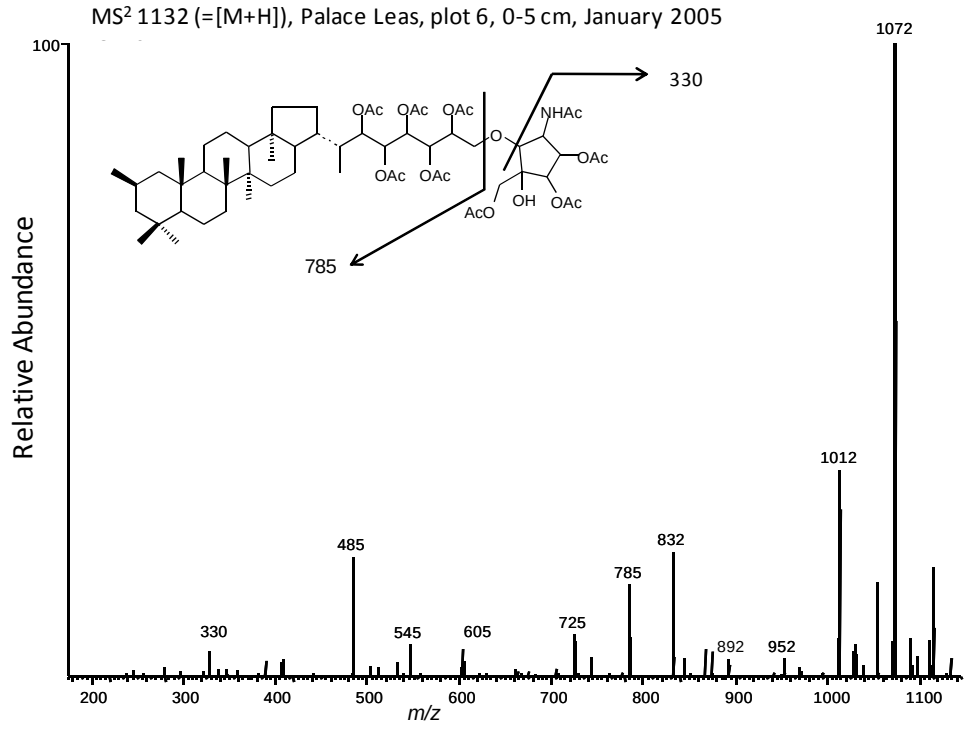


Fig. 2.25. APCI-HPLC-MS^2 Spectrum for 2methyl Bacteriohopanehexol cyclitol ether nona-acetate (**2k'**). Tentatively identified for the first time during this research.

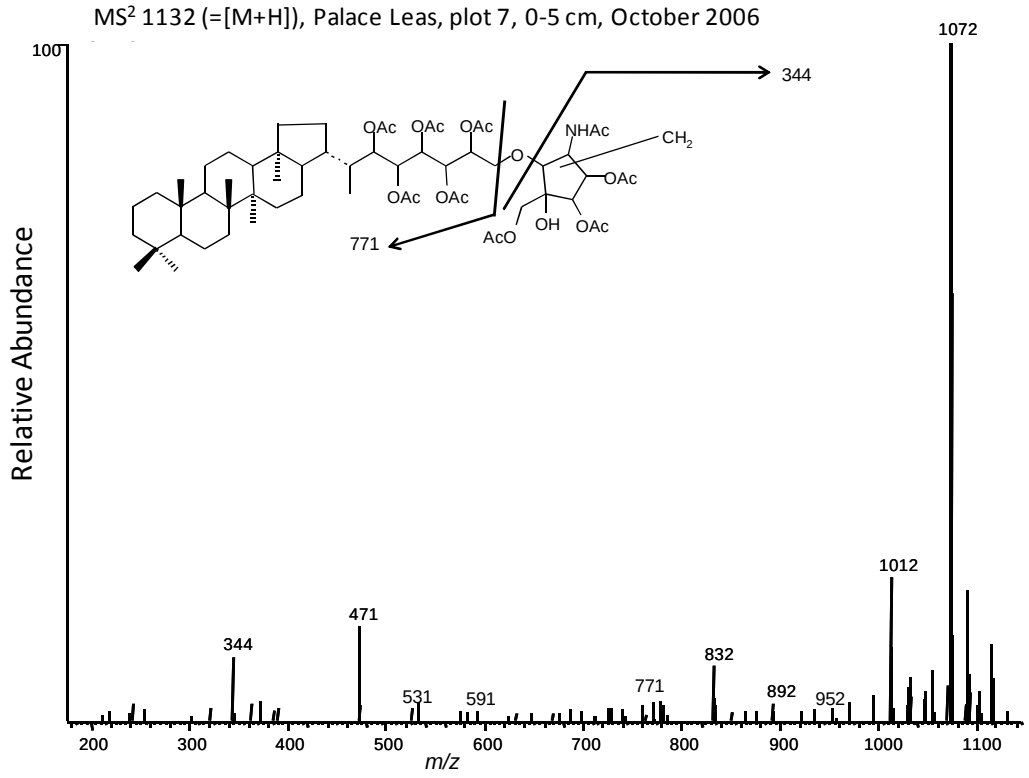


Fig. 2.26. APCI-HPLC-MS^2 Spectrum for Bacteriohopanehexol cyclitol ether nona-acetate with extra carbon on sugar (**1r'**). Tentatively identified for the first time during this research.

A final BHP structure in this group was identified as Guanidine-substituted bacteriohopanetetrol cyclitol ether (**1s**) (Fig. 2.26). This structure is readily protonated and produces a very stable characteristic ion of *m/z* 1086 ($[M+H]^+$) ion with little or no subsequent fragmentation observed in the MS^2 spectrum. In Fig. 2.27 the only observed fragmentation is the loss of a single hydroxyl giving *m/z* = 1026.

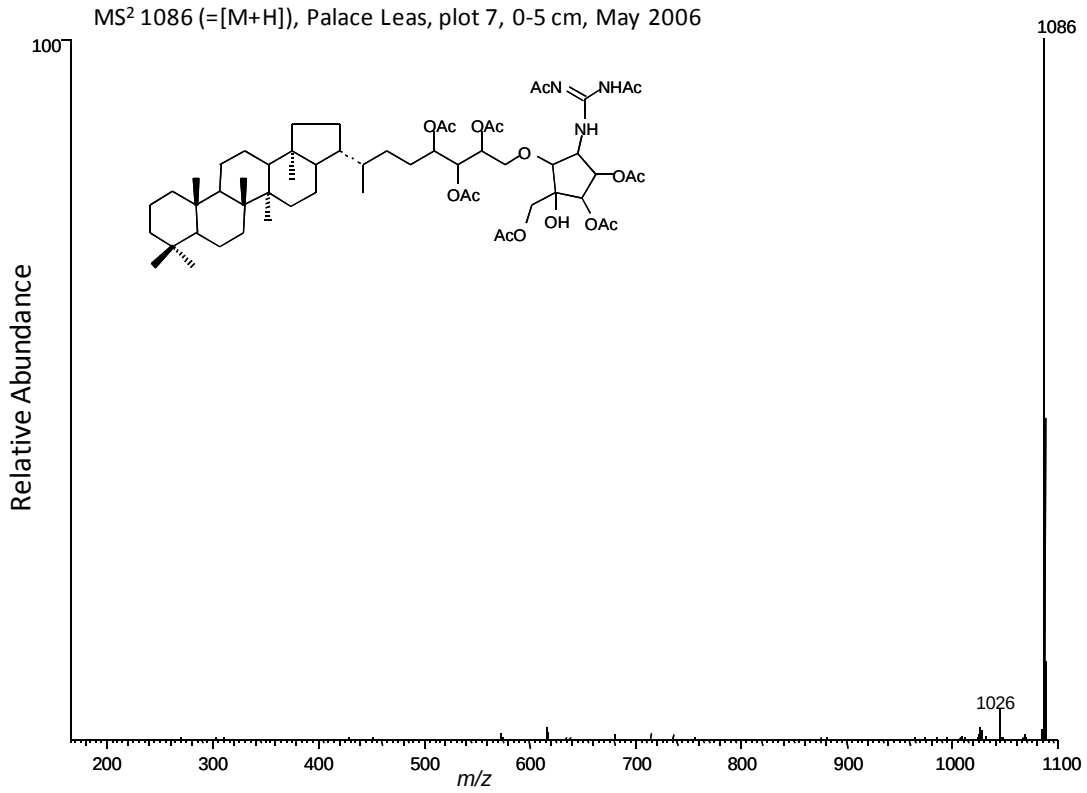


Fig. 2.27. APCI-HPLC-MS^2 Spectrum of Guanidine-substituted Bacteriohopanetetrol cyclitol ether octa-acetate (**1s'**).

2.7.4 35-Aminobacteriohopanepolyols

This group of BHPs contains an amino group at C-35 and are identified primarily by characteristic base peak ions of $[M+H]^+$ and by relative retention times (Talbot et al., 2003a,b). Tetra-, penta- and hexafunctionalised structures and also unsaturated and methylated structures were identified.

The most commonly observed structure is aminotriol (**1f**) (Fig 2.28). Once protonated this ion (m/z 714 = $[M+H]^+$) undergoes limited fragmentation due to the stability of the base peak ion, primarily in the side chain via loss of 3 acetylated hydroxyls (60 Da), to produce a characteristic sequence of *m/z* = 654, 594 and 534 (Fig. 2.28). An A+B fragment *m/z* = 191 is also sometimes seen.

The unsaturated aminotetrol (**5f**) has a very similar MS^2 spectrum (Fig. 2.29) with all ions 2 Da lower than the saturated form, giving fragments of *m/z* = 652, 592 and 532.

The two methylated forms (methylation at the C-2 (**2f'**) or C-3 position (**3f'**); Fig. 2.30) are 14 Da higher than the regular form giving fragments of *m/z* = 668, 608 and

548, with an A+B ring fragment of *m/z* = 205. The methylated forms are distinguished based on the differences in elution time (Fig. 2.6).

The pentafunctionalised aminotetrol (**1h**) (Fig. 2.30) and hexafunctionalised aminotetrol (**1c**) (Fig. 2.31) were also identified by the increase in base peak of +58 Da and +116 Da from the tetrafunctionalised form respectively, as seen in other structures, and in the sequential loss of -60Da for each loss of hydroxyl or -59Da for the amino group. The hexafunctionalised form (**1c'**) contains the m/z =471 fragment as seen in other hexafunctionalised structures (e.g. Fig. 2.24).

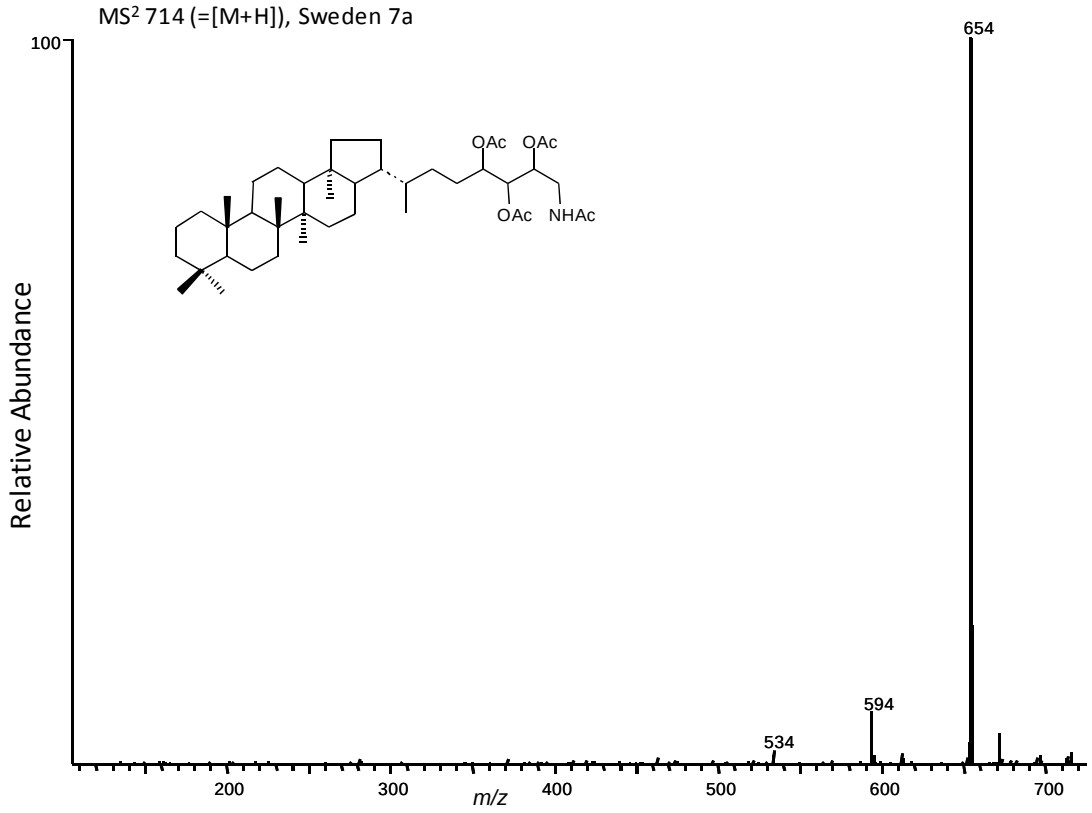


Fig. 2.28. APCI-HPLC-MS^2 Spectrum of Aminotriol (**1f'**) tetra-acetate. Identified by comparison with Talbot et al., 2001; 2003b.

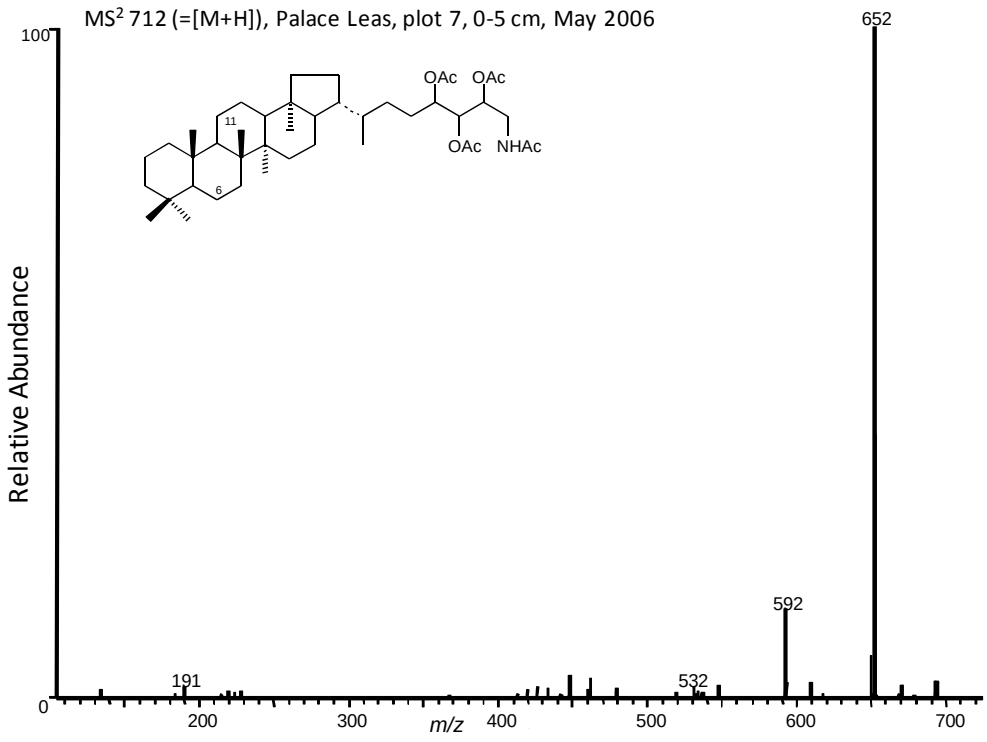


Fig.2.29. APCI-HPLC-MS^2 Spectrum of unsaturated Aminotriol tetra-acetate (**5f'**), Identified by comparison with Talbot et al., 2007b.

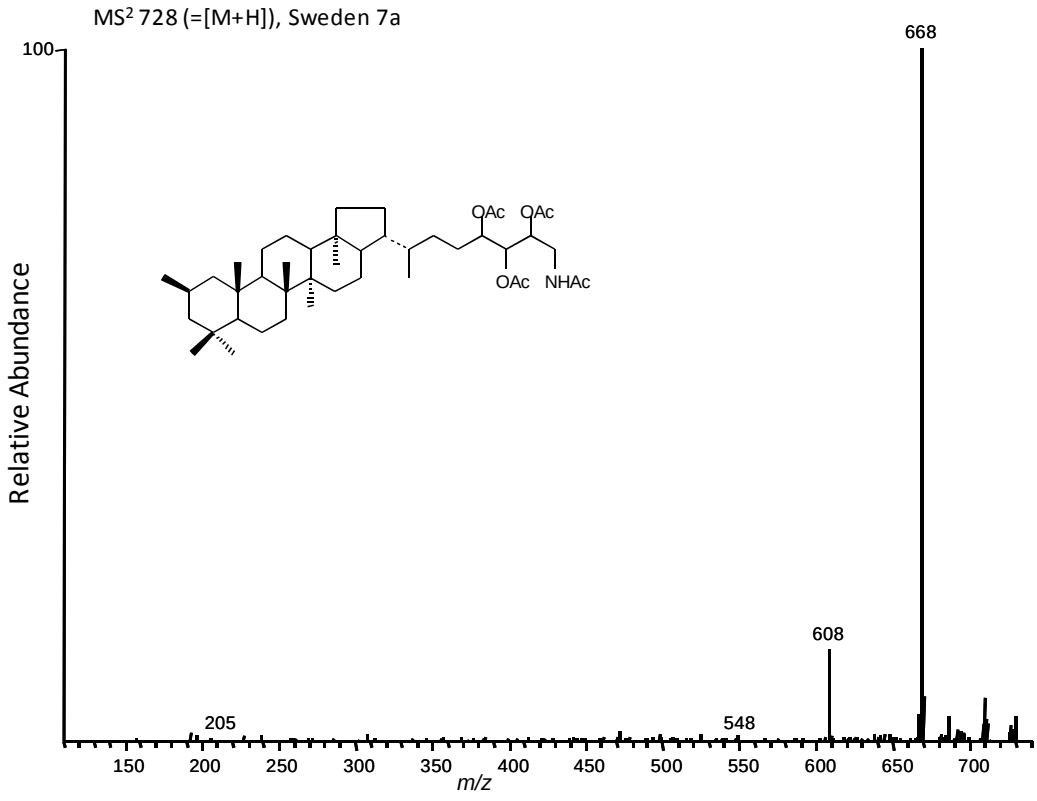


Fig. 2.30. APCI-HPLC-MS^2 Spectrum of 2 methyl aminotriol tetra-acetate (**2f'**). Identified by comparison with Talbot et al., 2008.

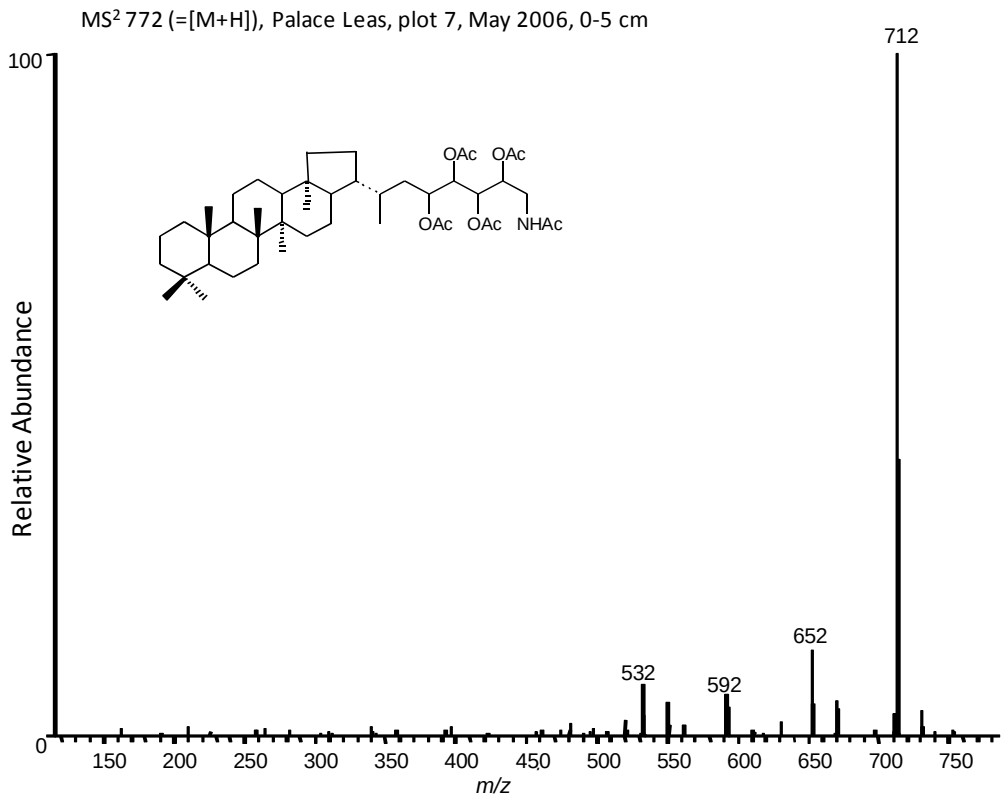


Fig. 2.31. APCI-HPLC-MS^2 Spectrum of aminotetrol penta-acetate (**1h'**). Identified by comparison with Talbot et al., 2003a.

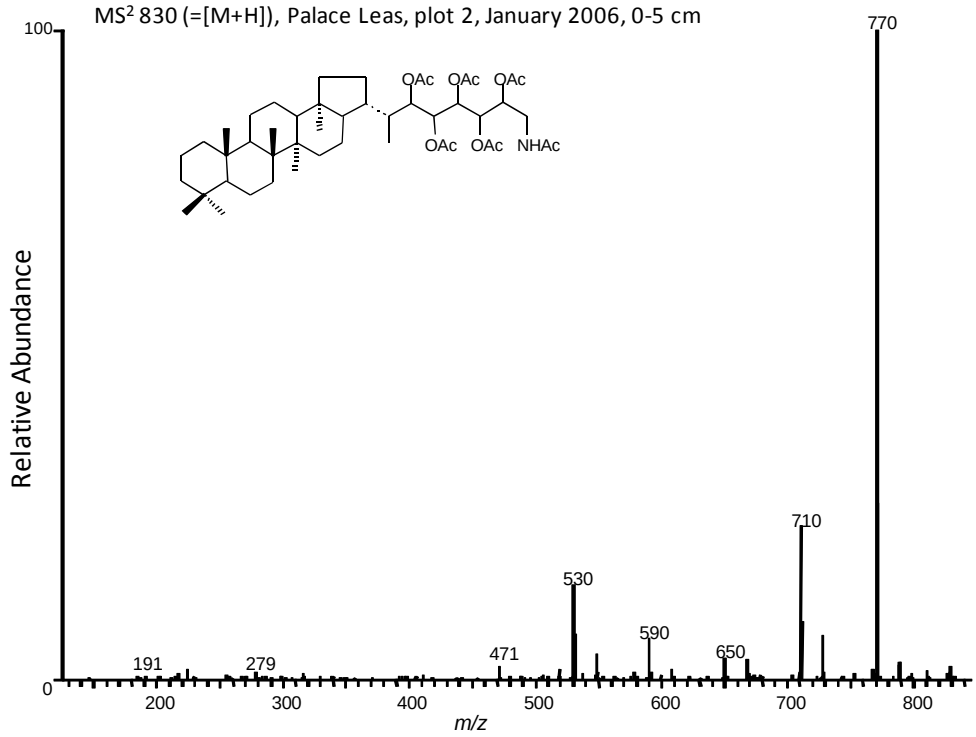


Fig. 2.32. APCI-HPLC-MS^2 Spectrum for aminopentol hexa-acetate (**1c'**). Identified by comparison with Talbot et al., 2003b.

2.7.5 Side-chain cyclised Bacteriohopanepolyols

This group of BHPs contains a cyclised side chain, and includes anhydroBHT (**1j**) (Fig. 2.33), ribonylhopane (**1s**) (Fig. 2.34) and a family of structures related to Adenosylhopane (**1e**) (Fig. 2.35). The base peaks of anhydroBHT (**1j**) and ribonylhopane (**1t**) and the adenosylhopane (**1e**) type structures are $[M+H]^+$, with the base peaks being formed by the protonation of the ether bond or by protonation of the amino group in the terminal group.

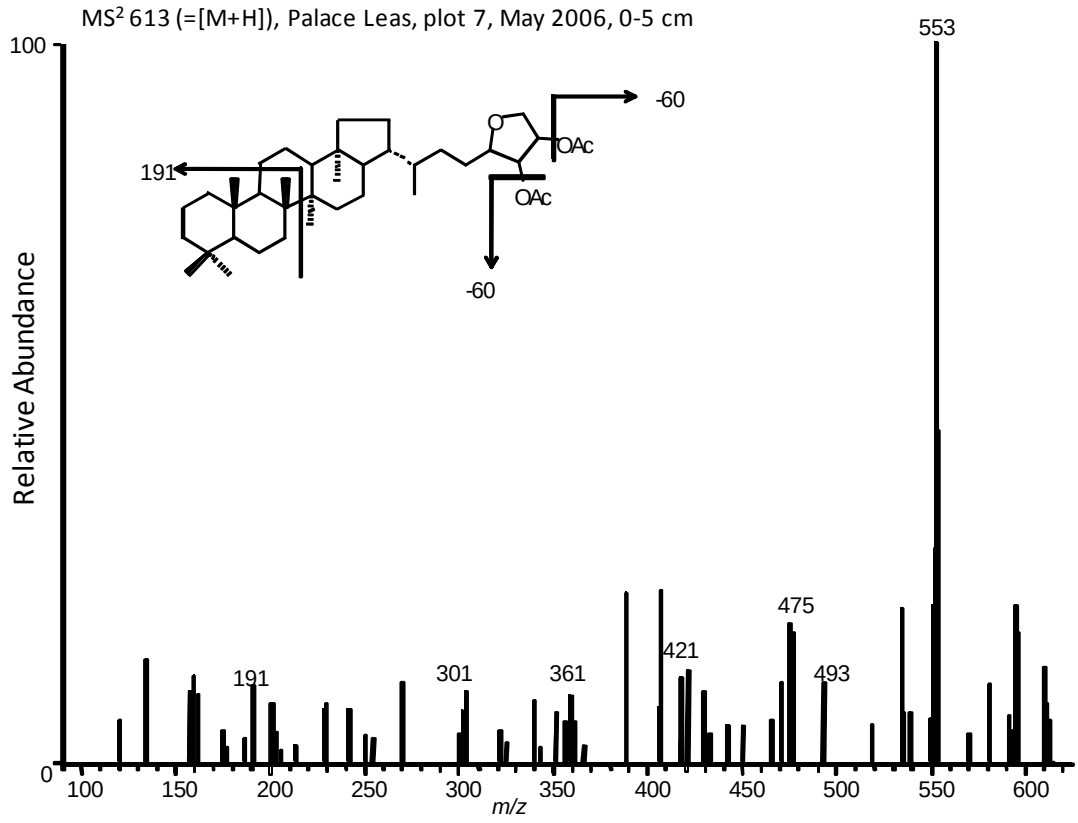


Fig. 2.33. APCI-HPLC-MS^2 Spectrum of AnhydroBHT diacetate (**1j'**). Identified by comparison with Talbot et al., 2005.

AnhydroBHT (**1j**) (Figure 2.3) was only identified rarely in the soils but was of significant importance in the sediments (Chapter 5). It is characterised by the loss of 2 hydroxyls to give *m/z* = 553 and 493, and the cleavage of the C-ring to give *m/z* = 191 and m/z = 421. The *m/z* = 421 can then undergo loss of 2 hydroxyls to give *m/z* = 361 and 301.

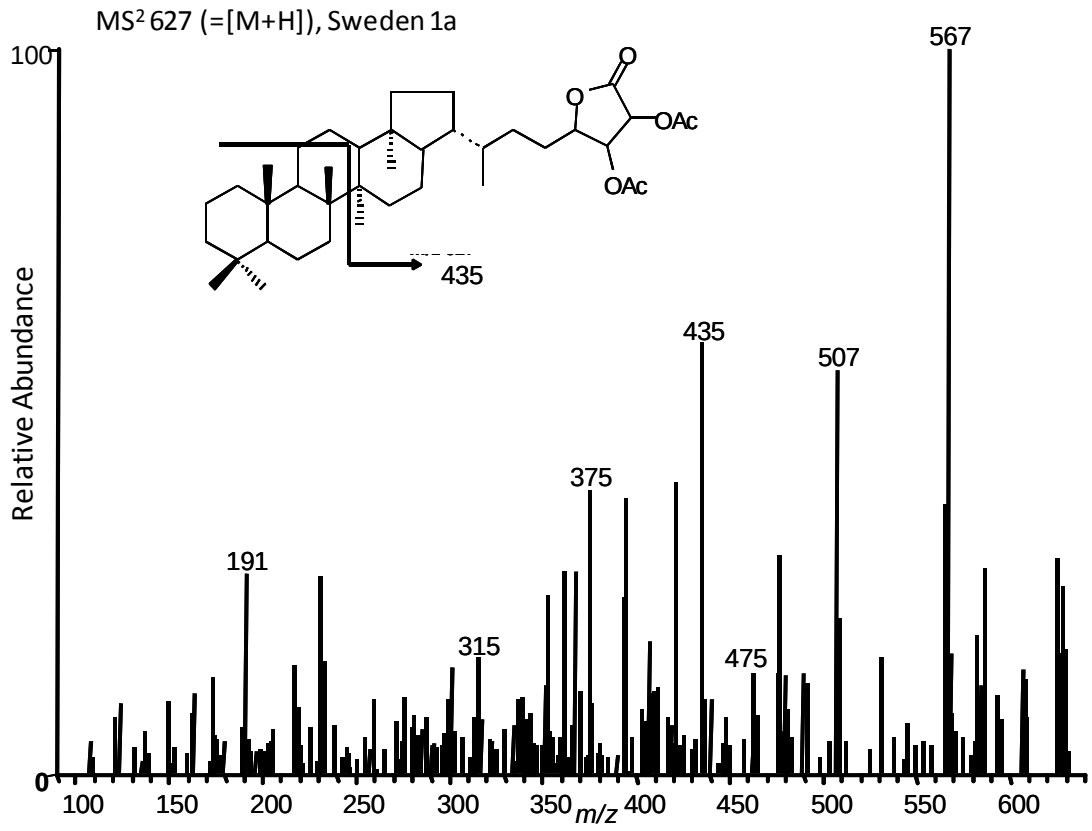


Fig. 2.34. APCI-HPLC-MS^2 Spectrum for Ribonylhopane diacetate (**1t'**). Identified by comparison with Talbot et al., 2007a.

Ribonylhopane (**1s'**) (Fig. 2.34) is characterised by the loss of 2 hydroxyls from the structure to give *m/z* = 567 and 507. The cleavage of the C-ring gives *m/z* =191 and the characteristic *m/z* = 435 from which 2 hydroxyls can be removed to give *m/z* = 375 and 315,

Adenosylhopane (**1e**) (Fig 2.35) is one of the most abundant structures found in soil and exists in the diacetate form and is characterised by the formation of 2 fragments generated by the loss of the terminal adenine group, *m/z* = 611 and 136, where *m/z* = 136 represents the adenine group. The *m/z* = 611 ion is stable due to the protonation of the ether bond. There is also the normal cleavage of the C-ring found in most BHPs giving ions of *m/z* = 191 and 419 after the removal of the terminal group. There is also a sequential loss of the 2 hydroxyls giving *m/z* = 551 and 491 and *m/z* = 359 and 299 from 419.

As with other BHPs such as BHT cyclitol ether (**1d**) (Fig.2.13) additional acetylation can occur. The triacetate form (**1e''**) (Fig. 2.36) contains the fragment *m/z* = 178,

which indicates that the extra acetylation is on the terminal group, normally *m/z* = 136 and seen in Fig. 2.35.

However a third spectrum (Fig. 2.37), with a base peak of *m/z* = 830, was also identified with an identical retention time to the other 2 forms of adenosylhopane (**1e'''**). The additional mass of +42 Da suggests a 4th acetylation, however, of the exact location of this acetylation is unknown, although a fragment, *m/z* =220, was observed indicating its presence on the adenine terminal group.

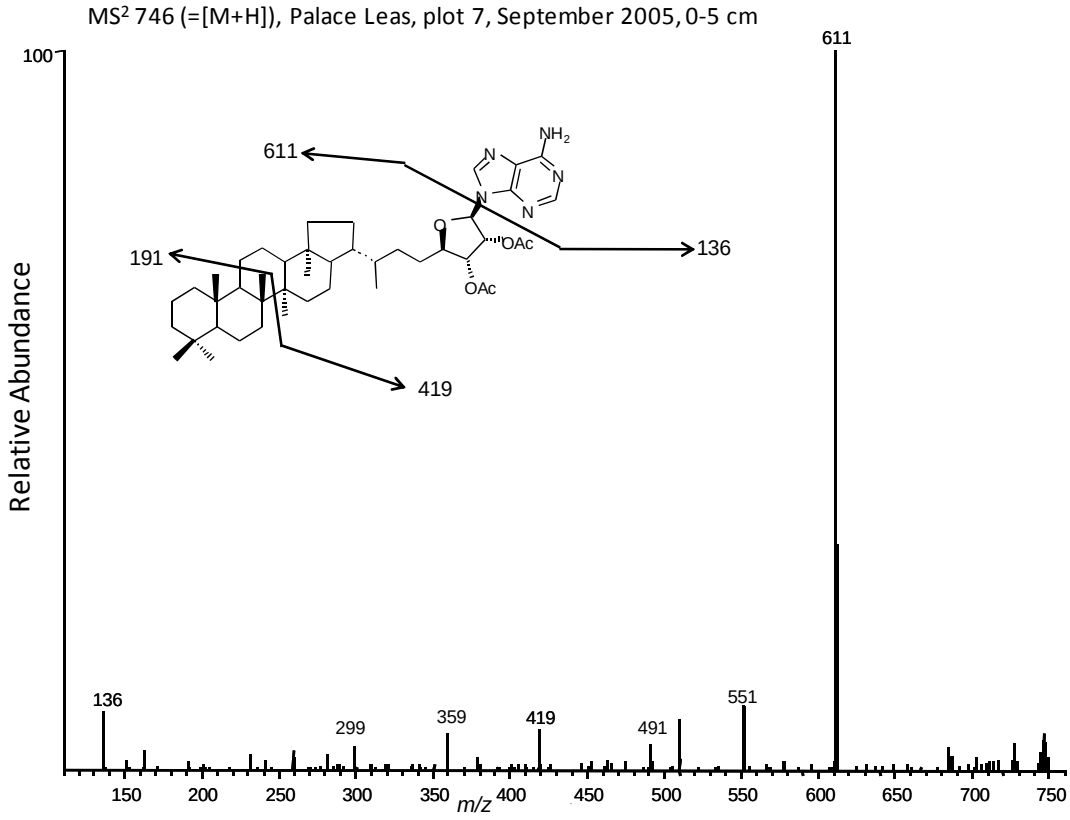


Fig. 2.35. APCI-HPLC-MS^2 Spectrum for diacetylated Adenosylhopane (**1e'**). Identified by comparison with Talbot et al., 2007a; Cooke et al., 2008b.

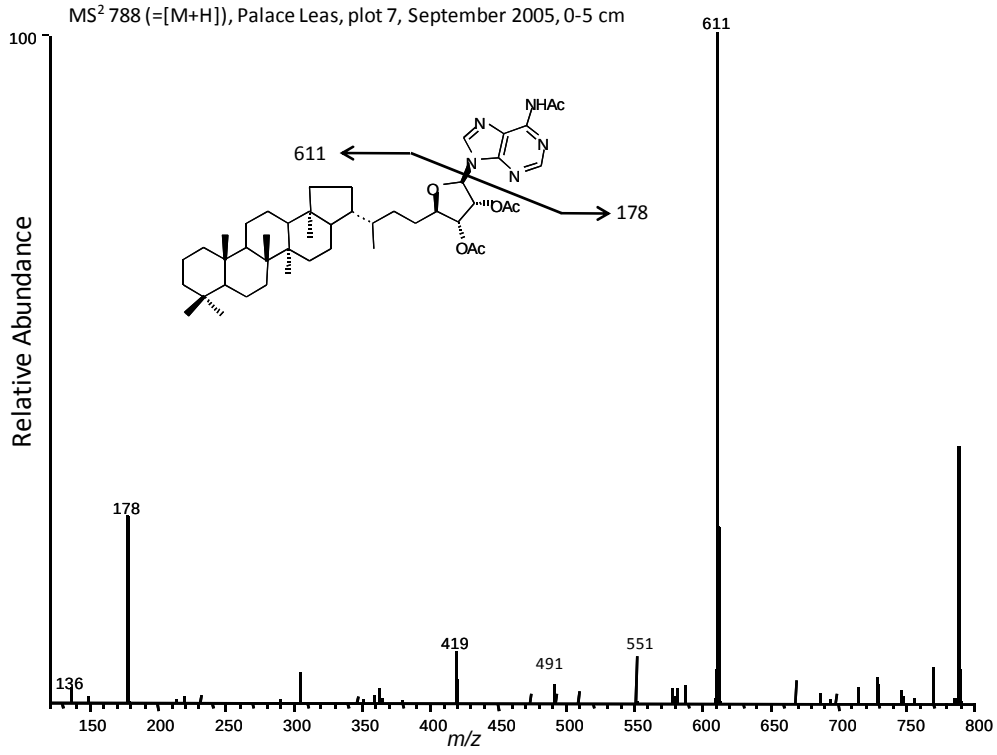


Fig. 2.36. APCI-HPLC-MS^2 Spectrum for triacetylated Adenosylhopane (**1e''**). Identified by comparison with Talbot et al., 2007a.

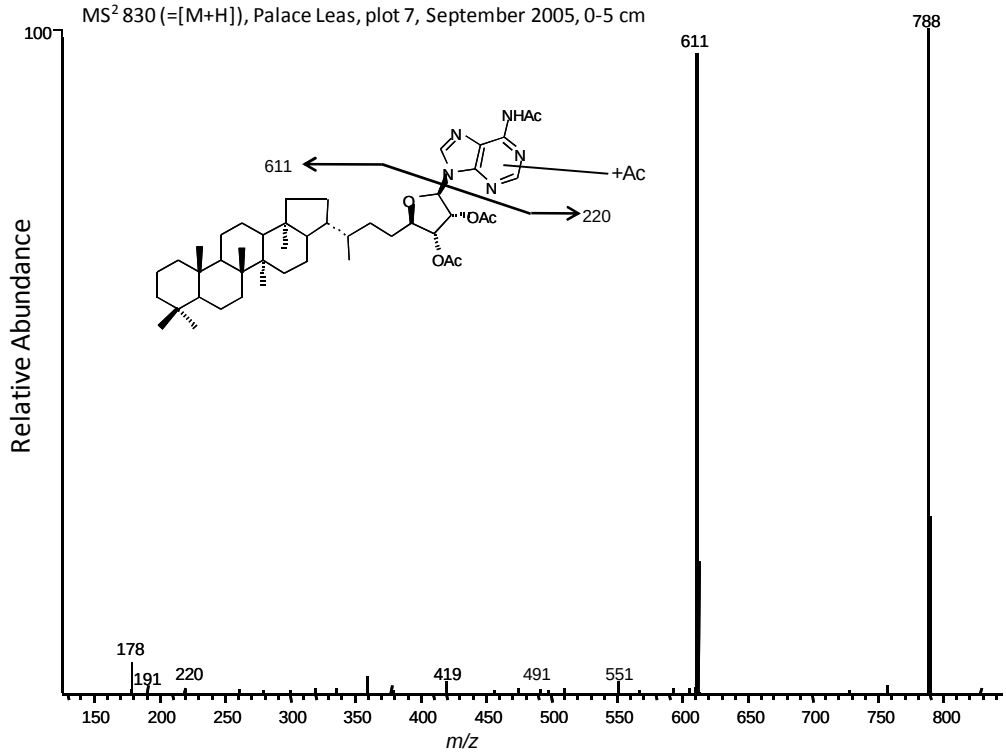


Fig. 2.37. APCI-HPLC-MS^2 Spectrum of Adenosylhopane tetra-acetate (**1e'''**). Identified by comparison with Fig. 2.35 and 2.36.

The methylated homologue of adenosylhopane (**2e**), most frequently observed as the triacetate form (**2e''**) (Fig. 2.38) is identified by the presence of *m/z* = 625 fragment ion compared with the *m/z* = 611 fragment observed in the non-methylated form (Fig. 2.35) indicating methylation on the ring system, probably at C-2. The presence of both *m/z* = 178 and 136 indicated the triacetate form. The diacetate form would have a base peak of *m/z* = 760 (**2e'**) and the fragment *m/z* = 178 would be absent.

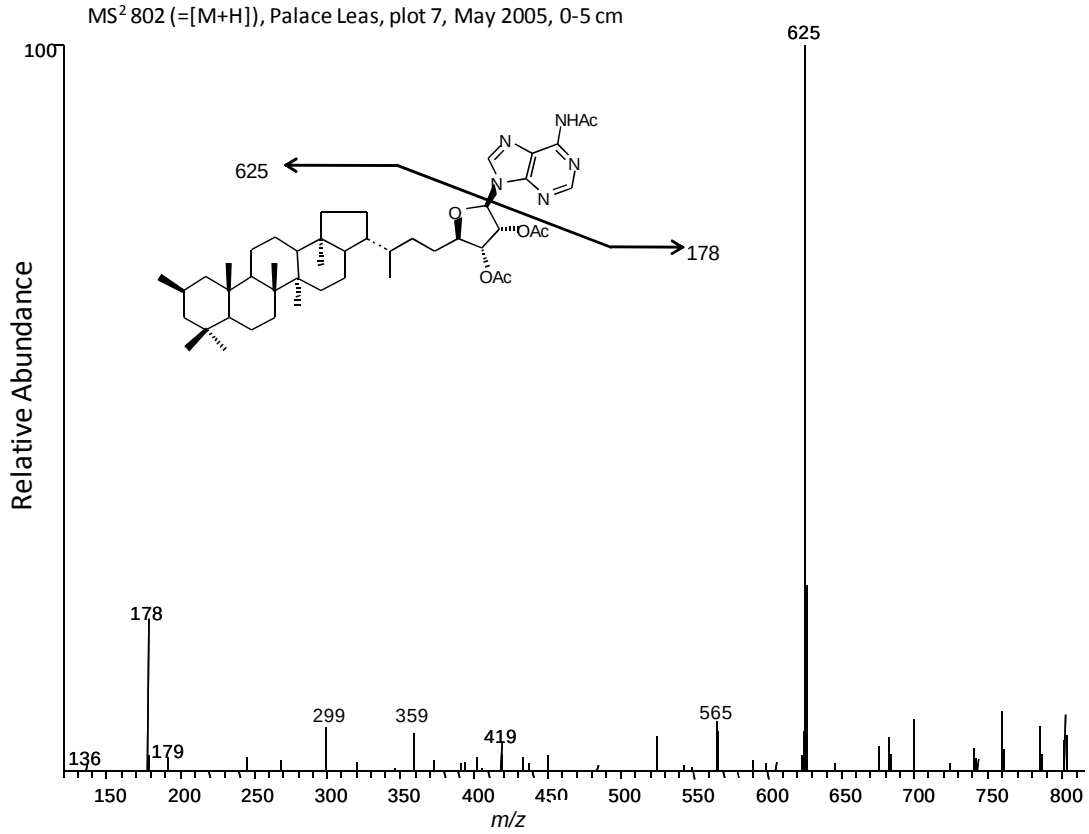


Fig. 2.38. APCI-HPLC-MS^2 Spectrum for triacetylated methyl adenosylhopane (**2e'**). Identified by comparison with Talbot et al., 2007a.

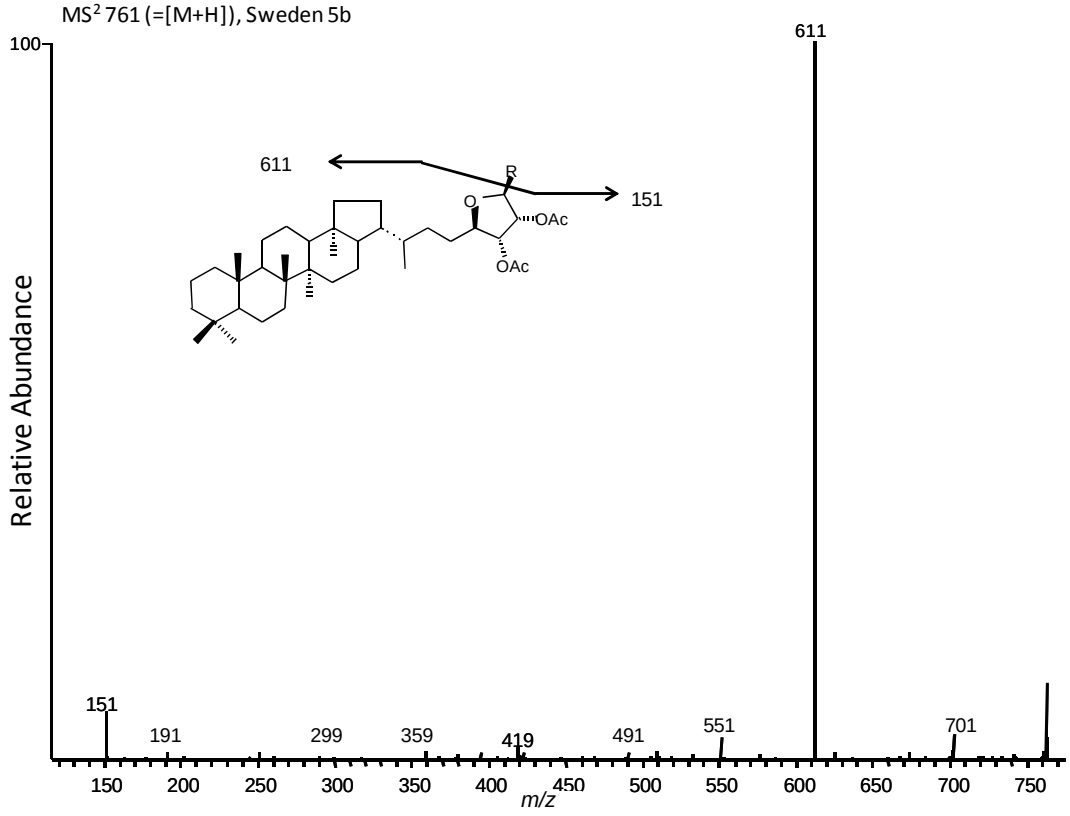


Fig. 2.39 APCI-HPLC-MS^2 Spectrum of Adenosylhopane-type 1(**1u'**). Identified by comparison with Talbot et al., 2007a; Cooke et al., 2008b.

A BHP, structurally related to adenosylhopane was identified (**1u**) (Fig. 2.39), as indicated by the dominant fragment ion of *m/z* = 611 (c.f. Fig. 2.30), with a base peak ion of *m/z* = 761 (**1t'**). The presence of the sequences *m/z* = 551, 491, and 419, 359 and 299 also confirms the similarity of adenosylhopane (Fig. 2.35). The terminal group is as yet unidentified and produced an ion of *m/z* = 151. This structure is described as "adenosylhopane-type-1".

The methylated homologue identified by the characteristic *m/z* = 625 ion (c.f. Fig. 2.38) and terminal group ion *m/z* = 151 was identified as 2-methyl adenosylhopane-type 1 (**2t'**) (Fig. 2.40).

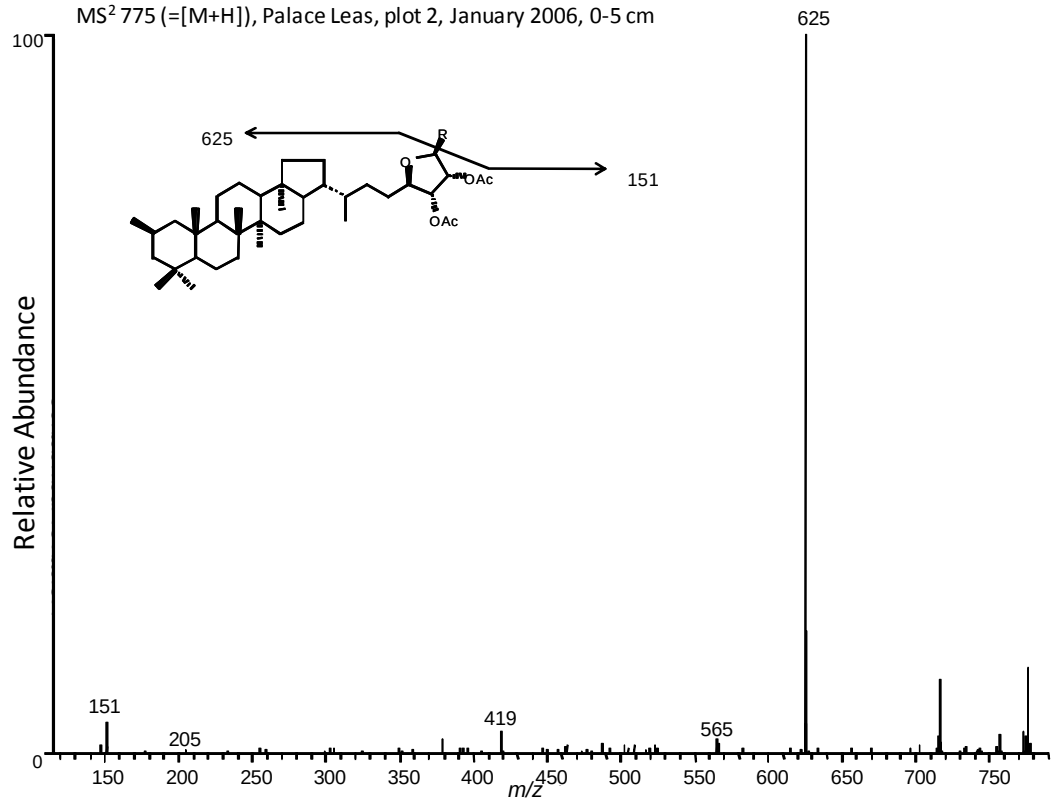


Fig. 2.40. APCI-HPLC-MS^2 Spectrum for 2-methyl Adenosylhopane type-1 (**2u'**). Identified by comparison with Cooke et al., 2008b.

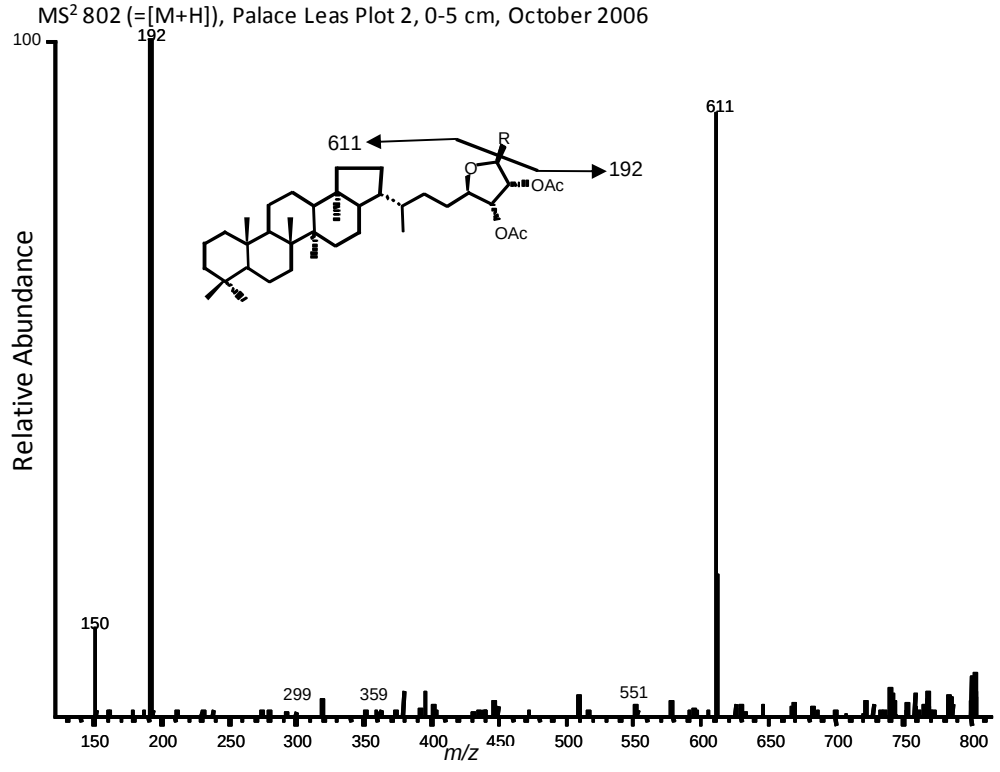


Fig. 2.41. APCI-HPLC-MS^2 Spectrum for Adenosylhopane type-2 (**1v'**). Tentatively identified for the first time during this research.

A second group structurally related to adenosylhopane, here termed adenosylhopane-type 2 (**1v**), (Figure 2.41) was identified. Although this structure has a parent ion of *m/z* = 802, identical to the triacetate of 2-methyladenosylhopane (**2e''**) (Fig. 2.38), the major ions clearly indicate that it is not a methylated structure, i.e. *m/z* =611 not *m/z* = 625. The terminal group ion has an *m/z* value of 192 and the presence of a second ion at *m/z* = 150 (i.e. loss of [CH_2CO or - 42 Da] suggests that there is an acetylated functional group on the terminal group and that this structure is therefore in the triacetate form (Fig. 2.41).

A methylated homologue of adenosylhopane-type 2 (**2v**), with a base peak of *m/z* = 816, was also identified by the MS^2 spectrum (Fig. 2.42) and assumed to be methylated at the C-2 position based on relative retention times. The identification was confirmed by the presence of an ion at *m/z* = 625 rather than 611 and the same terminal group indicated by *m/z* = 192 and 150)

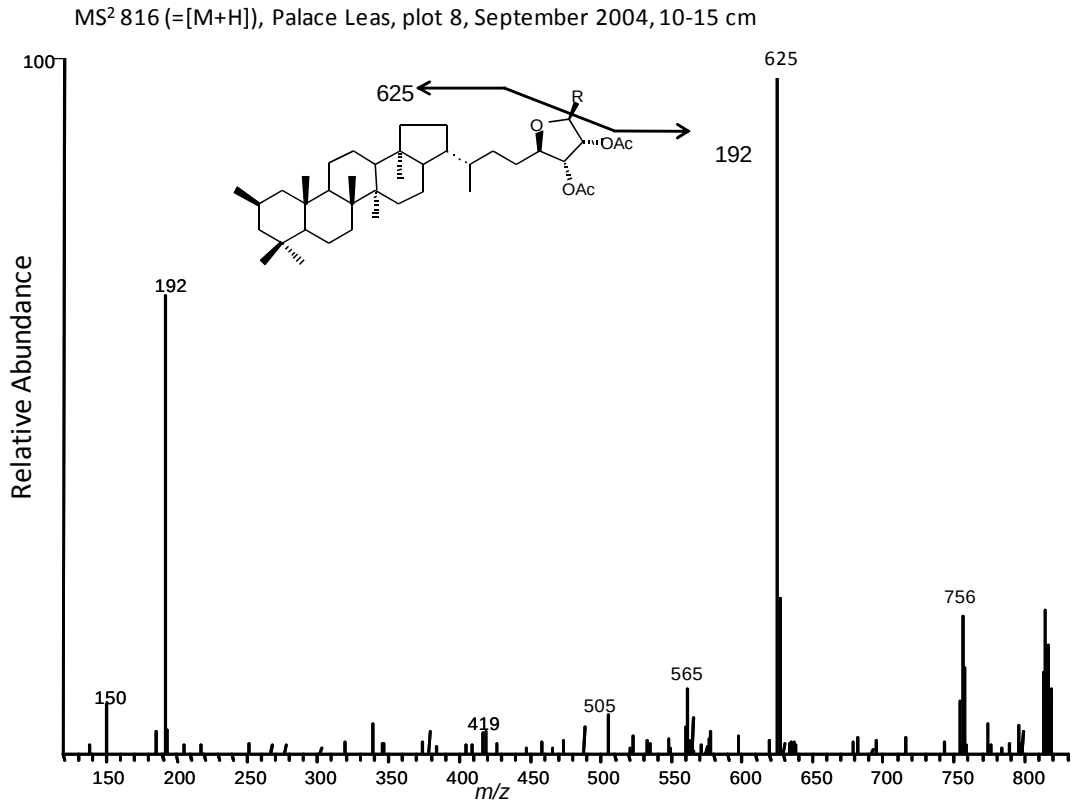


Fig. 2.42. APCI-HPLC-MS^2 Spectrum for 2-methyl adenosylhopane type-2 (**2v'**). Tentatively identified for the first time during this research.

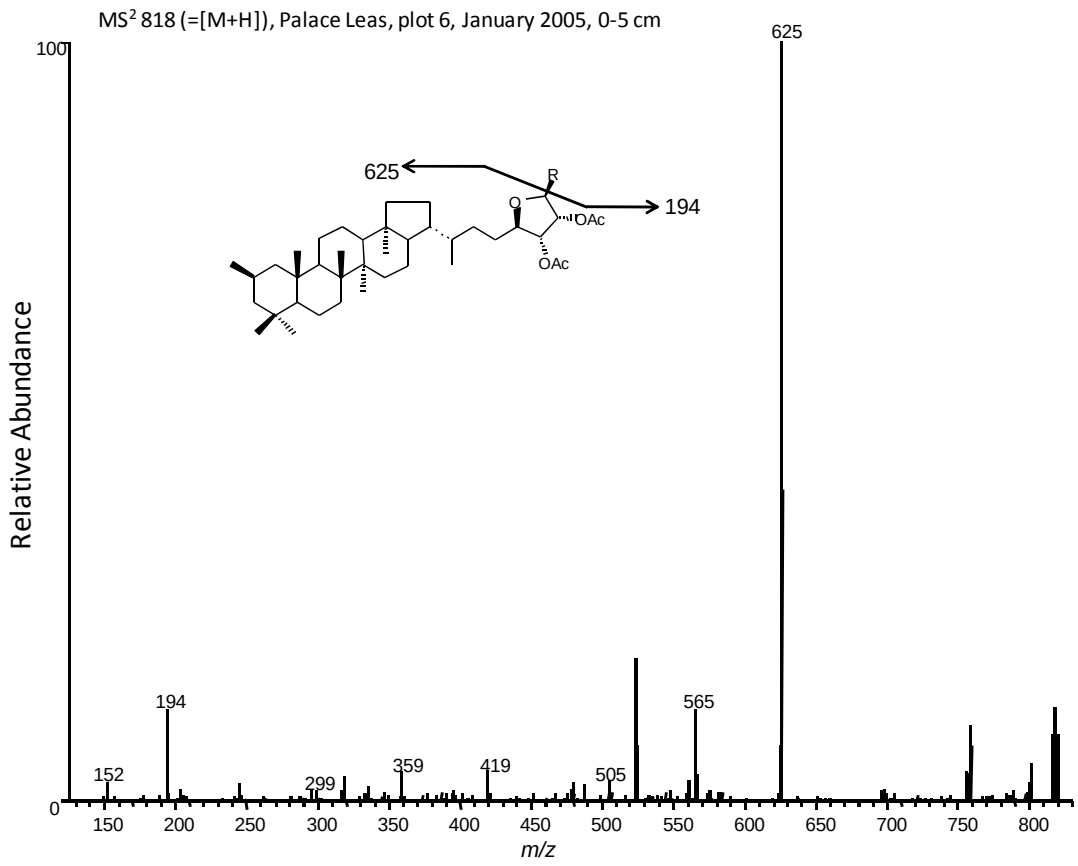


Fig. 2.43. APCI-HPLC-MS^2 Spectrum for 2-methyl adenosylhopane type-3 (**2w'**). Tentatively identified for the first time during this research.

A third related structure, adenosylhopane-type 3 (**2w**), (Fig. 2.43) was identified for the first time with a base peak of *m/z* = 818. This was only identified as a methylated structure (*m/z* = 625). The terminal group is indicated by fragment ions *m/z* = 194 and 152, indicating that the structure is the triacetate with additional acetylation on the terminal group.

A fourth related structure, adenosylhopane type-4 (**1x**) (Fig. 2.43) also with a base peak of *m/z* = 818 was also identified. The unidentified terminal group with an *m/z* = 208 was also believed to be in the triacetate form as indicated by the loss of 42 mass units between *m/z* = 208 and *m/z* = 166.

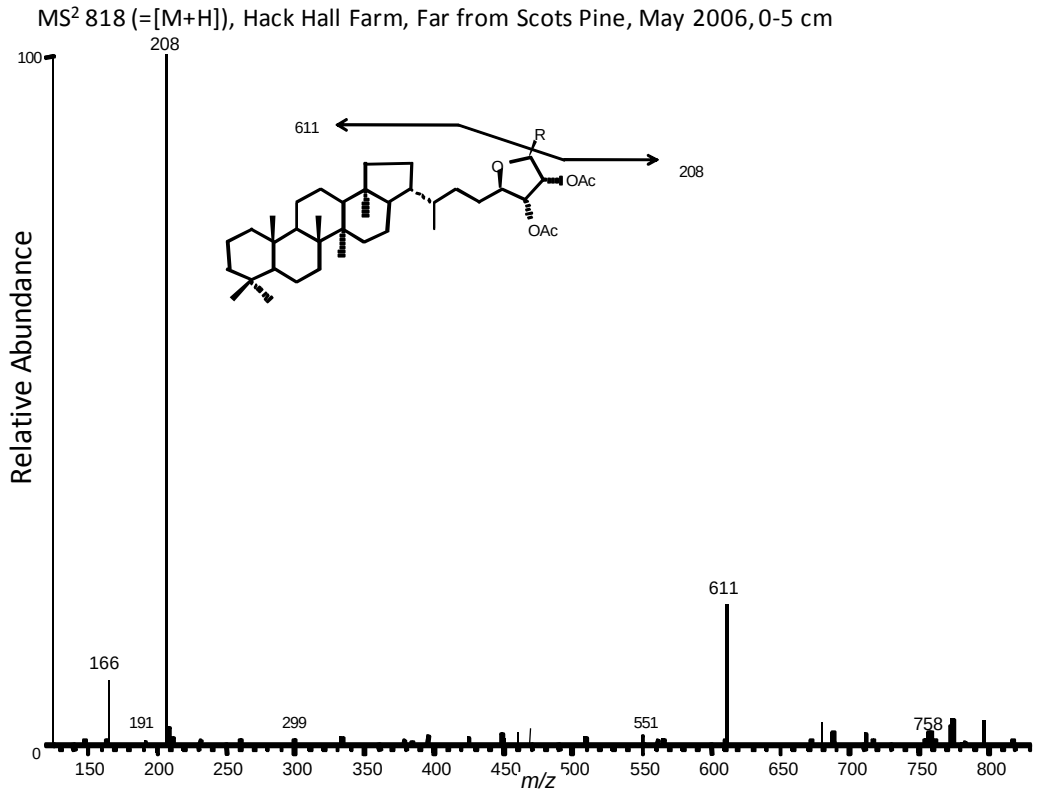


Fig. 2.44 APCI-HPLC-MS^2 Spectrum of adenosylhopane-type 4 (**1x'**). Tentatively identified for the first time during this research.

The structures of these terminal groups (Figs. 2.39 – 2.44 inclusive) cannot be identified using APCI-HPLC-MS and insufficient quantities could be obtained to enable analysis by other methods such as NMR.

2.8 Analysis by Gas Chromatography – Mass Spectrometry

Gas Chromatography-Mass Spectrometry (GC-MS) was not used extensively during this investigation, even though it has been widely used by many researchers to identify the presence and distribution of BHPs in a variety of bacteria and environments (Chapter 1).

The advantage in using HPLC-MS^n is that it enables the intact BHPs to be identified and semi-quantified whereas for many BHPs to be analysed by GC-MS the function group is removed using periodic acid, which cleaves carbon-carbon bonds between adjacent hydroxyls, followed by reduction by sodium borohydride to leave a terminal primary alcohol which can be analysed by GC-MS after acetylation (Fig 2.45) (e.g. Innes et al., 1997; 1998). Whilst this method enables distinction between tetra-, penta-

and hexafunctionalised structures, which will form C-32 hopanol (bishomohopanol) (**1y**), C-31 hopanol (homohopanol) (**1z**) and C-30 hopanol (hopanol) (**1aa**) respectively, any further information on the structure of the functional groups is lost.

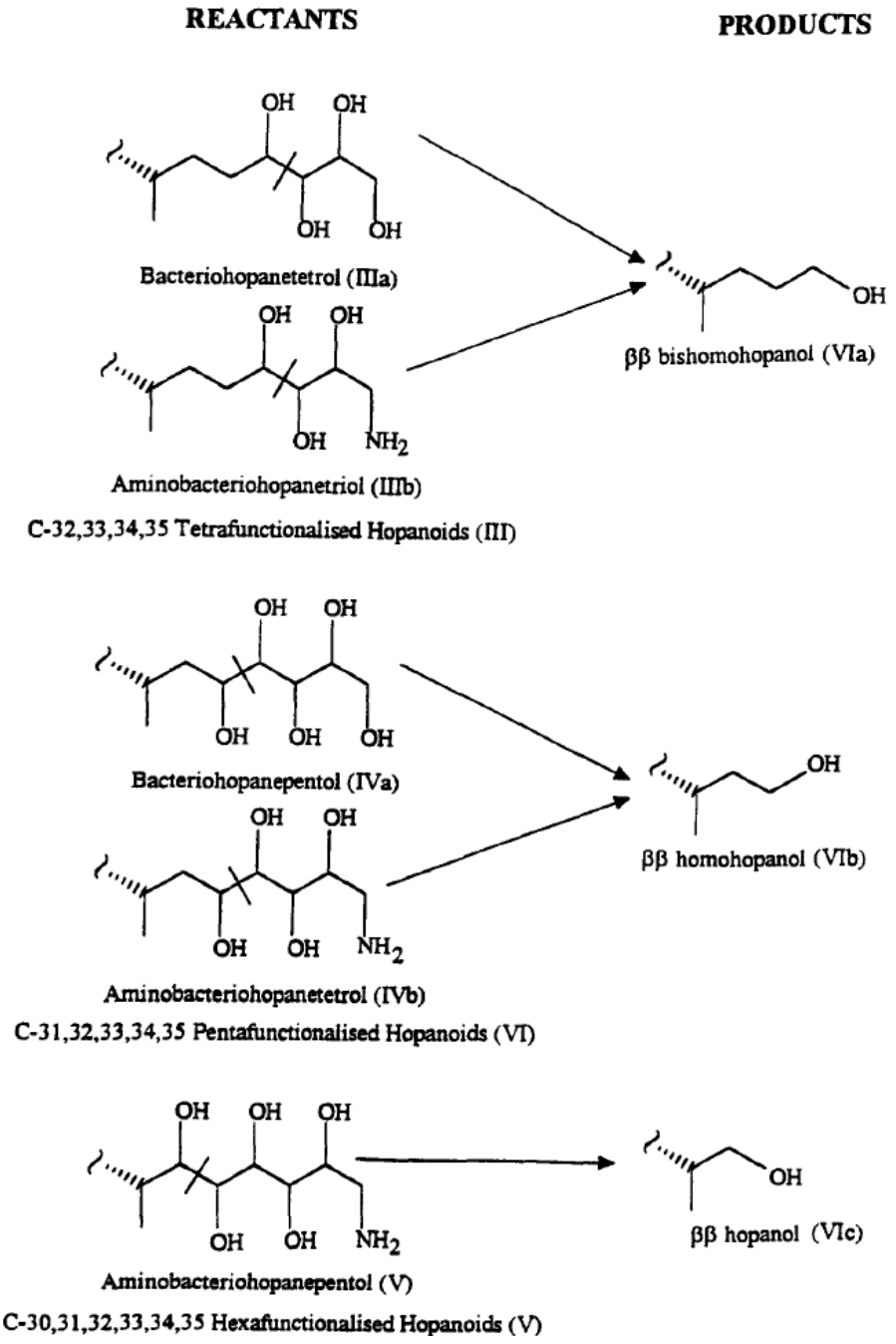


Fig. 2.45. Cleavage of BHP side chains by Periodic Acid / Sodium Borohydride Treatment. From Innes et al., 1997.

Whilst GC-MS could have provided confirmation on quantities and relative proportions of tetra-, penta- and hexafunctionalised BHPs Talbot and Farrimond

(2007) observed that adenosylhopane (**1e**) cannot be identified nor quantified by GCMS. As adenosylhopane (**1e**) and related structures comprised a significant proportion of the BHPs identified in soils it was clear that routine GCMS analysis of the samples could not provide significant additional information to the investigation.

2.8.1 Periodic Acid / Sodium Borohydride Treatment Method.

The method used is as described in Innes et al., 1997. An aliquot of the total lipid extract obtained by the modified Bligh and Dyer method (Section 2.3) was added to a conical flask, dried under a stream of nitrogen and stirred for l h at room temperature with 300 mg of periodic acid in 3ml of tetrahydrofuran/water (8:1 v/v). The products were then added to a separating funnel using 10ml of water and then 15ml of petroleum ether. The resulting biphasic mixture was shaken, allowed to separate and the petroleum ether fraction collected. Two addition sets of 15ml of petroleum ether were added to the separating funnel, shaken and the petroleum ether fraction extracted in each case. All 3 combined extracts were rotary evaporated to near dryness and any residual water was removed by further evaporation as an azeotropic mixture with acetone. The resulting extract was stirred for l h at room temperature with 100 mg of sodium borohydride in 3ml of ethanol.

15ml of 100 mM potassium dihydrogen phosphate was added and once effervescence had stopped the mixture was extracted with petroleum ether (15 ml; x3). The resulting extracts were combined, rotary evaporated to near dryness and transferred to large vials. The extracts were then acetylated prior to analysis on the GCMS using the procedure described in section 2.5.

The resultant hopanols were analysed by GC–MS using a Hewlett-Packard 5890 II GC system (split/splitless injector; 350°C) linked to a Hewlett-Packard 5972 mass-selective detector (electron energy 70 eV; filament current 220 mA; source temperature 270°C; multiplier voltage 2000 V; interface temperature 350°C). A 15 m DB5-HT fused silica column (0.25 mm i.d.; 0.1 mm film thickness) was used with helium as the carrier gas. The oven temperature was programmed from 50 to 200°C at 15°C/min (held for 1 min), from 200 to 250°C at 10°C/min (held for 1 min) and from 250 to 350°C at 5°C/min (held for 8 min).

Hopanols were identified from full scan (*m/z* 50–700) analysis of selected samples, by comparison with authentic standards and published spectra and by relative retention times. They were quantified from peak areas in the *m/z* 191 and m/z 205 (ring-A methylated species) mass chromatograms against the *m/z* 243 peak area response of the androstanol internal standard.

2.8.2 Identification of position of Methylation.

The periodic acid / sodium borohydride treatment is particularly useful when identifying the position of methylation on the A-ring of the BHP (Farrimond et al., 2004). A C-2 methylation on BHT (**2a**) will elute as a 2-methyl C-32 alcohol (**2x**) before the unmethylated C-32 (**1y**) alcohol formed from BHT, whereas C-3 methylated BHT (**3a**) will elute as a 3-methyl alcohol (**3y**) after the C-32 alcohol (**1y**) formed from BHT (Fig. 2.46).

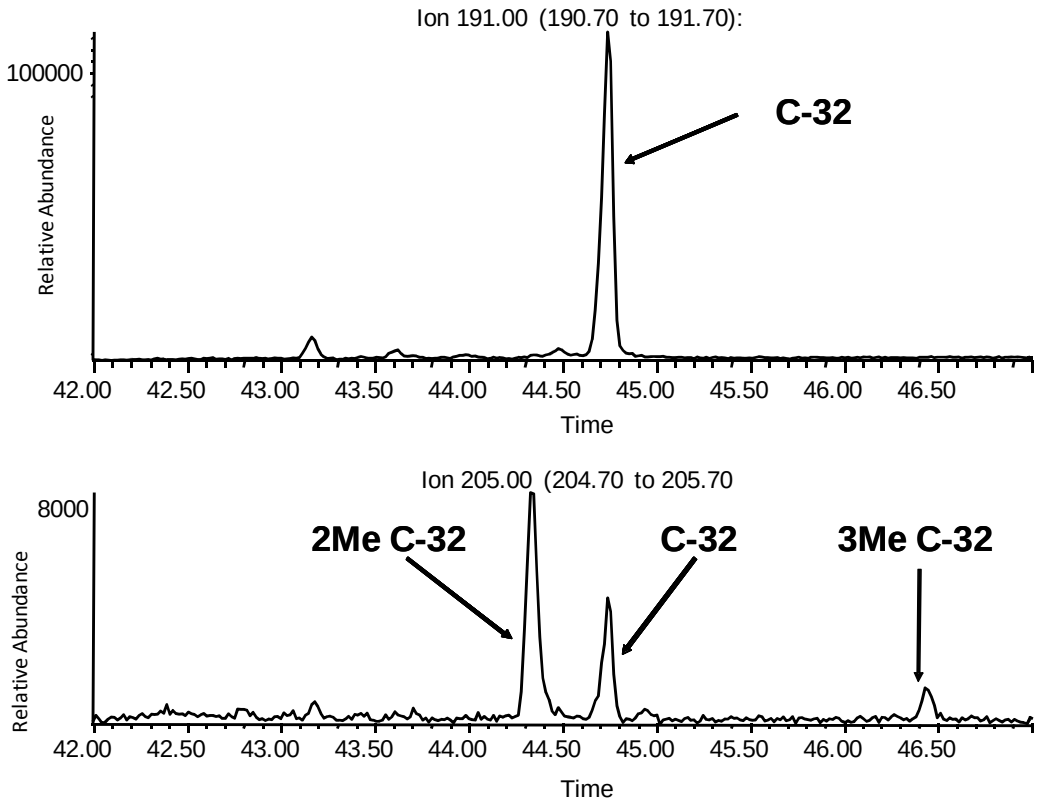


Fig. 2.46 GCMS chromatograms from Palace Leas plot 7 May 06 core 1. Highlighting presence of 2-methyl C-32 hopanol (**2y**) and 3-methyl C-32 hopanol (**2y**) and the relative elution times compared to unmethylated C-32 hopanol (**1y**). Identified by comparison with Farrimond et al., 2004.

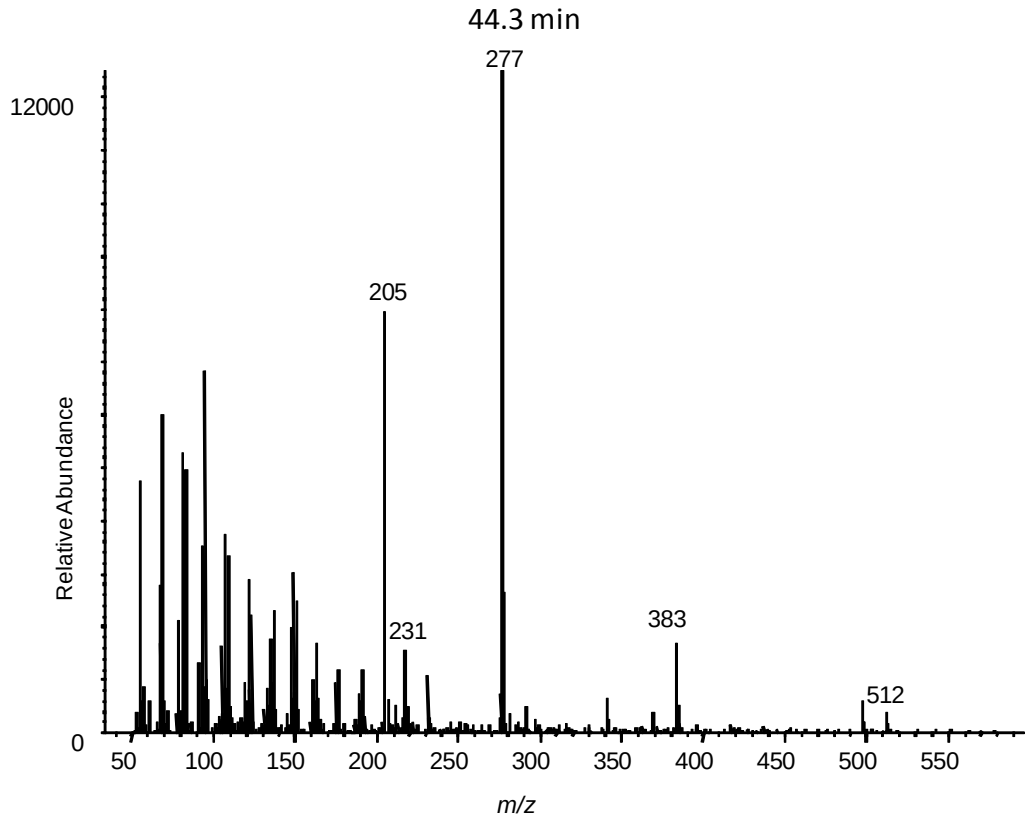


Fig. 2.47. Mass Spectrum from Palace Leas plot 8 Oct 06 core 1 for 2Me C-32 hopanol (**2y**).

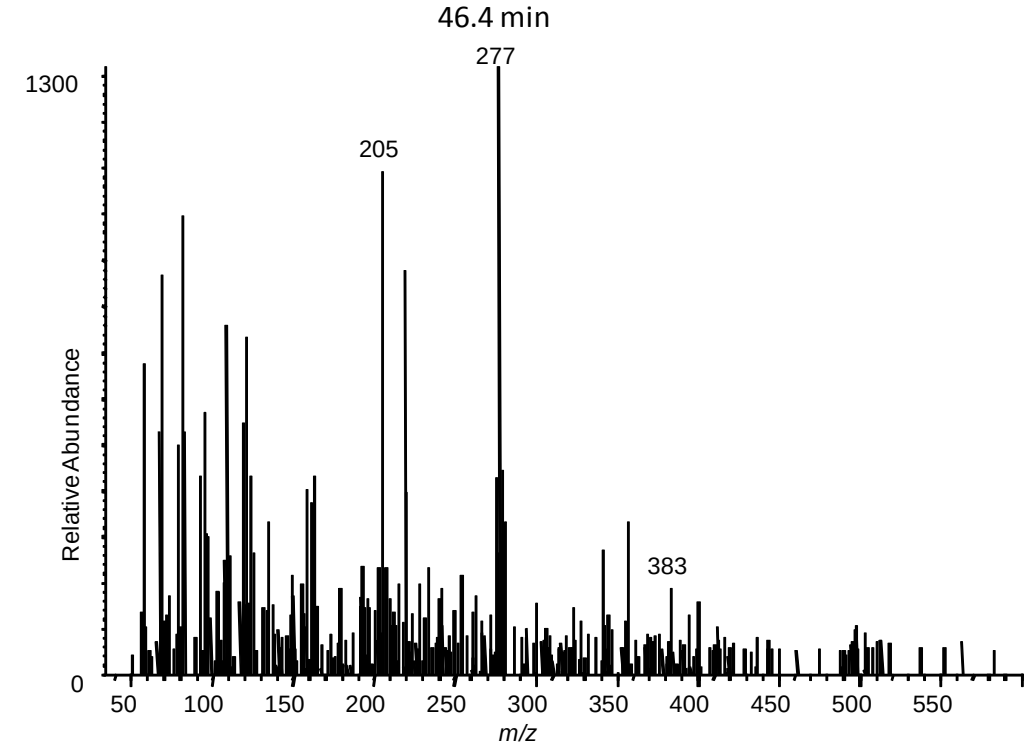


Fig. 2.48. Mass Spectrum from Palace Leas plot 7 May 06 core 1 for 3Me C-32 hopanol (**3y**).

The identification of the C-2 and C-3 methyl peaks was confirmed by analysis of the spectra (Figs. 2.47 and 2.46). Methylated C-32 bishomohopanol (**2y**) is identified by the ions formed by the cleavage of the C-ring giving ions of *m/z* = 205 for the methylated A+B rings and 277 for the D+E+side chain fragment. These fragments can clearly be observed in both spectra for C-2 and C-3 methylations (Figs. 2.47 and 2.48).

2.9 Method Development

The modified Bligh and Dyer extraction method used during the analysis and described in section 2.2 was not the first method used. Four other extraction methods (described below) were tested during the initial stage of the project to optimise the extraction of BHPs from the soil samples.

Three different soil samples were used; DSNM, a soil acquired from the Newcastle University garden, TFWC, acquired from a white clover field at Cockle Park Farm and WNBC9, a pasture soil collected adjacent to Lake Windermere (UK Lake District). These soils are the soils for which BHP data was reported in Cooke et al. (2008b). Previous analysis of the same soils (Yurdakok, 2003) had indicated that they contained differing quantities of BHPs with DSNM containing the least and WNBC9 the most.

2.9.1 Soxtherm Extraction

This method was developed by Innes et al., 1997, 1998 for analysis of BHPs in sediments and was subsequently used by Farrimond et al. (2000), Watson and Farrimond (2000) and Talbot et al, 2003a.

Briefly approximately 5 g of the freeze dried sample was added to a pre-extracted cellulose thimble and covered with pre-extracted cotton wool. Pre-extracted copper turnings were activated by immersing in HCl for 20 min. The acid was removed by rinsing with deionised was and the copper turnings rinsed in methanol to remove any residual water then 1-2 heaped spatulas were added to each Soxtherm beaker together with antibumping granules. The apparatus enabled 6 samples to be run simultaneously. The thimbles were placed in Soxtherm beakers and 150 ml

chloroform/methanol (2:1, v/v) added to each beaker. The samples were extracted on a Gerhardt soxtherm apparatus (Germany) for 6 h, at 150^{o}C with a 4 h immersion extraction followed by recovery and 2 h drip rinse. The resulting extracts were transferred to a 250 ml round bottom flask and the beaker rinsed using warm chloroform/methanol (2:1, v/v), to prevent the BHPs from sticking to the glassware. The extract was then rotary evaporated to near dryness, transferred to a glass vial using warm chloroform/methanol (2:1, v/v) and blown down under a stream of nitrogen to near dryness and stored in a fridge until required.

2.9.2 Double Soxtherm extraction

Following an initial 6 h soxtherm extraction using chloroform/methanol (2:1 v/v; Section 2.9.1), and identical extraction was carried out on following day. The two extracts were kept separate to allow determination of any BHPs extracted in the second extraction to measure the additional quantity of BHPs obtained during the second extraction.

2.9.3 Sonication extraction in water followed by Soxtherm extraction.

A slurry of approximately 5 g of freeze dried soil and 20 ml of 18MΩ water was sonicated for 45 min, freeze dried and subsequently extracted using the soxtherm apparatus as detailed above (section 2.9.1 only). Water is a more polar solvent than the other solvents used and would promote the release of the BHPs from the cells.
Also it was proposed that the adhesion of free BHPs and intact cells to clay particles in the soil was a potential limiting factor in the extraction efficiency. Therefore the sonication of the slurry would also promote the swelling of any 2:1 clays within the soil changing the adhesion geometry between the soil and the BHPs, and disaggregation of the clay particles within the soil. Also the water would compete with the BHPs for adsorption sites on the clay promoting the release of BHPs. The addition of water will also induce osmotic stress on intact cells, promoting lysing and increasing the extraction efficiency.

2.9.4 Sonic extraction with Soxtherm extraction.

A slurry of approximately 5 g of freeze dried soil and 30 ml of chloroform/methanol (2:1) was placed in a Teflon centrifuge tube, sonicated for 15 min at 40°C and centrifuged at 12,000 rpm for 15 min. The supernatant solvent was removed and transferred to a round bottomed flask. This was repeated three times and the extract collected (Extract 1). The soil residue was dried at room temperature for 48 h and subsequently extracted by Soxtherm for 6 h (Extract 2). Extracts 1 and 2 were then combined to give a total lipid extract.

2.9.5 Results

The key purpose of testing different extraction methodologies was to maximise the quantity of BHPs extracted and to enable the accurate identification and measurement of BHPs in the samples. As previously discussed in section 2.7, identification of BHPs is by comparison with known spectra and by relative retention times. To be confident of the relative retention times it is important to be able to identify by spectrum first. Initial comparison of the various extraction techniques indicated a wide variation in the number of identifiable spectra from each sample (Table 2.1).

Analysis of the Bligh and Dyer extract resulted in the largest number of identifiable spectra for each sample and maximised the actual number of BHPs that could be observed (Table 2.1).

The quality of the chromatograms is vitally important to the measurement of the BHPs. It is important that the signal to noise ratio on the chromatograms is high and the shape of the peaks is smooth and symmetrical to enable accurate integration. A comparison between the chromatograms generated as a result of analysis of the Bligh and Dyer (Figure 2.49) and Soxtherm extraction (Fig. 2.50) of sample TFWC clearly demonstrates that the quality of chromatograms produced from the Bligh and Dyer extract are much cleaner than those generated from analysis of the Soxtherm extract, with respect to peak shape and background noise level.

Table 2.1. Number of BHP structures identified by direct observation of the relevant spectrum or by comparison of relative retention time to a previously identified spectrum using the 5 different extraction techniques.

Sample	Method of peak identification	Bligh and Dyer	Soxtherm	Double Soxtherm	Water/soil sonication + Soxtherm	Sonic extraction + Soxtherm
DSNM	spectra	8	3	3	1	5
	Additional compounds identified by comparison of retention times	2	2	5	5	1
	Total number BHPs identified	10	5	8	6	6
TFWC	spectra	14	9	9	8	8
	Additional compounds identified by comparison of retention times	2	1	3	4	6
	Total number BHPs identified	16	10	12	12	14
WNBC9	spectra	14	3	3	6	4
	Additional compounds identified by comparison of retention times	0	12	12	2	4
	Total number BHPs identified	14	15	15	8	8

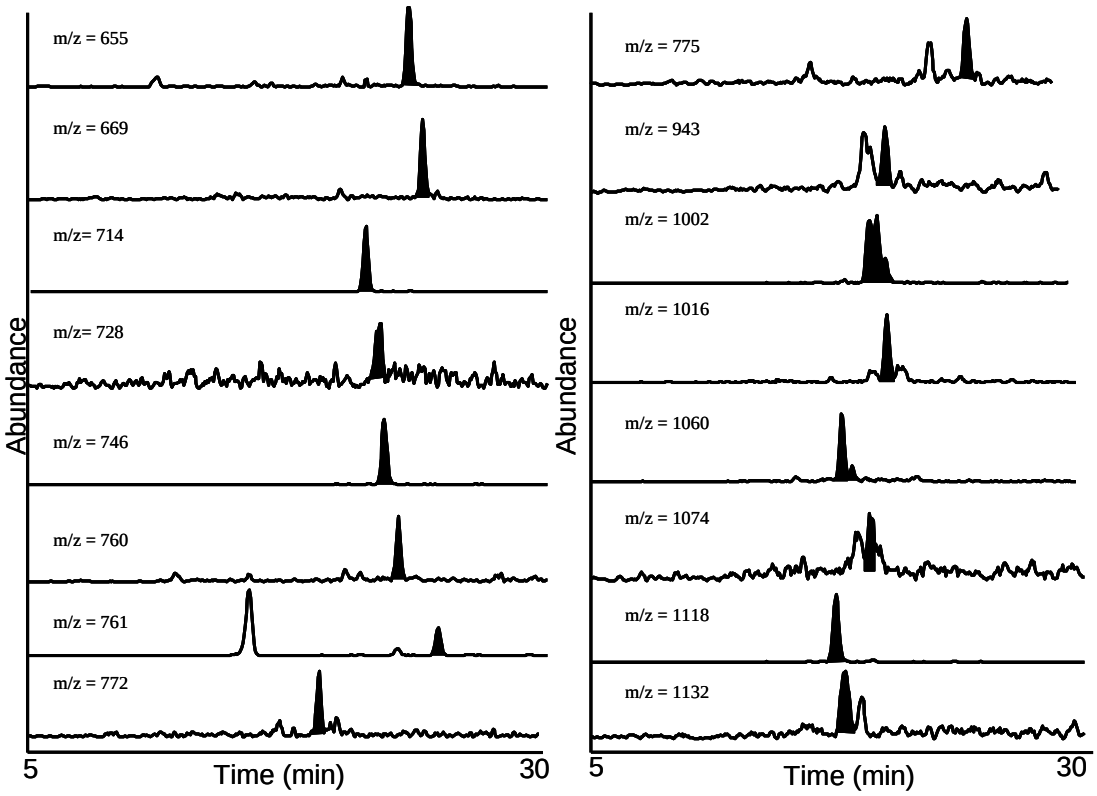


Fig. 2.49 Partial APCI base peak chromatograms of BHPs identified in sample TFWC following Bligh and Dyer Extraction. BHPs identified by base peak ion.

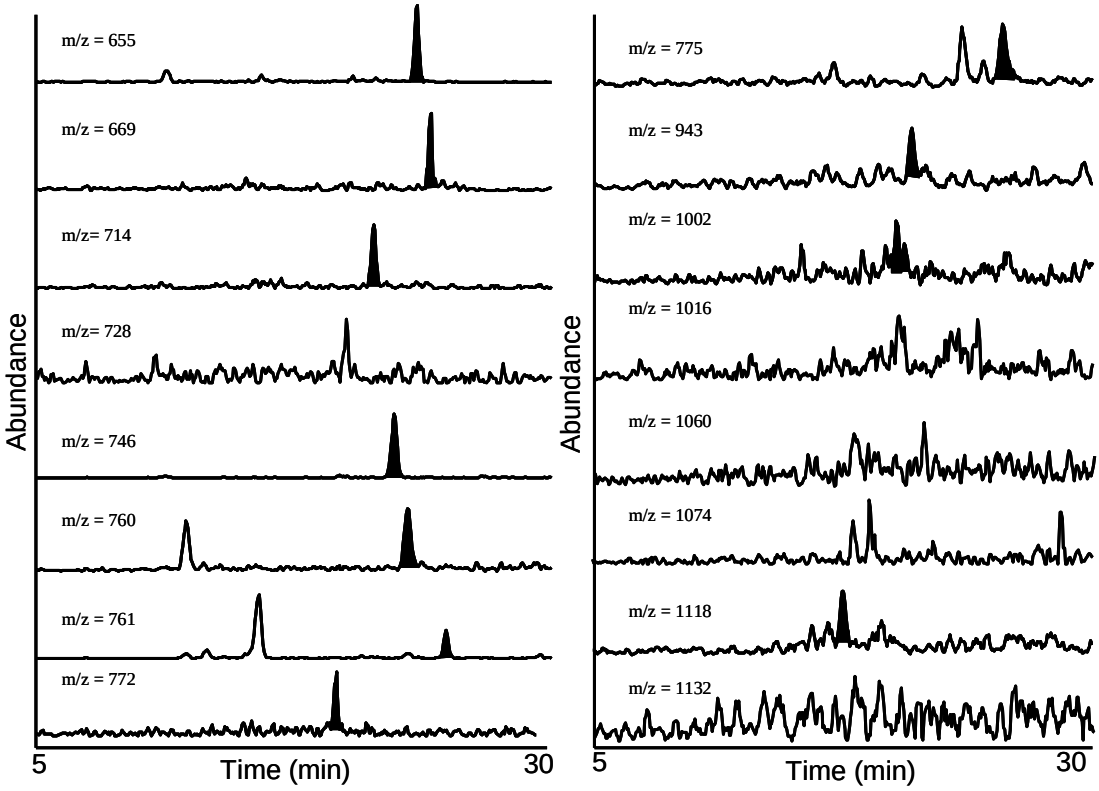


Fig. 2.50. Partial APCI base peak chromatograms of BHPs identified in sample TFWC following Soxtherm Extraction. BHPs identified by base peak ion.

The final test for the quality of the extraction techniques is on the quantity of BHPs extracted. Each of the three samples showed a slight increase in the levels of BHPs extracted using the Bligh and Dyer extraction techniques (Figs. 2.51, 2.52, 2.53) compared to the standard Soxtherm analysis, a significant increase compared to the water and soil extraction and results comparable to the sonication followed by soxtherm extraction.

The result of these tests clearly demonstrated that the Bligh and Dyer technique optimised the extraction efficiency, ease of identification and measurement of the BHPs. This method was used in all subsequent analyses and represented a step change in the ability to extract and analyse BHPs from environmental samples.

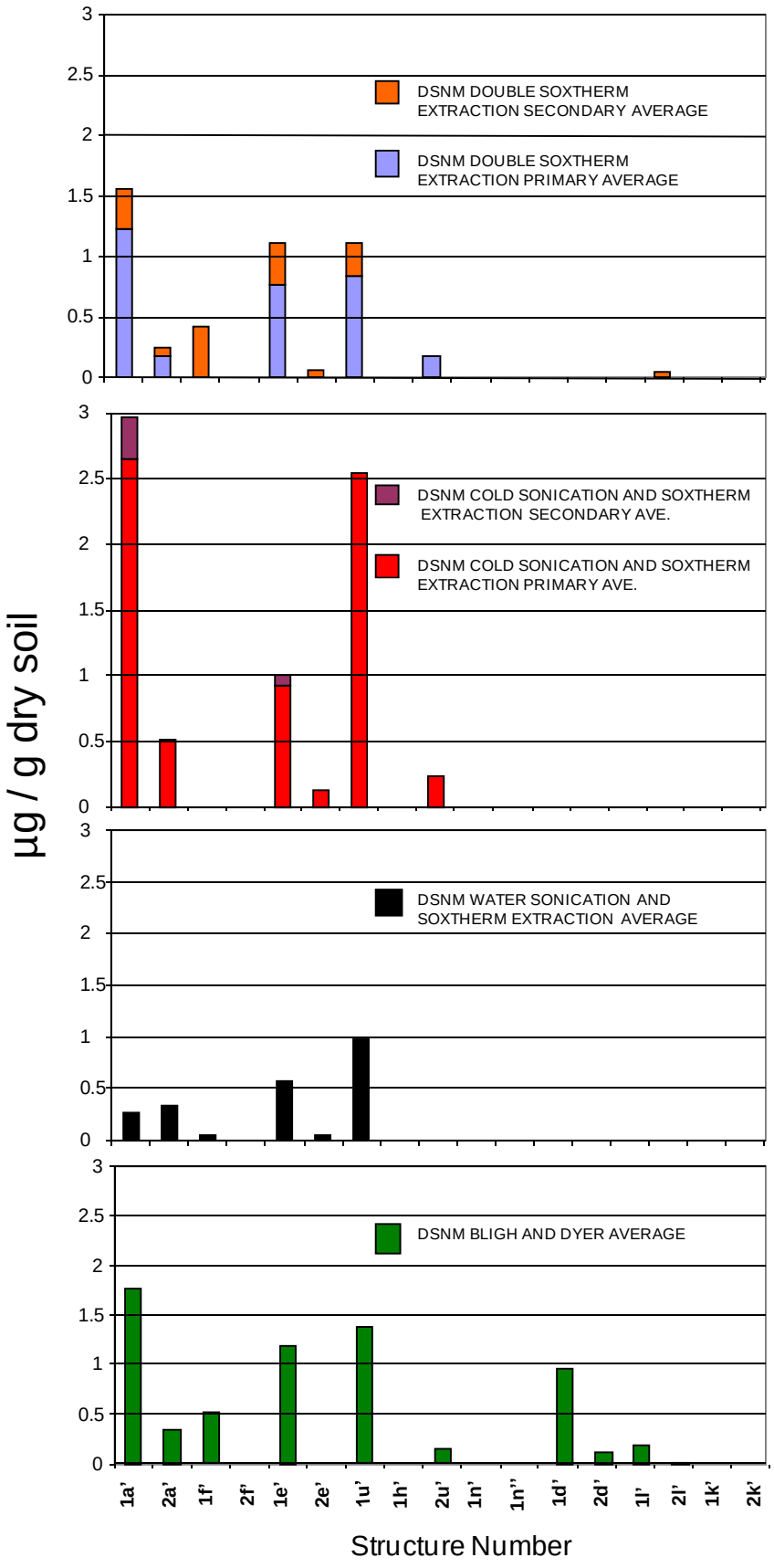


Fig. 2.51. Comparison of BHP extraction semi-quantitative concentrations from DSNM, 3 replicates. BHPs identified by base peak ion, see table 2.2.

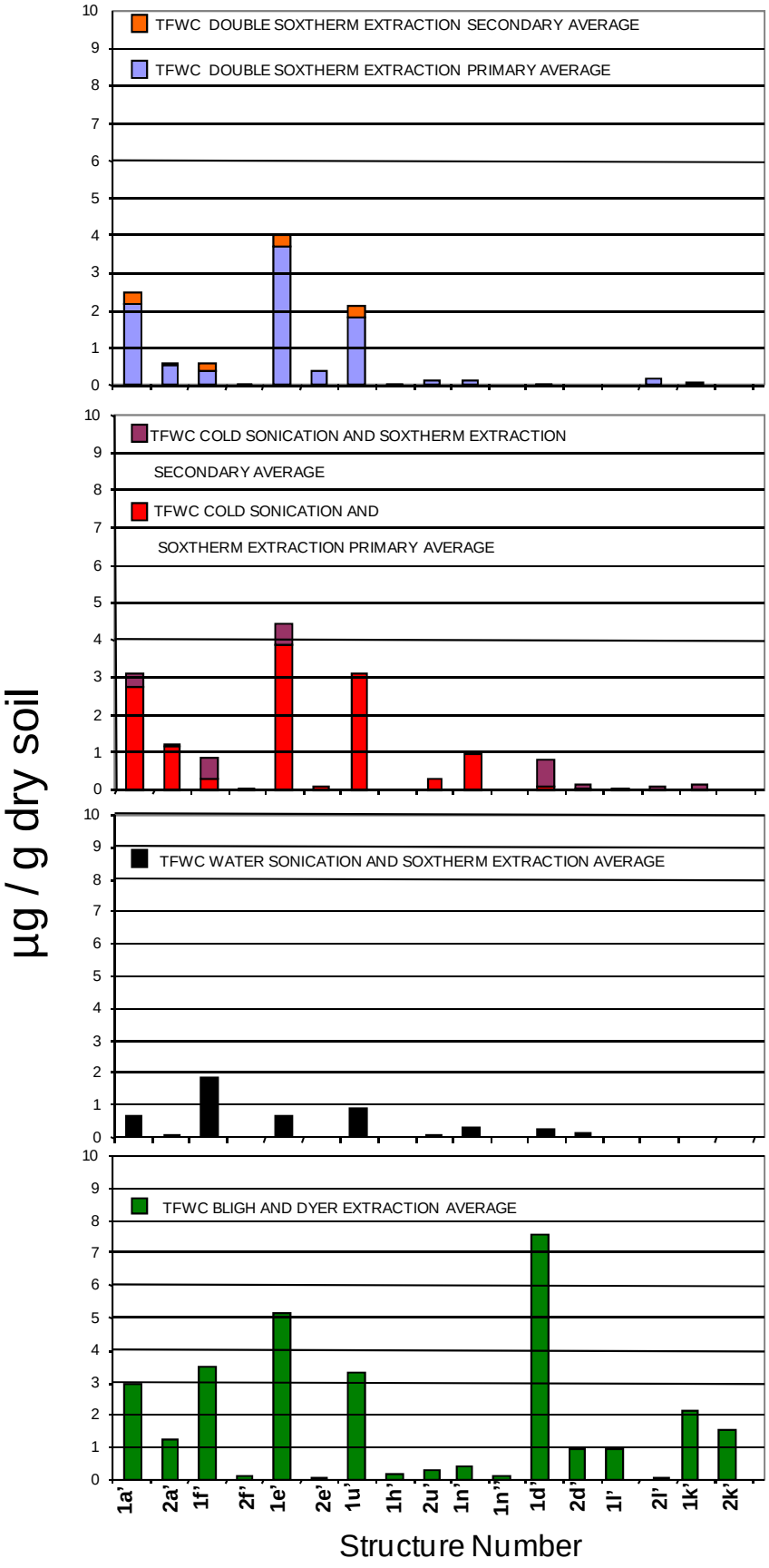


Fig. 2.52. Comparison of BHP extraction semi-quantitative concentrations from TFWC, 3 replicates. BHPs identified by base peak ion, see table 2.2.

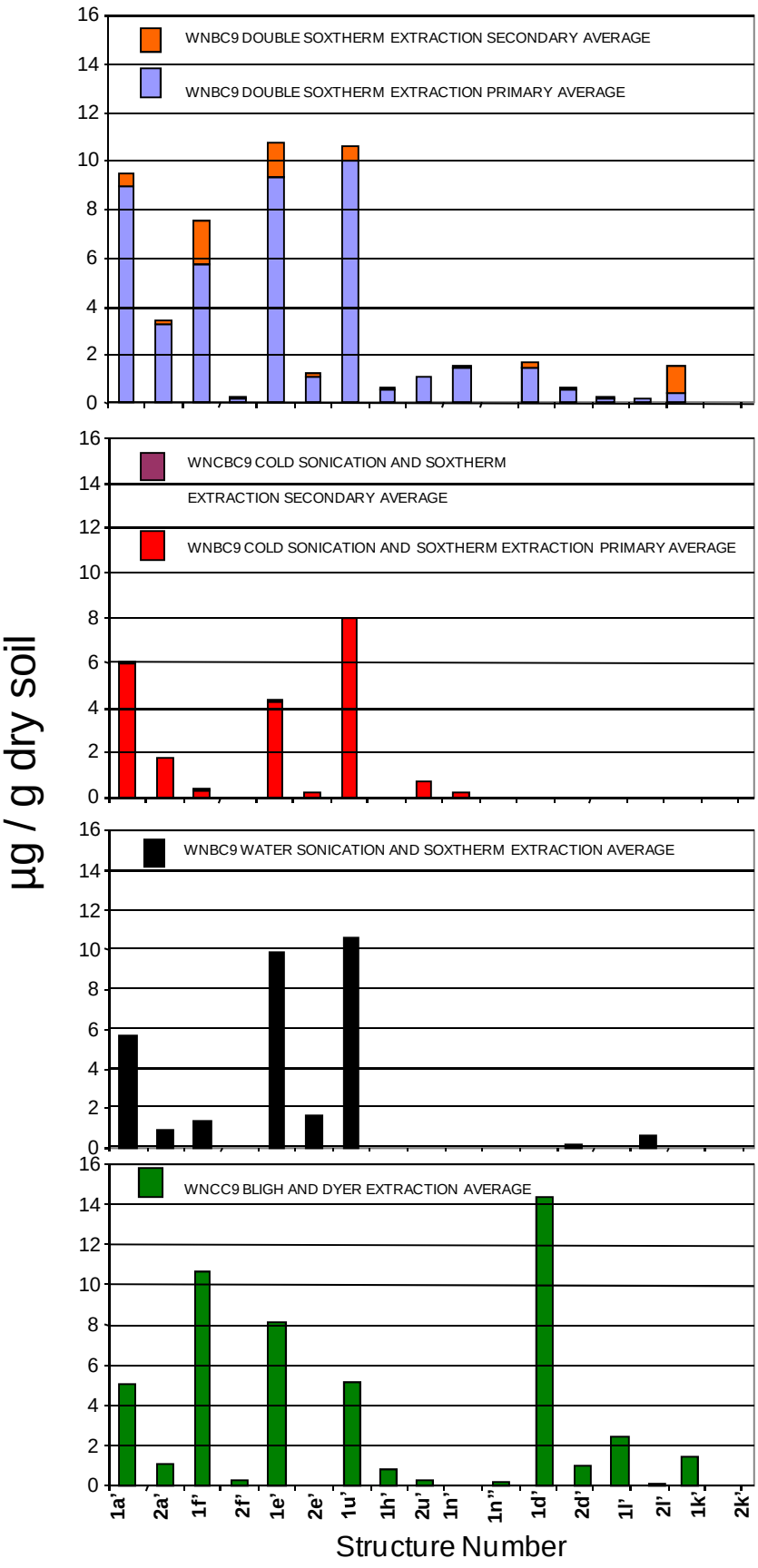


Fig. 2.53. Comparison of BHP extraction semi-quantitative concentrations from WNBC9, 3 replicates. BHPs identified by base peak ion, see table 2.2.

2.10 Relationship between BHPs, Bacteria and Environment

The relationship between laboratory cultured bacteria and BHPs, certain environments and BHPs is now well described and has been discussed in Chapter 1. These results are summarised in Table 2.2a (BHPs and bacteria) and Table 2.2b (BHPs and environment) and are used to indicate the presence of specific bacteria and bacterial processes in the samples.

Table 2.2a. Summary Table of relationship between BHPs and Source Bacteria.

Abbreviated name	Base Peak *m/z*	Structure Number	Known Source organisms[b]
AnhydroBHT	**613**	**1j'**	Possibly *R. acidophilus* [47]
Hopane lactone	**627**	**1t'**	Ammonia oxidising bacteria[39,47] PNSB[47]
$\Delta^{6 \text{ or } 11}$-BHT	**653**	**5a', 6a'**	Acetic acid bacteria[27,48]
BHT	**655**	**1a'**	Various[2,3,4,12,15,18,19,21,23,25,27,30-32,34,35,36,37,40,41,42,43,44,47,48,50,52]
2-me BHT	**669**	**2a'**	Cyanobacteria[3,13,40,44,50,55] *Rhodopseudomonas palustris*[32] *Methylobacterium organophilum*[34]
3-me BHT	**669**	**3a'**	Acetic Acid Bacteria,[48]
12-me BHT	**669**	**4a'**	Unknown
Unsat. aminotriol	**712**	**5f', 6f'**	*Rhodopseudomonas palustris*[47]
BHpentol	**713**	**1m'**	Cyanobacteria[36,50]
Aminotriol	**714**	**1f'**	Various[4,6,14,24,25,26,31,32,39,42,44,47,50,52]
2-me aminotriol	**728**	**2f'**	Cyanobacteria[56]
3-me aminotriol	**728**	**3f'**	Methanotrophs[57]
Adenosylhopane	**746**	**1e'**	Purple non-sulfur bactéria1[4,22,26,36,47] *Nitrosomonas europaea*[39,47] *Bradyrhizobium japonicum* [46]
2-me adenosylhopane	**760**	**2e'**	*Bradyrhizobium japonicum*[47]
'Adenosylhopane type- 1'[a]	**761**	**1u'**	Purple non-sulphur bacteria[47]
BHhexol	**771**	**1b'**	Unknown
Aminotetrol	**772**	**1h'**	Methanotrophs[11,23,24,36,42,43,56] *Desulfovibrio* sp.[4]
'2-me adenosylhopane type-1'	**775**	**2u'**	Unknown
'Adenosylhopane type-2'	**802**	**1v'**	Unknown
'2-me adenosylhopane type-2'	**816**	**2v'**	Unknown
'2-me adenosylhopane type-3'	**818**	**2w'**	Unknown
'Adenosylhopane type-4'	**818**	**1x'**	Unknown
Unsat, Aminopentol	**828**	**5c', 6c'**	Methanotrophs[11]
Aminopentol	**830**	**1c'**	Methanotrophs[11,23,36,42,43,56]
3-me aminopentol	**844**	**2c'**	Methanotrophs[11,23,43]
Unsat. BHT pentose	**941**	**5n', 6n'**	Cyanobacteria[50]
BHT pentose	**943**	**1n'**	Cyanobacteria[36,50]
2-me BHT pentose	**957**	**2n'**	Cyanobacteria[50]
Unsat. BHT ce	**1000**	**5d', 6d'**	Cyanobacteria[50] *B. cepacia*[47]
BHpentol pentose	**1001**	**1o'**	Unknown
BHT ce	**1002**	**1d'**	Various[4,12,14,15,17,18,19,21,26,34,36,42,43,47,48,50,52]
BHT glu	**1002**	**1g'**	Various [14,19,21,34,35,36,40,42,43,47,48,50,52]
Unsat. 2-me BHT ce	**1014**	**7d'**	Unknown
Unsat. BHT ce methylated on sugar	**1014**	**5p'**	Unknown
2-me BHT ce	**1016**	**2d'**	Cyanobacteria[50]
3-me BHT ce	**1016**	**3d'**	*Gluconacetobacter xylinus*[48]
'BHT ce methylated on sugar'	**1016**	**1p'**	Unknown
Unsat. BHpentol ce	**1058**	**5l', 6l'**	*Gluconacetobacter xylinus*[48]
BHpentol ce	**1060**	**1l'**	various[47,50]
BHpentol glu	**1060**	**1q'**	Unknown
2-me BHpentol cyclitol ether	**1074**	**2l'**	Unknown
3-me BHpentol ce	**1074**	**3l'**	*Gluconacetobacter xylinus*[48]
Guanidine sub. BHT ce	**1086**	**1s'**	Methylotrophic bacteria[36,37]
BHhexol ce	**1118**	**1k'**	Unknown
2-me BHhexol ce	**1132**	**2k'**	Unknown
3-me BHhexol ce	**1132**	**3k'**	Unknown
'BHhexol ce methylated on sugar'	**1132**	**1r'**	Unknown

Table 2.2b(i) Summary Table of relationship between BHPs and environment.

Abbreviated name	**Base Peak *m/z***	**Structure Number**	**Known locations where observed[b]**
AnhydroBHT	**613**	**1j'**	Recent and ancient soils and sediments[1,7,9,29,59], diagenetic product[7,38] Hot springs[28,46]
Hopane lactone	**627**	**1t'**	Hot springs[46] Soils[54,59]
$\Delta^{6 \text{ or } 11}$-BHT	**653**	**5a', 6a'**	Soils[8,54,59] Peat[58] Sediments[50,59]
BHT	**655**	**1a'**	Water column[5,53] Sediments[7,9,10,29,45,49,50,59] Soils[8,29,33,54,59] Peat[58,59] Hot springs[16,28]
2-me BHT	**669**	**2a'**	Soils[8,29,33,54,59] Water column[5,53] Sediments[9,10,29,45,49,59] Hot springs[16]
3-me BHT	**669**	**3a'**	Soils[8,59] Sediment [59]
12-me BHT	**669**	**4a'**	Sediment samples[58,59]
Unsat. aminotriol	**712**	**5f', 6f'**	Soils[8,54,59] Water column[53]
BHpentol	**713**	**1m'**	Hot spring samples[28,46] Soils[29] Sediments[29,45,50]
Aminotriol	**714**	**1f'**	Water column[5,53] Sediments[7,9,10,29,49,50,59] Soils[8,29,33,54,59] Hot springs[16]
2-me aminotriol	**728**	**2f'**	Soils[8,33,54,59] Hot springs[16] Sediments[49,59] Peat[59]
3-me aminotriol	**728**	**3f'**	Soils [59]
Adenosylhopane	**746**	**1e'**	Sediments[7,9,29] Soils[8,29,33,54,59] Peat[59]
2-me adenosylhopane	**760**	**2e'**	Sediments[7,9,59] Soils[8,33,59] Peat[58,59]
'Adenosylhopane type-1'	**761**	**1u'**	Soils[8,33,54,59] Sediments[9,59] Peat[58,59]
BHhexol	**771**	**1b'**	Sediments[49] Soils[59]
Aminotetrol	**772**	**1h'**	Sediments[7,9,10,29,45,49,50,51,59] Peat[58,59] Water column[5,53] Soils[8,29,33,54] Hot springs[16]
'2-me adenosylhopane type-1'[a]	**775**	**2u'**	Soils[8,54,59] Sediments[9,59] Peat[58,59]
'Adenosylhopane type-2'	**802**	**1v'**	Soils[59]
'2-me adenosylhopane type-2'	**816**	**2v'**	Soils[59]
'2-me adenosylhopane type-3'	**818**	**2w'**	Soils[59]
'Adenosylhopane type-4'	**818**	**1x'**	Soils[59]
Unsat, Aminopentol	**828**	**5c', 6c'**	Peat[59]
Aminopentol	**830**	**1c'**	Sediments[9,10,45,49,50,59] Water column[5,53] Hot springs[16] Peat[58,59] Soils[59]
3-me aminopentol	**844**	**2c'**	Hot springs[16] Sediment [45]
Unsat. BHT pentose	**941**	**5n', 6n'**	Soils[8,59] Sediments[50,59]
BHT pentose	**943**	**1n'**	Soils[8,59] Sediments[49,50,59] Peat[58,59]
2-me BHT pentose	**957**	**2n'**	Soils[8,59] Sediments[49,50,59]

Table 2.2b(ii) Summary Table of relationship between BHPs and environment.

Abbreviated name	**Base Peak *m/z***	**Structure Number**	**Known locations where observed[b]**
Unsat. BHT ce	**1000**	**5d', 6d'**	Soils[8,59]
BHpentol pentose	**1001**	**1o'**	Sediments[49,50,59]
BHT ce	**1002**	**1d'**	Sediments[7,9,10,29,43,49,50,59] Water column[5] Soils[8,29,33,54,59] Peat[59]
BHT glucosamine	**1002**	**1g'**	Sediments[7,10,49,50 ,59]
Unsat. 2-me BHT ce	**1014**	**7d'**	Soils[8,59]
Unsat. BHT ce methylated on sugar	**1014**	**5p'**	Soils[59]
2-me BHT ce	**1016**	**2d'**	Sediments[7,9,49,50,59] Soils[8,33,54,59] Peat[59]
3-me BHT ce	**1016**	**3d'**	Soils[59]
'BHT ce methylated on sugar'	**1016**	**1p'**	Sediment[59]
Unsat. BHpentol ce	**1058**	**5l', 6l'**	Soils[59]
BHpentol ce	**1060**	**1l'**	Sediments[7,9,49,50,59] Soils[8,33,54,59] Peat[58,59]
BHpentol glu	**1060**	**1q'**	Soils[59] Sediments[10]
2-me BHpentol cyclitol ether	**1074**	**2l'**	Soils[8,33,54,59] Peat[59]
3-me BHpentol ce	**1074**	**3l'**	Soils[59]
Guanidine substituted BHT ce	**1086**	**1s'**	Soils[8,33,54,59] Sediments[43,49,50]
BHhexol ce	**1118**	**1k'**	Sediments[7,9,49,50,59] Soils[8,54,59] Peat[58,59]
2-me BHhexol ce	**1132**	**2k'**	Soils[8,59] Sediments[9,59] Peat[58,59]
3-me BHhexol ce	**1132**	**3k'**	Soils[59]
'BHhexol ce methylated on sugar'	**1132**	**1r'**	Soils[59]

[a] Structures named in italics are tentative based on interpretation of mass spectrum in comparison to spectra of authentic standards of other known BHP structures (cf. Talbot et al., 2003b, 2007a, 2007b and unpublished)

[b] References related to hopanoid production: [1]Bednarczyk et al. 2005; [2]Berry et al., 1991; [3]Bisseret et al., 1985; [4]Blumenberg et al., 2006; [5]Blumenberg et al., 2007; [6]Bravo et al., 2001; [7]Cooke et al., 2008a; [8]Cooke et al., 2008b; [9]Cooke et al., 2009; [10]Coolen et al., 2008; [11]Cvejic et al., 2000a; [12]Cvejic et al., 2000b; [13]Doughty et al., 2009; [14]Flesch and Rohmer, 1988; [15]Flesch and Rohmer, 1989; [16]Gibson et al., 2008; [17]Herrmann et al., 1996; [18]Joyeux et al., 2004; [19]Knani et al., 1994; [20]Langworthy 1976; [21]Moreau 1997; [22]Neunlist and Rohmer, 1985a; [23]Neunlist and Rohmer, 1985b; [24]Neunlist and Rohmer, 1985c; [25]Neunlist et al., 1985; [26]Neunlist et al., 1988; [27]Ourisson and Rohmer 1992; [28]Pancost et al., 2006; [29]Pearson et al., 2009a; [30]Pieseler and Rohmer, 1992; [31]Poralla et al., 2000; [32]Rashby et al., , 2007; [33]Redshaw et al., 2008; [34]Renoux and Rohmer, 1985; [35]Rohmer 1988; [36]Rohmer, 1993; [37]Rosa-Putra et al., 2001; [38]Schaeffer et al., 2008; [39]Seemann et al., 1999; [40]Simonin et al., 1996; [41]Sinninghe Damsté et al., 2004; [42]Talbot et al., 2001; [43]Talbot et al., 2003a; [44]Talbot et al., 2003b; [45]Talbot et al., 2003c; [46]Talbot et al., 2005; [47]Talbot et al., 2007a; [48]Talbot et al, 2007b; [49]Talbot and Farrimond, 2007; [50]Talbot et al, 2008a; [51]Talbot et al, 2008b; [52]Vilcheze et al., 1994; [53]Wakeham et al., 2007; [54]Xu et al., 2009; [55]Zhao et al., 1996; [56]Zhou et al., 1991; [57]Birgel et al., unpublished data; [58]Talbot et al., unpublished data; [59]Cooke et al., this thesis.

3. Palace Leas Meadow Hay Plots Case Study

3.1 Introduction

The main focus of this research was to identify variations in BHP distributions in soil samples. For a useful comparison to be made a location was needed where only one parameter was different between the various samples. The Palace Leas meadow hay plots were chosen as the plots are all identical but for the last 110 years each plot had been treated with a different fertiliser regime. This enabled direct comparisons to be made between the plots with respect to BHP distribution without other environmental factors having to be considered.

The Palace Leas pasture plots, Cockle Park Farm (Figs. 3.1 and 3.2), are a long term experiment to investigate the effect of different fertiliser treatments on soil productivity and hay yield. The size of the site is approximately 100 m by 200 m, with a uniform northern exposure, no other surrounding vegetation (Fig 3.3) and an original uniform soil type of clay loam to a depth of 15 – 20 cm on top of a stiff clay subsoil.

The investigation of the Palace Leas soils took place between September 2004 and January 2007, it representes the first long term analysis of the BHP content of soils to be undertaken over a number of years and is the largest single study of BHP distribution in soil ever undertaken. Its purpose was to investigate the quantity, diversity and fate of BHPs in soil by comparing different sites under identical environmental conditions and to monitor variations in BHP levels over time to identify seasonal variations and the long term fate of the BHPs.

Work by other researchers (Chapter 1 and Table 2.2) has clearly linked the production of specific BHPs to specific bacteria. This enables the relationship between BHPs and bacteria to be used to identify differences in the BHP producing bacterial population of each plot.

3.2 Site Background Information

The Palace Leas plots are located on Cockle Park Farm, Hebron, approximately 20 km north of Newcastle upon Tyne (Fig. 3.1), grid reference NZ202915. The field was an organically fertilised pasture until 1896 when it was converted to use as a long

term pasture experiment (Hopkins and Shiel, 1996). The field is laid out in a series of 14 parallelogram shaped plots, each approximately 12 m wide and 100 m long, (Fig. 3.2) with an open North facing, exposure (Fig 3.3).

Each of the plots is subjected to a different fertiliser regime (Table 3.1) to investigate the effect of different fertilisers on the soil. It must be noted that the plots are used to graze livestock and therefore receive a superficial deposit of manure and urine. This may have an effect on the fertiliser regime but the overall effect should be consistent across the plots. All plots therefore can be considered to be treated with surficial manure as opposed to being untreated.

The original soil was a clay loam over clay of the Hallsworth Series, pelo stagnogley, with the hard clay layer at approximately 15 cm below the soil surface across the site (Shiel and Rimmer, 1984). Stagnogley soils are slowly permeable seasonally waterlogged soils also known as surface water gleys.

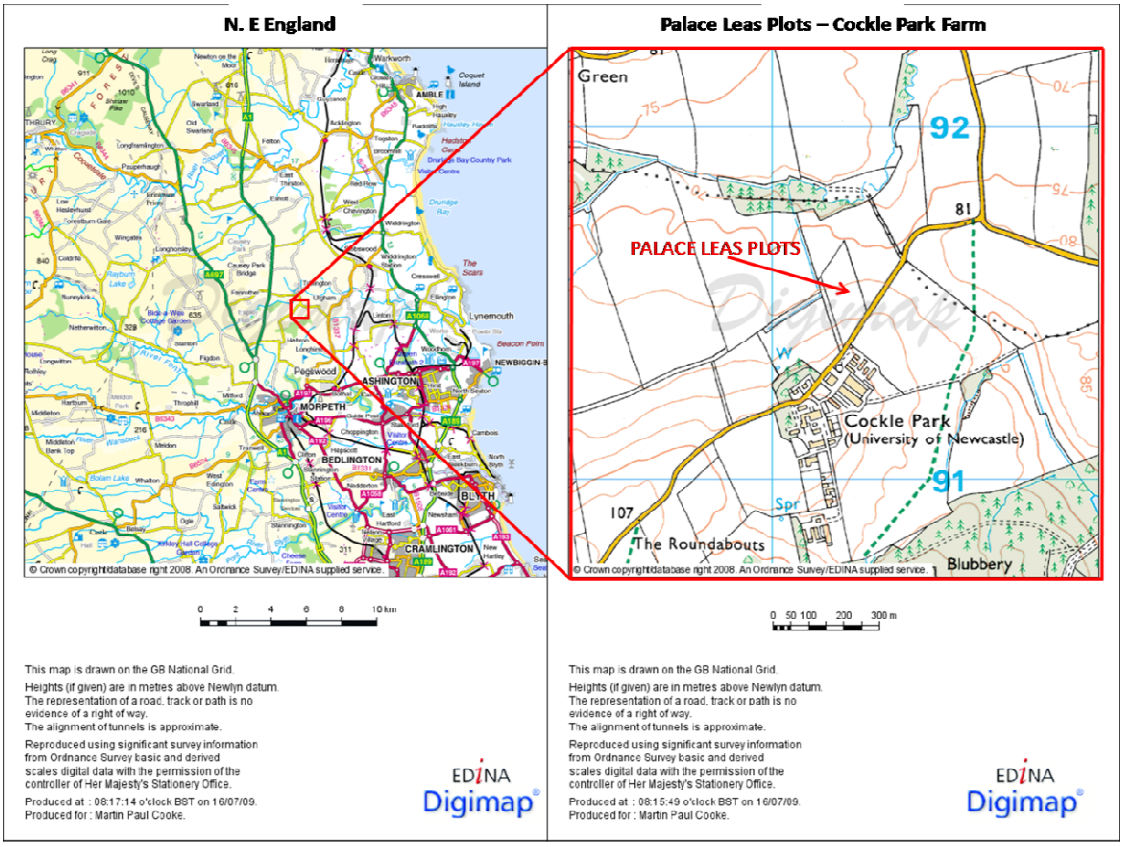


Fig. 3.1. Location of Palace Leas Plots

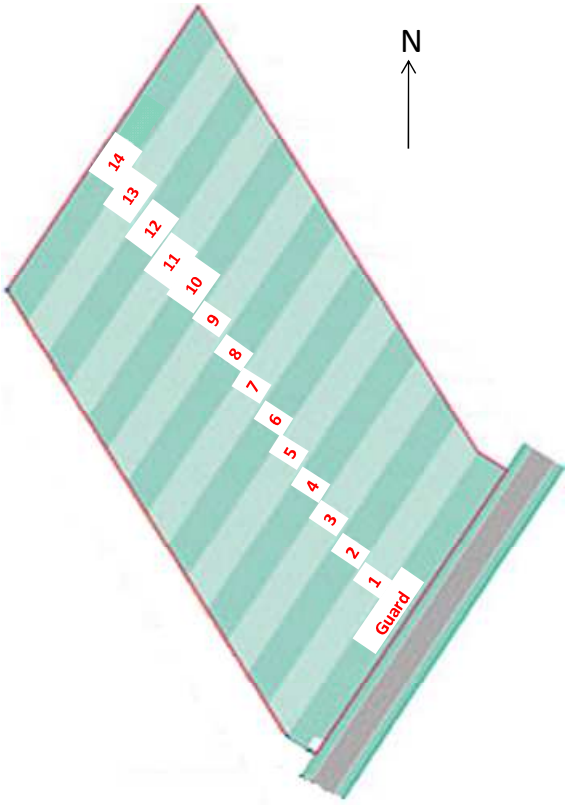


Fig. 3.2 Palace Leas Plots layout. http://www.staff.ncl.ac.uk/r.s.shiel/Palace_Leas/layout.htm

Fig. 3.3 Palace Leas. January 2006, taken from SW corner of field facing north.

The addition of ammonium sulphate (N on Table 3.1) to the soil has resulted in the lowering of the soil pH on some of the plots whereas the pH in all the other plots remained relatively constant at pH 4.7 – 5.5. Plot 7 (ammonium sulphate treatment only Table 3.1) has a lower pH, 3.8, than the other plots except plot 11, pH 3.7, which received ammonium sulphate and potassium chloride (muriate of potash) only.

The result of the lowering of the pH in plot 7 from pH 5.0 has been a redistribution of the organic material in the soil with an increase in surface organic matter on this plot (Shiel and Rimmer, 1984). The organic matter content in the top 9 cm is higher than the other plots but the organic matter between 9 cm and 15 cm is lower than the other plots (Shiel and Rimmer, 1984). Shiel and Rimmer also identified a reduction in biological activity of this organic layer. This was also attributed to the acidification of the soil which will reduce the bacterial, floral and faunal activity in the soil; biological activity is known to increase following the addition of lime (e.g. Wakelin et al., 2009).

The differences between the fertilisers can also be seen in the hay yields (Table 3.1). The control plot 6 produced an average of 2554 kg ha^{-1} whereas plot 2, subjected to a manure fertiliser only, produced 5290 kg ha^{-1} and plot 9, potassium fertiliser only, produced only 2317 kg ha^{-1} of hay per year (Coleman et al., 1987). This indicates significant variation in soil fertility with respect to hay yield, the manure only plot being the most fertile of the plots analysed in this study.

The maximum rate of glucose induced respiration (V_{max}), a bacterial population size indicator, has also been investigated (Hopkins and Shiel, 1996) and is significantly higher in the manure treated plots, e.g. plot 2, indicating higher bacterial activity, than in the other plots, especially plot 7 (Table 3.1). The implication of this is that the microbial population of plot 7 is lower than the other plots. This is confirmed by the ratio of microbial carbon mass to organic carbon mass which is an order of magnitude less on plot 7 than on other plots (Table 3.1).

Plot 7 has been shown to have the highest rate of carbon dioxide respiration per unit biomass (qCO_2) but glucose respiration was lower in plot 7 than the other plots (Table 3.1). This indicates that the smaller bacterial population in plot 7 is respiring at a greater rate than the other plots. This indicates that the population is stressed, probably due to the lower pH on plot 7 than the other plots, and this could contribute to the accumulation of organic matter on this plot due to lack of bacterially induced turnover (Hopkins and Shiel, 1996).

From these observations it is apparent that different fertiliser regimes affect the soil properties and bacterial activity. This investigation will look at the effect of the different fertilisers with respect to BHP signatures from various depths within the soil. The 5 different plots investigated are highlighted in yellow on table 3.1 and are the extremes of the fertilisation regimes.

Table 3.1. Fertiliser and manure application regime.

Plot	Year of Cycle	Farmyard manure t ha^{-1}	Fertilizer (kg nutrient ha^{-1})			Hay Yield (kg ha^{-1})	[a]V_{max} (µmol CO_2 g^{-1} soil h^{-1})	[a]*q* CO2 (µmol CO_2 mg^{-1} C_{mic} h^{-1})	[a]Ratio of microbial C to organic C (C_{mic}:C_{org})
			N	P_2O_5	[b]K_2O				
1		20	17	30	34				
2		20				5290	1.09	0.27	0.017
3	1	20							
	2		17	30	34				
4	1	20							
	2								
5	1	40							
	2		17	30	34				
	3		17	30	34				
	4		17	30	34				
6						2554	0.658 (0.0082)	0.37 (0.044)	0.014
7			35			3049	0.248	1.23	0.003
8				60		3463	No Data	No Data	No Data
9					67	2317	No Data	No Data	No Data
10			35	60					
11			35		67				
12				60	67				
13			35	60	67				
14			100	66	100				

Adapted from http://www.staff.ncl.ac.uk/r.s.shiel/Palace_Leas/treatments.htm

[a]From Hopkins and Shiel, 1996

[b]K_2O is an equivalent and actually KCl (muriate of potash) is applied to the soil.

3.3 Goals

The purpose of the Palace Leas investigation was to test hypothesis 1;

The BHP fingerprint of a soil indicates the current bacterial population.

by pursuing 4 different goals. Goals 1-3 test the ability to identify differences in the soil BHP distributions and to use those differences to infer the bacterial population. Goal 4 tests the recalcitrance of BHPs in soils describing the age of the BHPs in the soil and therefore the relevance to the current bacterial population.

Goal 1

Identify the BHP profile of a soil from different plots.

For BHPs to be useful as a biomarker for bacterial activity it must be possible to identify different bacterial populations directly from the BHP profile of the soil. Analysis by many researchers has shown that different bacteria produce different BHPs (see Chapter 1 and table 2.2 and the references therein). The presence of these BHPs in the soil will therefore indicate the presence of the bacteria without the need to directly culture the bacteria or the use of culture dependent DNA marker techniques.

Goal 2

Identify any differences in soil BHP profile due to differences in fertilizer regime.

From the length of time of the Palace Leas' fertiliser treatments, approximately 110 years, it can be assumed that the bacterial populations on each plot are likely to be at or approaching equilibrium and that differences in the bacterial population due to fertiliser differences are well established (Hopkins and Shiel, 1996; Shiel and Rimmer, 1984). Therefore these differences in BHP producing bacterial population can be measured by differences in the BHP distribution.

However for BHPs to be a useful biomarker in measuring fertiliser effect there must be significant and measurable differences between the BHP results for each plot and not between the results within the plots. If the variation within the plots is comparable to the variation between the plots then BHPs cannot be used to measure changes in the bacterial population due to the fertiliser regime as the natural variation within the soil BHP profile is greater than that induced by the fertiliser.

The fertiliser regime has also lead to differences in the overlying vegetation (Birch, 1985) on the plots invstigated. This will also have an influence on the underlying soil bacterial population and therefore the BHP distribution. These changes in the BHP population due to changes in plant population can therefore be indirectly related to the fertiliser regime

Goal 3

Identify differences in BHP profile between stressed and non-stressed plots.

If BHPs were produced uniformly by different bacterial populations then the concentration and distribution of BHPs would be a direct indicator of bacterial population size and variation. However under different environmental conditions BHP production may vary significantly (e.g. Welander et al., 2009). A low bacterial population under environmental stress could therefore produce relatively high levels of BHPs when compared to a less stressed but greater bacterial population.

In the Palace Leas plots, plot 7 is known to contain a relatively small and stressed bacterial population when compared to plot 2 (Hopkins and Shiel, 1996).

If the level of BHPs is directly proportional to the bacterial population then the level of BHPs in plot 7 would be expected to be the lower than in plots 2 and 6 (Table 3.1). However if bacteria produce BHPs in response to environmental stress then it would be expected that the level of BHPs in plot 7 would be higher or equivalent to that in plots with a higher bacterial population, i.e. plots 2 and 6 (Hopkins and Shiel, 1996).

Goal 4

Identify any temporal differences in the BHP profile.

Variations in the BHP producing bacterial population and activity within a plot due to seasonal variation will be expressed either by changes in the distribution of BHPs or by changes in the total concentration of BHPs.
If any significant variation is observed then it must also be true that the BHP profile of the soil represents changes in the current bacterial activity of the soil and is not as a result of preservation within the soil of much older BHPs, where BHPs would be expected to accumulate over time.

3.4 Sampling Methodology

Three 15 cm cores (identified as cores 1, 2 and 3) were taken from each of the plots to be analysed at approximately 4 month intervals between September 2004 and January 2007. Administration and collection issues resulted in there being no cores taken for May 2005 and October 2005. To counteract this problem the sampling was extended to January 2007 to ensure a continuous year's data was obtained. Samples were therefore collected and analysed from September 2004, January 2005, January 2006, May 2006, October 2006 and January 2007. Cores were collected as described in section 2.1 and stored at -22^{o}C. Cores 1 and 2 were then sectioned in depth layers of 0-5, 5-10 and 10-15 cm, freeze dried, ground, extracted and analysed as described in chapter 2.
In addition to the analysis of each core, one complete core from each sampling date was extracted and analysed in duplicate and one in every 15 extracts was analysed twice to measure reproducibility. All results shown are the average of all the analyses for each plot, both from the 2 cores and the repeat extractions and analyses.
Sampling of the soils indicated that the soil is a uniform brown colour with little evidence of the expected mottling and colour changes that would be expected from a surface water gley (Fig 3.4). This implies that the oxygen penetration into the soil is significant to a depth of at least 15 cm and there is little waterlogging within the soil due to the underlying clay layer.

3.5 Results

3.5.1 Comparison between cores 1 and 2

Analysis of the relative standard deviation (RSD) between the BHP analysis of the Palace Leas samples for cores 1 and 2, and repeats, for each plot gave an RSD of 17.8% when compared to the experimental RSD of 17%, where samples were repeatedly analysed by HPLC-MS^n (section 2.7).

3.5.2 Results

A detailed analysis of the results from September 2004 for each plot will be used as a baseline set of results and variations identified in other samples will be compared to the results for September 2004. The sample cores from January 2006 clearly show a uniform brown colour to the soil (Fig 3.4) with no observable differences down the core or between cores. This profile was consistent throughout the period of investigation.

Analysis of the BHPs from each plot for September 2004 (Fig 3.5) identified 34 different BHPs in the 5 plots. The BHP distribution was dominated by 3 different BHPs, aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**), in each of the 5 plots and at all depths. These three BHPs account for approximately 60 – 70% of the BHPs in each soil profile (Appendix). In all the soils a fourth BHP is a significant contributor to the BHP total. For plots 2, 6, 8 and 9 the fourth BHP is adenosylhopane type-1 (**1u**), whereas for plot 7 the fourth most abundant BHP is BHT (**1a**).

The individual plots each contain between 21 (plot 9) and 25 different BHPs (plots 7 and 8), with the greatest number of BHPs in each plot being identified in the 0-5 cm layer. The BHPs identified in the September 2004 samples include 6 BHPs with previously unknown structures; 2-methyl adenosylhopane type-1 (**2u**), (subsequently reported in Cooke et al., 2008b), adenosylhopane type-2 (**1v**), 2-methyl adenosylhopane type-2 (**2v**), unsaturated 2-methyl BHT cyclitol ether (**7d**) (subsequently reported in Cooke et al., 2008b), unsaturated BHT cyclitol ether with extra $–CH_2$ on the terminal sugar (**5p**) and 3-methyl BHhexol cyclitol ether (**3k**). The identification of these structures is based on comparison with known structures and is

only tentative at this time. A fuller description of these structures is given in Section 2.8.

The total concentration of BHPs in September 2004 varies between the plots with a maximum of 1818 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 0-5 cm layer of plot 7 and a minimum concentration of 288 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 10-15 cm layer of plot 7. Plots 6 and 7 contain the highest concentration of BHPs with plot 2 the lowest overall concentration.

Palace Leas Core 2, January 2006.

Palace Leas Plot 6 January 2006

Palace Leas Plot 7 January 2006

Palace Leas Plot 8 January 2006

Palace Leas Plot 9 January 2006

Fig. 3.4 Palace Leas soil cores. January 2006. Core length approximately 15 cm

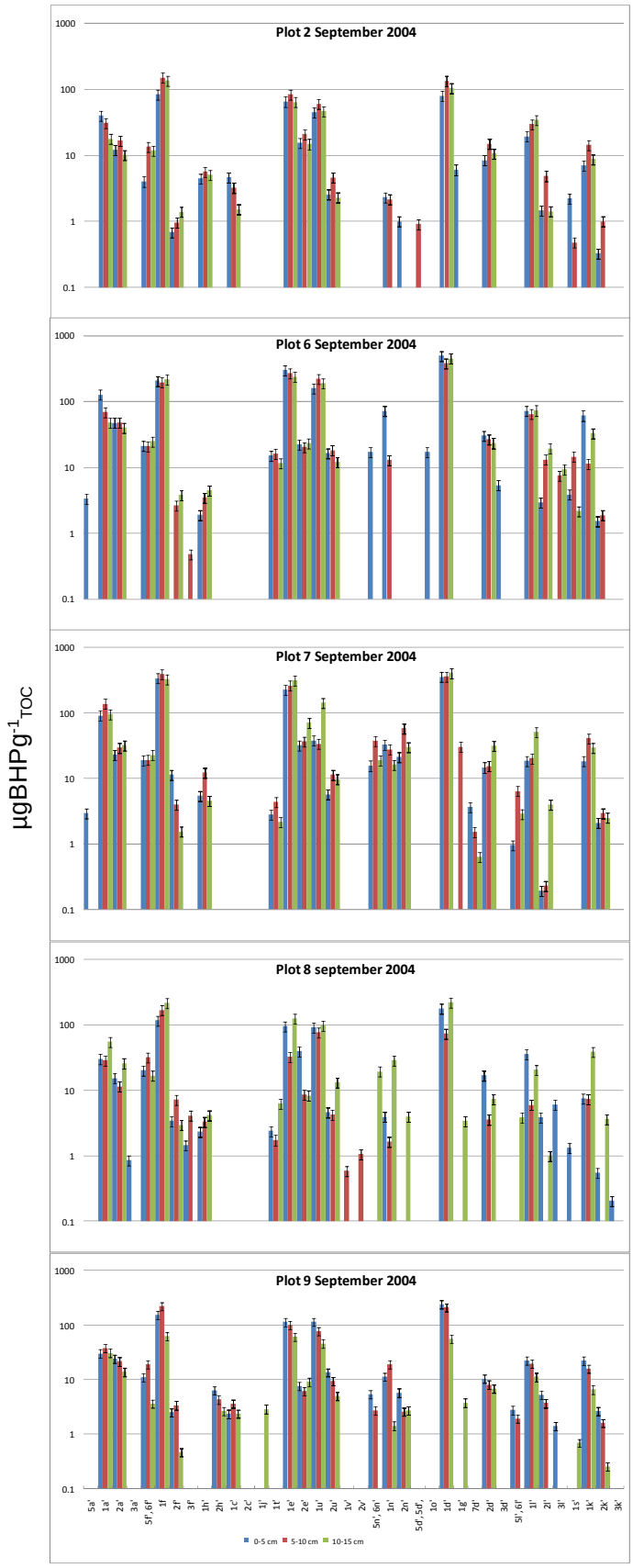


Fig. 3.5 Log scale of averaged Palace Leas BHP semi quantitative concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) from September 2004. BHPs identified by structure number (see table 2.2 for details).

3.5.2.1 Palace Leas Plot 2.

The TOC in plot 2, September 2004, is high in the 0-5cm layer, 9.1 %, (Appendix) probably due to the application of manure to the site which has a high organic carbon content. The TOC then drops to 5.2% (5-10 cm) and 3.9% (10-15 cm). In the 0-5 cm layer extracted BHPs account for 404 µg out of the total of 91 mg of organic carbon per g dry soil (0.4%). In the 5-10 cm layer BHPs account for 1.1% of OC per g dry soil and in the 10-15 cm layer 1.2%.

The results for plot 2, September 2004 (Fig. 3.5), showed 21 different BHPs within the core, with 21 in 0-5 cm layer, 20 in 5-10 cm and 16 in 10-15 cm. The BHP profile was dominated by 4 BHPs, aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type 1 and BHT cyclitol ether (**1d**) accounting for 68% (0-5 cm layer), 72% (5-10 cm) and 75% (10-15 cm) of the BHP total (Fig 3.5 and appendix) with aminotriol (**1f**) the most abundant, followed by BHT cyclitol ether (**1d**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**).

BHT (**1a**) and BHpentol cyclitol ether (**1l**) make up the largest proportion of the remaining BHPs, accounting for 15% (0-5 cm), 10% (5-10 cm) and 11% (10-15 cm) of the total (Fig 3.5 and appendix).

The other minor BHPs comprise between 14 and 18% of the BHP total, with a wide variety of structures including aminopentol (**1c**) and 6 different C-2 methylated BHPs, including 2-Me BHT (**2a**) and 2-Me aminotriol (**2f**). In all cases the methylated forms are less abundant than the non-methylated form. Unsaturated structures are also present, including unsaturated aminotriol (**5f or 6f**) and unsaturated BHT cyclitol ether (**5d or 6d**), again at lower levels than the saturated form. Bacteriohopanehexol cyclitol ether was observed in both the C-2 methylated form and the form where there is an extra $–CH_2$ on the terminal group. These 2 structures co-elute and can not be separated so the quantitative results were combined as *m/z* = 1132 but the presence of both structures was identified by their characteristic APCI MS^2 spectrum (Figs. 2.25 and 2.26). Analysis of the variation in BHP distribution with depth shows the number of structures identified decreases with depth with 4 structures, BHT pentose (**1n**), BHT glucosamine (**1g**), unsaturated BHpentol cyclitol ether (**5l or 6l**) and guanidine substituted BHT cyclitol ether (**1s**), all observed less frequently moving down the soil profile.

Many of the BHPs show a trend of increase in concentration in the 5-10 cm band followed by return to levels similar to those observed in the 0-5 cm band in the 10-15 cm band, e.g. 2-Me BHT (**2a**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**). However, 4 structures deviate significantly from this pattern, BHT (**1a**) shows a continual decrease with depth, BHpentol cyclitol ether (**1l**) shows a continual increase with depth and both aminotriol (**1f**) and BHT cyclitol ether (**1d**) show rises greater than 50% in the 5-10 cm band which fall only slightly in the 10-15 cm band.

Analysis of all the plot 2 samples during the entire sampling period showed that the BHP profile continues to be dominated by the 4 main BHPs (Fig 3.6) that were identified in September 2004 (Fig 3.5). The general BHP profile and relative distributions of the BHPs remain relatively consistent within the plot (Fig 3.6) when compared to each other and to September 2004 (Fig. 3.5). There are however some temporal differences that were observed between the plot 2 samples.

A further 11 BHPs were, however, identified in plot 2 during the course of the investigation. In all cases these BHPs were found at low levels and only in some samples indicating a minor contribution to the BHP profile (Table 3.2). The exception to this is the presence of 3-methyl BHpentol cyclitol ether (**3l**) which was observed in all samples except September 2004.

Table 3.2 Date and location of BHPs identified in plot 2 samples other than Plot 2 September 2004

Abbreviated name	**Base Peak m/z**	**Structure Number**	**Sample – Plot 2 only**
AnhydroBHT	**613**	**1j**	October 2006 (0-5 cm)
Hopane lactone	**627**	**1t**	January 2006 (0-5, 5-10 cm), January 2007 (0-5 cm)
Δ^6-BHT	**653**	**5a**	January 2007 (5-10 cm)
'Adenosylhopane type-2'	**802**	**1v**	October 2006 (0-5 cm)
'2-methyl adenosylhopane type-2'	**816**	**2v**	October 2006 (0-5 cm), January 2006 (5-10 cm)
2-methyl aminopentol	**844**	**2c**	May 2006 (0-5, 5-10 cm), October 2006 (0-5 cm)
Unsaturated 2-methyl BHT cyclitol ether	**1014**	**7d**	May 2006 (0-5 cm)
3-methylBHT cyclitol ether	**1016**	**2d**	May 2006 (0-5 cm), January 2007 (0-5, 5-10 cm)
Unsaturated BHpentol cyclitol ether	**1058**	**5l or 6l**	January 2005 (0-5, 5-10 cm), January 2006 (0-5 cm)
3-methylBHpentol cyclitol ether	**1074**	**3l**	All samples except September 2004
3-methylBHhexol cyclitol ether	**1132**	**3k**	October 2006 (5-10 cm)

The second major difference is changes in the absolute levels of BHPs identified in the cores. All plot 2 samples (Fig 3.6) show similar levels of BHPs with the exception of October 2006 where the levels of BHPs are approximately double that observed in the other cores.

The final difference is in changes in the levels of BHPs at different depths down the core. The levels in January 2005 are approximately equal at all levels down the core (Fig 3.6) indicating an even distribution of the BHPs. This pattern is also seen in January 2007. January 2006 (Fig 3.6) clearly indicates an increase in BHPs with depth. Both May 2006 and October 2006 clearly show that the highest levels of BHPs are found in the 5-10 cm layer. In all these cases the changes in distribution is dominated by changes in the 4 main BHPs; aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**).

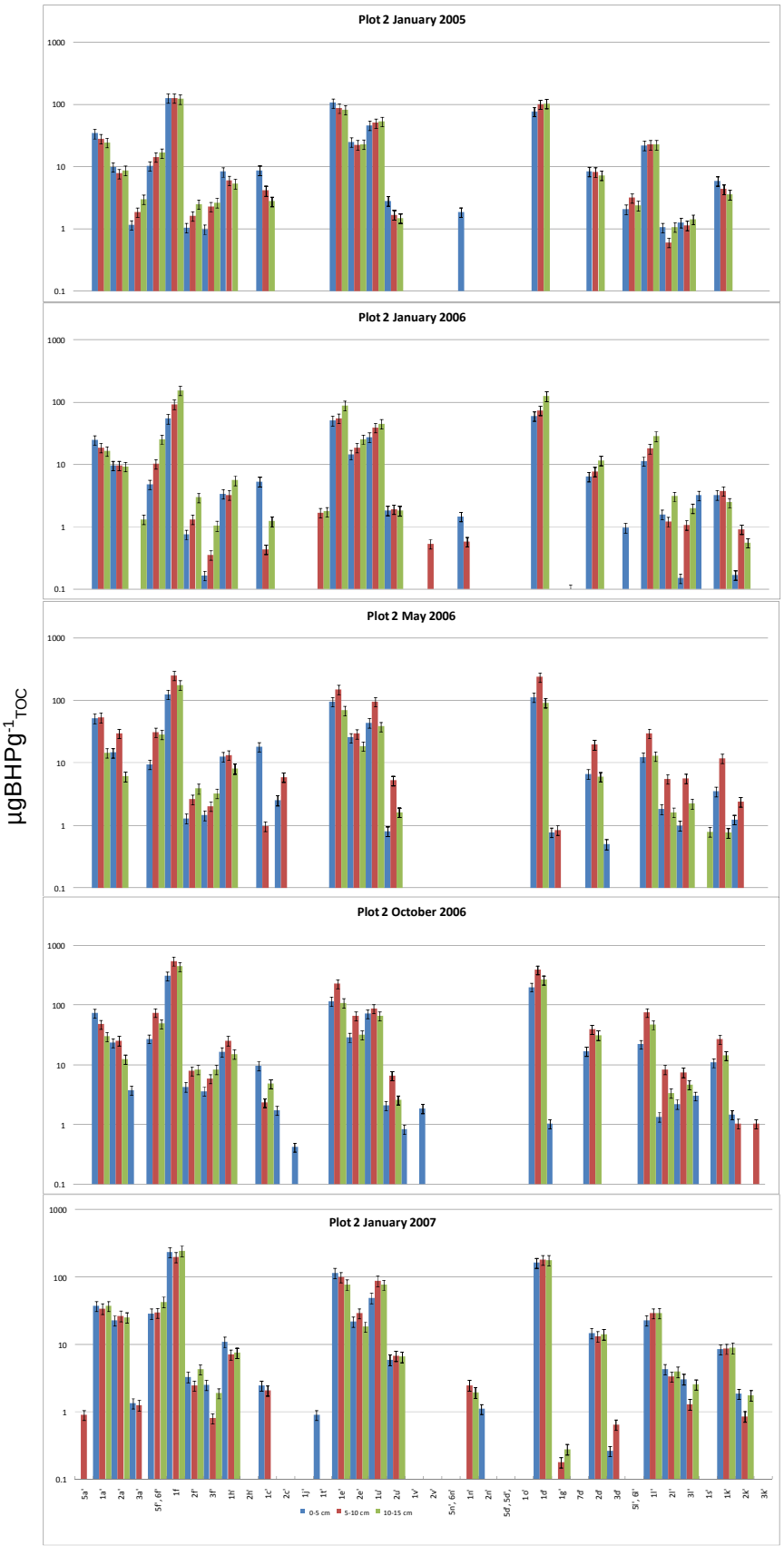


Fig 3.6 Log scale of averaged Palace Leas Plot 2 BHP semi quantitative concentrations ($\mu g_{BHP}\ g^{-1}{}_{TOC}$). BHPs identified by structure number (see table 2.2 for details).

3.5.2.2 Palace Leas Plot 6.

Plot 6, September 2004, has approximately 50% of the TOC of plot 2 (Appendix), and the BHP content is approximately 3-4 times greater in plot 6 than plot 2 (relative to organic cabon), indicating a much higher percentage of BHPs in the organic layer of the sample with BHPs accounting for 3% of OC, 0-5 cm layer, 4.9%, 5-10 cm, and 5.7%, 10-15 cm layer (Fig 3.5).

A total of 23 different BHPs were identified in plot 6 September 2004 (Fig 3.5), with 22 in 0-5 cm layer, 21 in 5-10 cm and 18 in 10-15 cm. Aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**) are again the most abundant. The most obvious difference from plot 2 is that BHT cyclitol ether (**1d**) is the most abundant BHP, followed by adenosylhopane (**1e**), then adenosylhopane type-1 (**1u**) and aminotriol (**1f**), at approximately the same level. These 4 structures account for 69% (0-5 cm), 75% (5-10 cm) and 77% (10-15 cm) of the BHP total.

There are also significant differences in the concentrations of the minor BHPs when compared to plot 2, September 2004, with aminopentol (**1c**), BHT glucosamine (**1g**) and unsaturated BHT pentose (5**n or 6n**) being absent in plot 6. In addition to these differences in identified BHPs, and the significant increase in BHP concentration, the concentration of BHT pentose (**1n**) in the 0-5 cm layer for plot 6 to 4% of total BHP content compared to only 0.2% in plot 2.

Analysis of the other plot 6 results (Fig 3.7) indicates that there is no clear trend in the distribution of BHPs down the cores nor in the total concentrations of the majority of the BHPs throughout the sampling period. However the level of aminotriol (**1f**) in the 0 – 5 cm layer of May 2006 (Fig. 3.7) is very high, 1436 $\mu g_{BHP}\ g^{-1}{}_{TOC}$, and in the January 2007 0 – 5 and 10 – 15 cm layers (772 and 789 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ respectively). The level of BHT cyclitol ether (**1d**) in January 2007 (0 – 5 cm) is also extremely high when compared to the other samples (682 $\mu g_{BHP}\ g^{-1}{}_{TOC}$).

In addition to the 23 BHPs identified in plot 6, September 2004, a further 6 BHPs were identified in the other plot 6 samples (Table 3.3). These BHPs were identified at low levels and infrequently, indicating a minor contribution to the BHP total.

Table 3.3 Date and location of BHPs identified in Plot 6 samples other than in plot 6 September 2004

Abbreviated name	Base Peak m/z	Structure Number	Sample – plot 6 only
2-methylBHT pentose	**957**	**2n**	January 2005 (0-5, 5-10 cm), January 2006 (0-5, 5-10, 10-15 cm), January 2007 (0-5, 10-15 cm)
Unsaturated BHT cyclitol ether	**1000**	**5d or 6d**	January 2005 (0-5, 10-15 cm), January 2006 (0-5, 10-15 cm)
BHT glucosamine	**1002GLU**	**1g**	January 2005 (0-5, 10-15 cm), January 2006 (0-5, 5-10 cm) May 2006 (0-5, 5-10, 10-15 cm), October 2006 (0-5, 5-10, 10-15 cm), January 2007 (10-15 cm)
Unsaturated 2-methyl BHT cyclitol ether	**1014**	**7d**	May 2006 (0-5, 5-10, 15-15 cm)
Unsaturated BHpentol cyclitol ether	**1058**	**5l or 6l**	January 2005 (0-5, 5-10 cm)
3-methylBHhexol cyclitol ether	**1132**	**3k**	May 2006 (0-5, 5-10 cm), October 2006 (0-5, 5-10 cm)

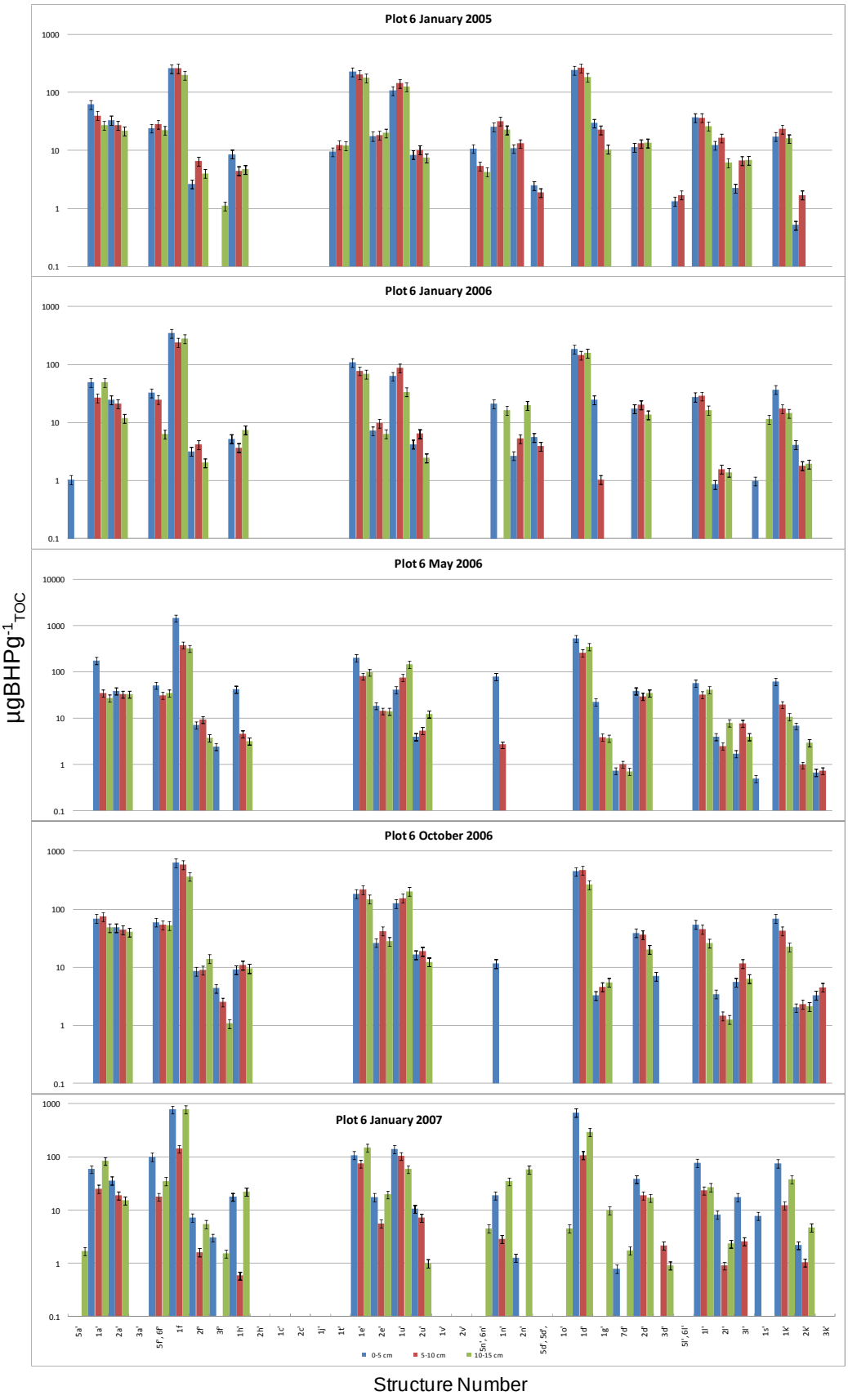


Fig 3.7 Log scale of averaged Palace Leas Plot 6 BHP semi quantitative concentrations ($\mu g_{BHP}\ g^{-1}{}_{TOC}$). BHPs identified by structure number (see table 2.2 for details).

3.5.2.3 Palace Leas Plot 7.

In plot 7, September 2004, the 0-5 cm layer of the core has a very high TOC (16%) (Appendix). This is in accordance with the observations of Shiel and Rimmer (1984) and related to the formation of an organic rich, almost peat like layer in the top 0-5 cm of the soil profile.

A total of 25 BHPs were identified during analysis of plot 7, September 2004. These include the novel unsaturated BHT cyclitol ether with an extra $–CH_2$ (**5p or 6p**) located on the terminal sugar (see Fig. 2.19). Unsaturated BHpentol cyclitol ether (**5l** or **6l**) was also detected in the plot 7 samples

The distribution of BHPs is significantly different to plot 2 and 6; here adenosylhopane type-1 (**1u**) is significantly less abundant and is classed as a minor contributor to the BHP total, whereas the concentration of BHT (**1a**) has increased (Fig. 3.5). BHT cyclitol ether (**1d**) is again the most abundant BHP followed by aminotriol (**1f**), adenosylhopane (**1e**) and then BHT (**1a**). The 4 major BHPs account for 72% (0-5 cm), 80% (5-10 cm) and 77% (10-15 cm).

There is a significant concentration of BHT pentose (**1n**) and its unsaturated (**5n or 6n**) and methylated form (**2n**) in plot 7 which decreases dramatically with depth from 11% (0-5 cm) to 1% (10-15 cm). This is similar to the occurrence of these structures in plots 2 and 6, where there is also a decrease with depth (Fig. 3.5).

Plot 7 shows a dramatic variation in BHP concentration with depth that is mirrored by a similar variation in TOC. The concentration and number structures decreases from 1817 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ to only 288 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 10-15 cm layer (Fig. 3.5). The concentration in the 0-5 cm layer is especially high when the high TOC (16.0%) is taken into account. The decrease with depth is reflected in the reduction of each of the BHP structures except adenosylhopane type 1 which increases in the 5-10 cm layer and unsaturated aminotriol (**5f or 6f**) which shows a significant increase in the 5-10 cm layer when compared to the other 2 layers. The number of observed structures decreases from 25 in the 0-5 cm to 15 in the 10-15 cm. The % contribution of BHPs to the TOC varies with depth; 1.1% (0-5 cm), 4.8% (5-10 cm), and 1.7% (10-15 cm).

Analysis of the other plot 7 samples (Fig 3.8 and appendix) clearly shows that in all the other samples there is a decrease in BHP concentration with depth, except for September 2004, where the concentrations remain relatively constant throughout the core. The concentration of BHPs in the samples also varies dramatically with

September 2004, January 2005 and January 2006 all having similar concentrations (Figs. 3.5 and 3.8) whereas May 2006 has approximately triple the BHP concentration (Fig. 3.8) and October 2006 approximately double the BHP concentration (Fig. 3.8). There is also a rise in the level of aminotriol (**1f**) in May 2006 (0-5 cm) to 1615 μg_{BHP} $g^{-1}{}_{TOC}$, which is far in excess of the rises observed in the other BHPs in this sample.
In total a further 10 BHPs were identified from all the plot 7 samples (Table 3.4) that were not identified in the September 2004 samples. Again these were all minor components and occurred infrequently.

Table 3.4. Date and location of BHPs identified in plot 7 samples other than in plot 7 September 2004

Abbreviated name	**Base Peak m/z**	**Structure Number**	**Sample – plot 7 only**
AnhydroBHT	**613**	**1j**	January 2006 (0-5, 5-10 cm), May 2006 (0-5, 5-10 cm)
3-methylaminotriol	**728**	**3f**	January 2005 (0-5 cm), May 2006 (10-15 cm), January 2007, 0-5, 5-10, 10-15 cm)
Unsaturated BHT cyclitol ether	**1000**	**5d or 6d**	May 2006 (0-5 cm)
BHpentol pentose	**1001**	**1o**	January 2005 (0-5 cm), May 2006 (0-5 cm), October 2006 (0-5, 5-10 cm)
3-methylBHT cyclitol ether	**1016**	**3d**	January 2007 (5-10 cm)
3-methylBHpentol cyclitol ether	**1074**	**3l**	May 2006 (10-15 cm), January 2007 (5-10 cm)
Guanidine substituted BHTcyclitol ether	**1086**	**1s**	January 2005 (10-15 cm), May 2006 (10-15 cm)
3-methylBHhexol cyclitol ether	**1132**	**3k**	January 2005 (5-10 cm), October 2006 (0-5 cm), January 2007 (5-10 cm)

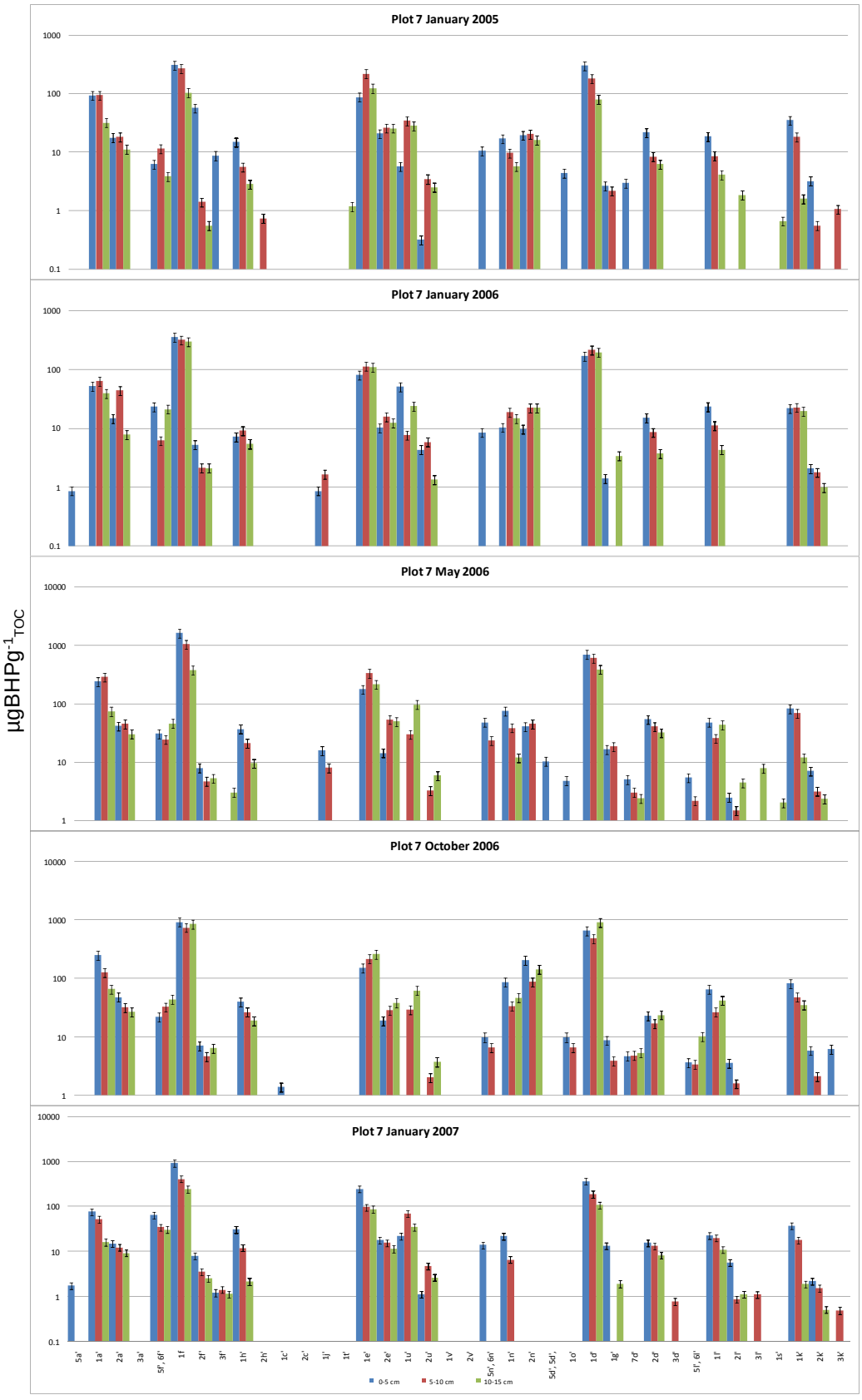


Fig. 3.8 Log scale of averaged Palace Leas Plot 7 BHP semi quantitative concentrations ($\mu g_{BHP}\ g^{-1}{}_{TOC}$). BHPs identified by structure number (see table 2.2 for details).

3.5.2.4 Palace Leas Plot 8

The TOC levels in Plot 8, September 2004, are similar to the other locations, except plot 7, with a gradual decrease moving down the soil profile from 4.3% (0-5 cm), 3.5% (5-10 cm) to 2.9% (10-15 cm). This results in the BHP contribution to the TOC being 3.7% (0-5 cm), 1.9% (5-10 cm), and 1.6% (10-15 cm).

The BHP profile is again dominated by 4 BHPs, BHT cyclitol ether (**1d**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and aminotriol (**1f**) which account for 71% (0-5 cm and 5-10 cm) and 74% (10-15 cm).

A total of 25 BHP structures were identified during the analysis of plot 8 September 2004 with 20 in the 0-5 cm layer, 23 in the 5-10 cm layer and 19 in 10-15 cm layer. There is a significant decrease in the level of BHPs with depth from 1609 $\mu g BHP g^{-1}_{TOC}$ (0-5 cm) down to 472 $\mu g_{BHP} g^{-1}_{TOC}$ (10-15 cm). For all the BHPs structures there was a decrease in level moving down the soil profile but the rate of decrease was lowest of aminotriol (**1f;** Fig. 3.5).

The analysis of the other plot 8 samples (Fig. 3.9) indicated that the overall concentration of the BHPs remained relatively consistent throughout the period of investigation. All plots showed a general slight decrease in the concentration of BHPs with depth except for October 2006 and January 2007 which showed a slight increase with depth (Fig 3.9).

During the analysis of all the plot 8 samples only 1 additional BHP, 3-methyl BHT cyclitol ether (**3d**), was identified (Appendix), in 2 samples, January 2005 (0-5 cm) and January 2007 (5-10 cm).

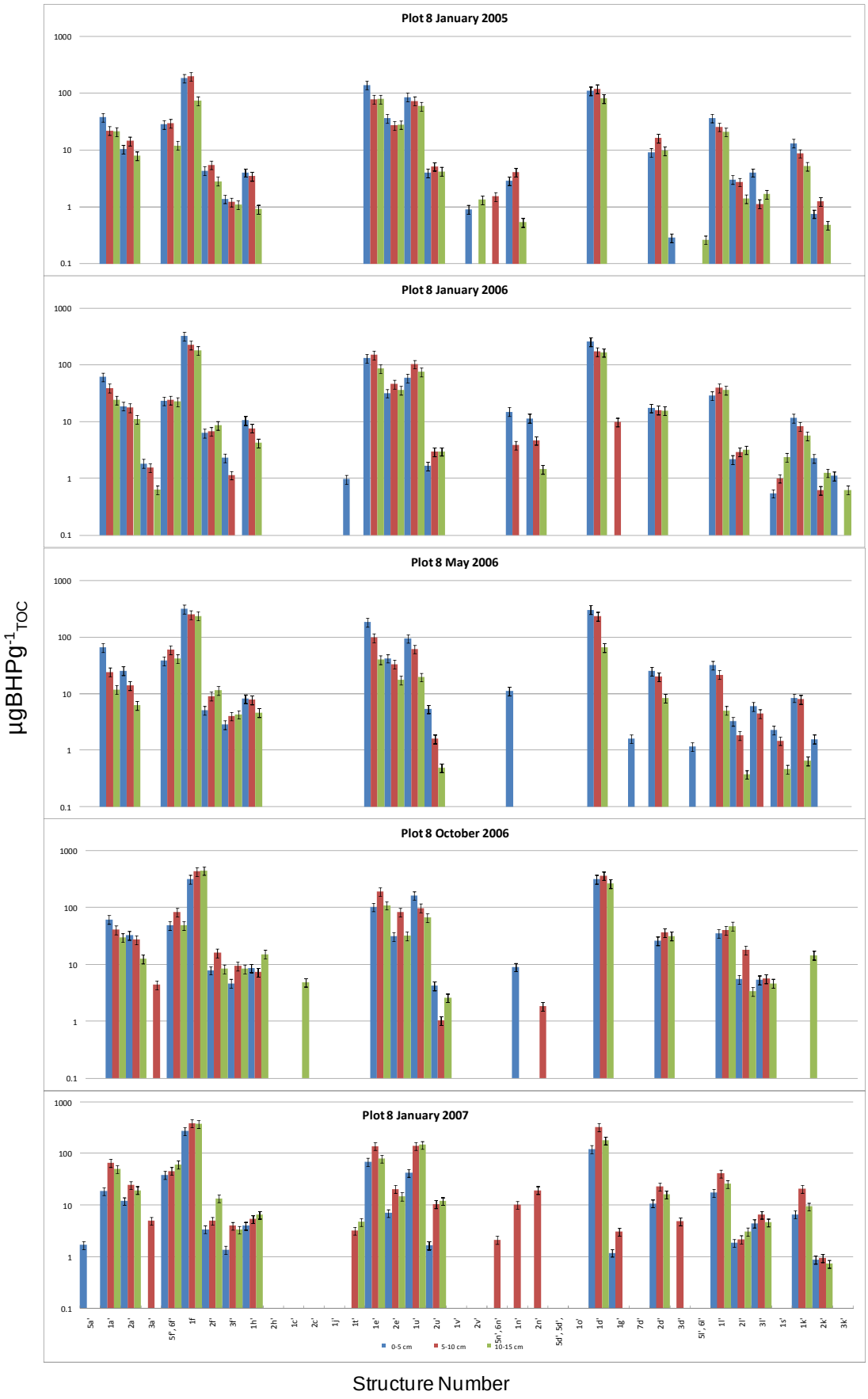


Fig. 3.9 Log scale of averaged Palace Leas Plot 8 BHP semi quantitative concentrations ($\mu g_{BHP}\ g^{-1}_{TOC}$). BHPs identified by structure number (see table 2.2 for details).

3.5.2.5 Palace Leas Plot 9.

The TOC (%) in plot 9, September 2004, is again comparable with the other plots, except plot 7, being 5.3% (0-5 cm), 3.1% (5-10 cm) and 4.6% (10-15 cm) although there is a slight increase in the 10-15 cm layer relative to the 5-10 cm layer is unusual. In plot 9, September 2004, 4 BHPs again dominate the profile, BHT cyclitol ether (**1d**), aminotriol (**1f**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**) accounting for 71% (0-5 cm and 5-10 cm) and 74% (10-15 cm) of the total BHPs (Fig. 3.5). The concentrations of 3 of these 4 BHPs increase in the 5-10 cm layer before returning to concentrations similar to those in the top layer in the 10-15cm layer (Fig 3.5). Aminotriol (**1f**) decreases in the middle layer before significantly increasing in 10-15 cm layer. The concentration of BHPs remains relatively consistent throughout the core of the September 2004 samples, with BHPs comprising 1.4% (0-5 cm), 2.6% (5-10 cm) and 1.7% (10-15 cm) of the soil OC. 22 different BHP structures were identified during the analysis of the September 2004 samples with 21 BHPs identified in both the 0-5 cm and 5-10 cm layers and 20 in the 10-15 cm.

Analysis of the other plot 9 samples (Fig. 3.10) indicates that the concentration of BHPs decreases with depth in January 2006, May 2006 and October 2006 whereas there is a decrease with depth in January 2005 and January 2007. The concentration also varied during the investigation with January 2005 and January 2006 having concentrations similar to September 2004. May 2006, October 2006 and January 2007 all had BHP concentrations approximately twice that observed in September 2004 (Fig. 3.5 and Fig 3.10).

During the investigation 7 additional BHP were identified as minor components of the BHP profile (Table 3.5 and Appendix). Their occurrence was infrequent and they represented a minor contribution to the total BHPs.

Table 3.5 Location of BHPs identified in plot 9 samples but not in plot 9 September 2004

Abbreviated name	Base Peak m/z	Structure Number	Sample – plot 9 only
$\Delta^{6 \text{ or } 11}$-BHT	**653**	**5a or 6a**	January 2006 (0-5 cm)
3-methylaminotriol	**728**	**3f**	January 2005 (10-15 cm), January 2006 (0-5, 5-10 cm) May 2006 (0-5, 5-10, 10-15 cm), October 2006 (0-5, 5-10, 10-15 cm), January 2007 (10-15 cm)
BHpentol pentose	**1001**	**1o**	May 2006 (0-5 cm)
Unsaturated 2-methyl BHT cyclitol ether	**1014**	**7d**	May 2006 (0-5 cm)
3-methylBHT cyclitol ether	**1016**	**3d**	May 2006 (5-10, 10-15 cm), October 2006 (5-10, 10-15 cm), January 2007 (0-5, 5-10 cm)
3-methylBHhexol cyclitol ether	**1132**	**3k**	May 2006 (0-5, 5-10, 10-15 cm), October 2006 (5-10 cm)

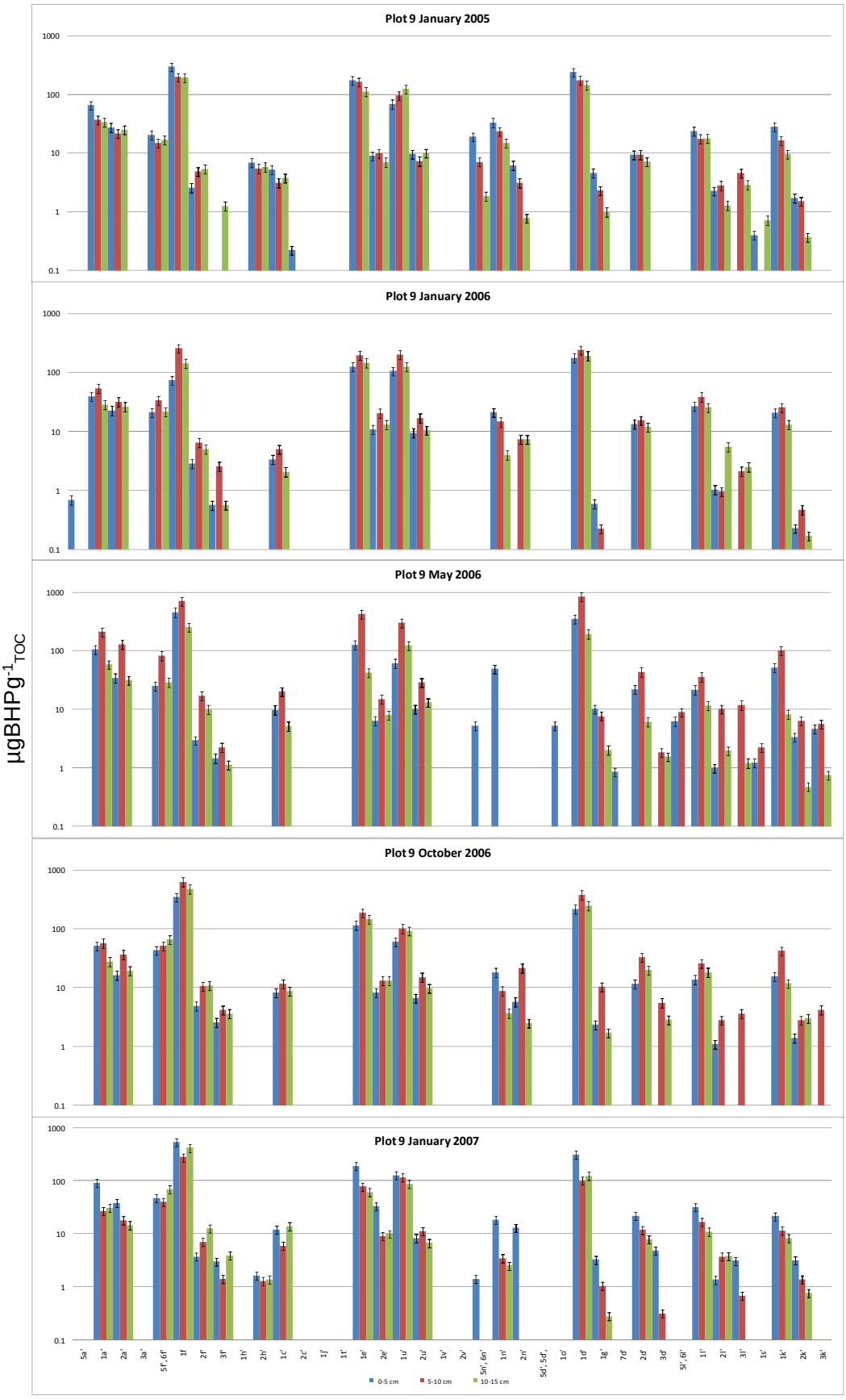


Fig. 3.10 Log scale of averaged Palace Leas Plot 9 BHP semi quantitative concentrations (μg_{BHP} g^{-1}_{TOC}). BHPs identified by structure number (see table 2.2 for details).

3.5.6 Temporal Differences in BHP Distribution

Temporal variation in BHP concentration on the Palace Leas plots was monitored between January 2006 and January 2007. There was significant variation in the total BHP content of each plot throughout the year (Figs 3.6 – 3.10), with each plot showing an increase in total BHP content in May and October before a decrease in January except for Plot 8 where the concentration remained relatively consistent (Fig 3.11).

Plot 2 clearly shows an increase in total BHPs from January 2006 to October 2006 before declining in January 2007 (Fig. 3.11), with the 5-10 cm layer containing the highest BHP concentration. Plot 7 shows the highest level of activity in May 2006, whereas for plots 2 and 6 the peak is in October (Fig. 3.11). For plot 8 the level of BHPs in the 0-5 cm layer remains constant from January 2006 until October 2006 before dropping in January 2007. The lower 2 layers both show decreases in concentration in May 2006, rises in October 2006 before again dropping in January 2007 (Fig. 3.11).

In plot 9 the total BHP concentration in the 5-10 cm layer is the highest. The overall pattern to the distribution is similar to the other plots with a general increase in concentration in the summer and autumn and a decrease in January. However the 0-5 cm layer shows a significant increase in concentration in January 2007.

In most cases the individual BHP concentration tended to mirror the change in total BHP concentration, e.g. compare the concentration of aminotriol (**1f**) in Plot 2 (Fig. 3.12) with the total BHP concentration in plot 2 (Fig 3.11), and similarly BHT cyclitol ether (**1d**; Fig. 3.13). However in plot 2 this change in concentration differed slightly from the overall change in total BHP concentration due to a drop in the levels of BHPs in the 10-15 cm layer in the May 2006 sample (Figs 3.12 and 3.13). BHT (**1a**), 2me BHT (**2a**), adenosylhopane type-1 (**1u**) and 2-Me adenosylhopane type-1 (**2u**) all show significant concentration rises in the 5-10 cm layer, May 2006, when compared to the other layers (Figs. 3.14 to 3.17). Aminopentol (**1c**) has a significant rise in BHP concentration in the 0-5 cm layer, May 2006, when compared to the other layers (Fig. 3.18).

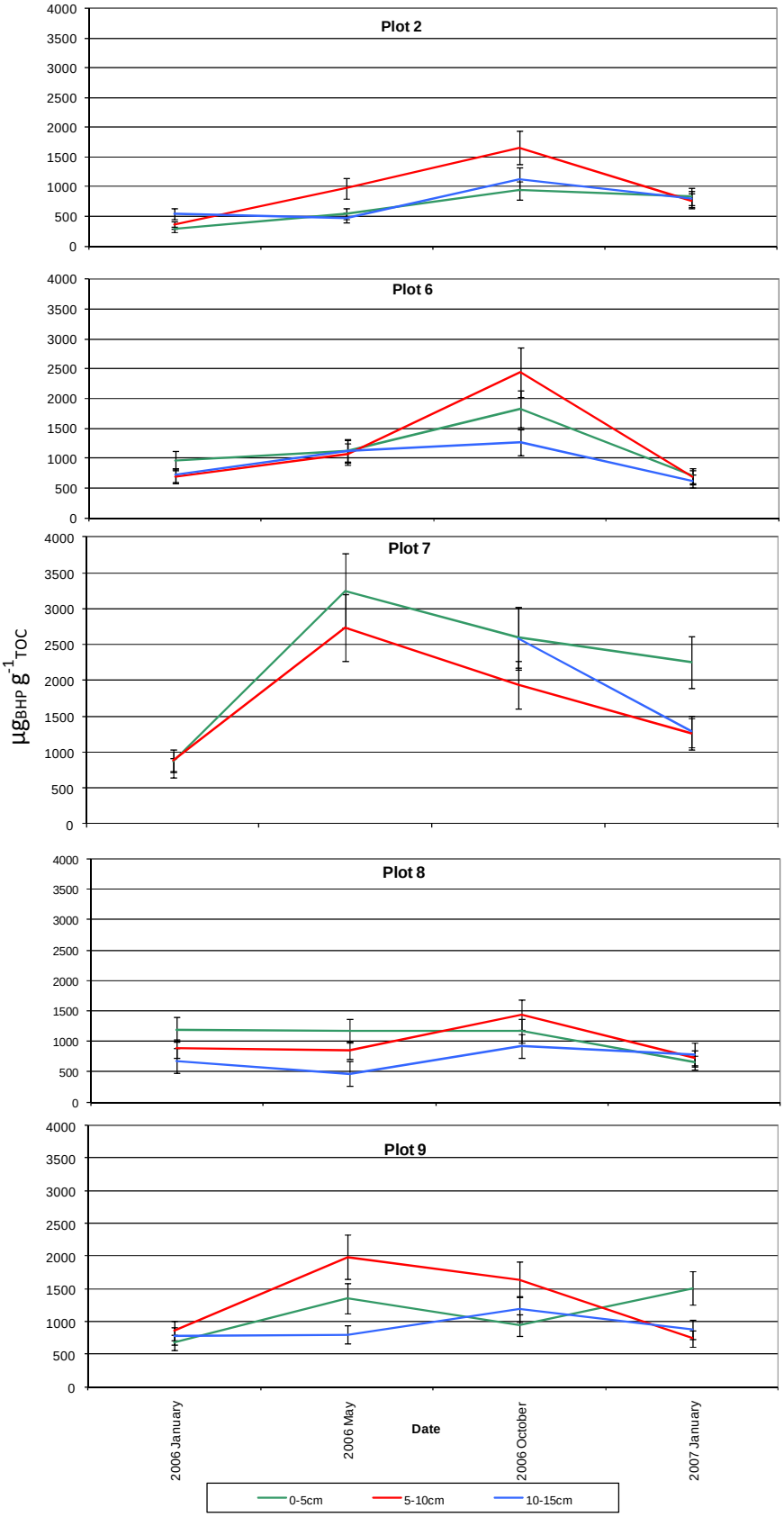


Fig 3.11 Seasonal variation in averaged total BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), Palace Leas.

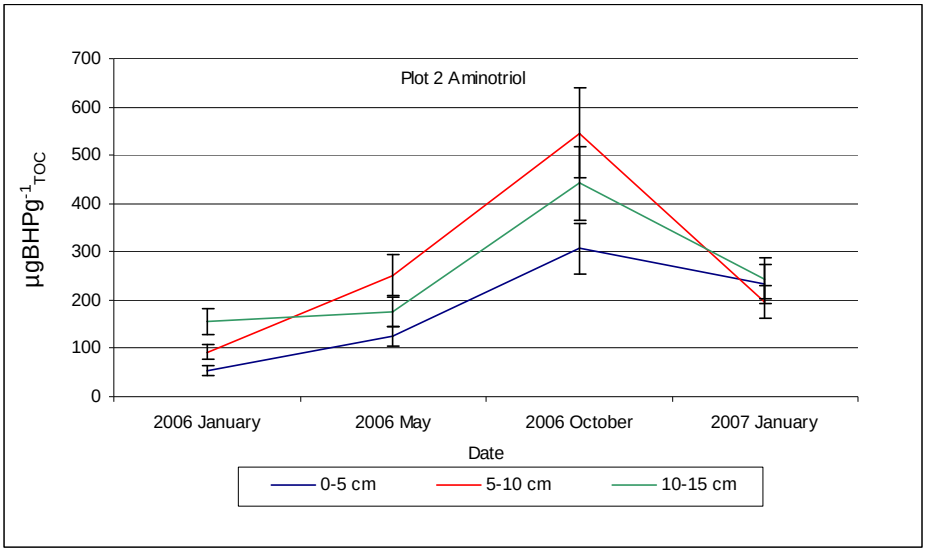


Fig. 3.12 Seasonal variation in Aminotriol (**1f**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 2.

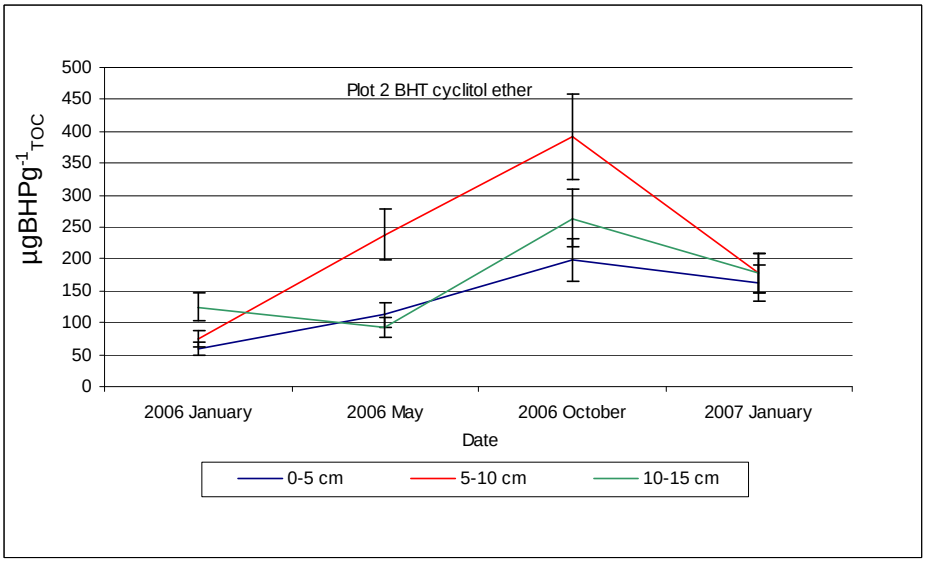


Fig. 3.13 Seasonal variation in BHT cyclitol ether (**1d**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 2.

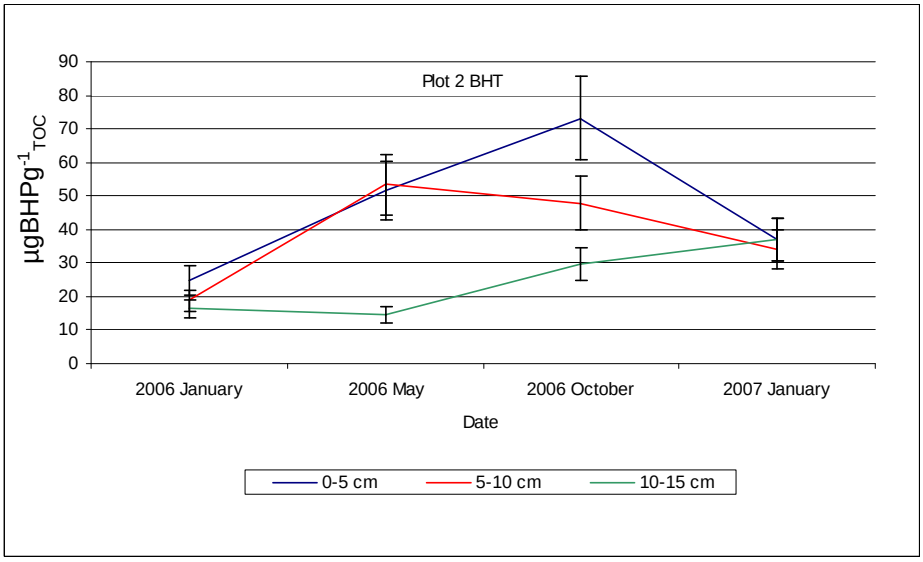


Fig. 3.14 Seasonal variation in BHT (**1a**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 2.

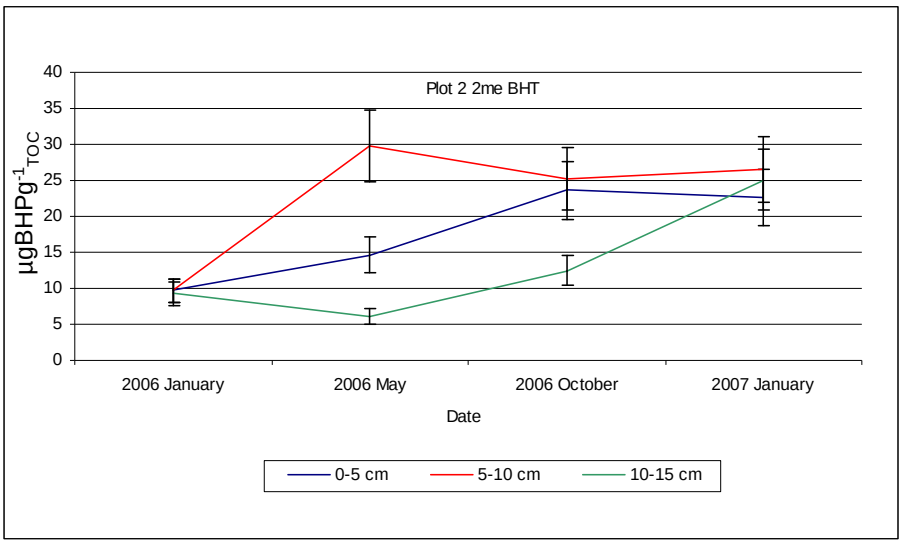


Fig. 3.15 Seasonal variation in 2me BHT (**2a**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 2.

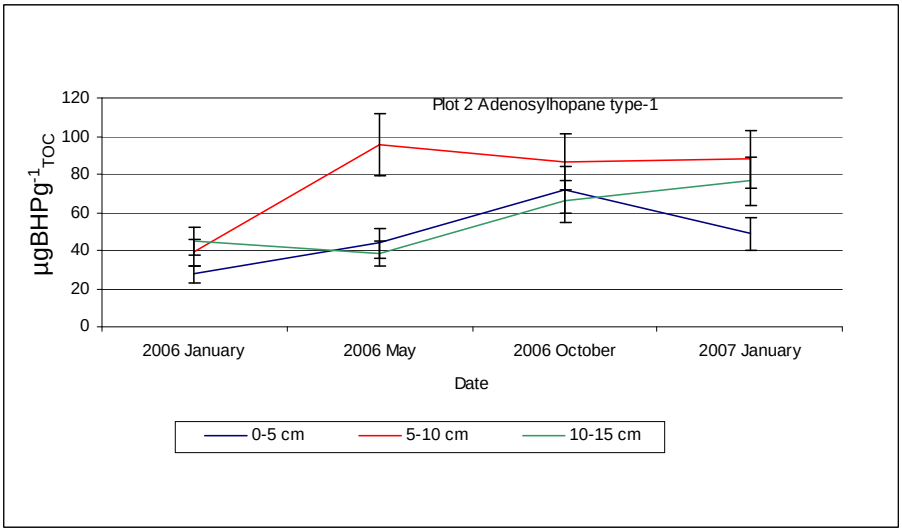


Fig. 3.16 Seasonal variation in Adenosylhopane type-1 (**1u**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 2.

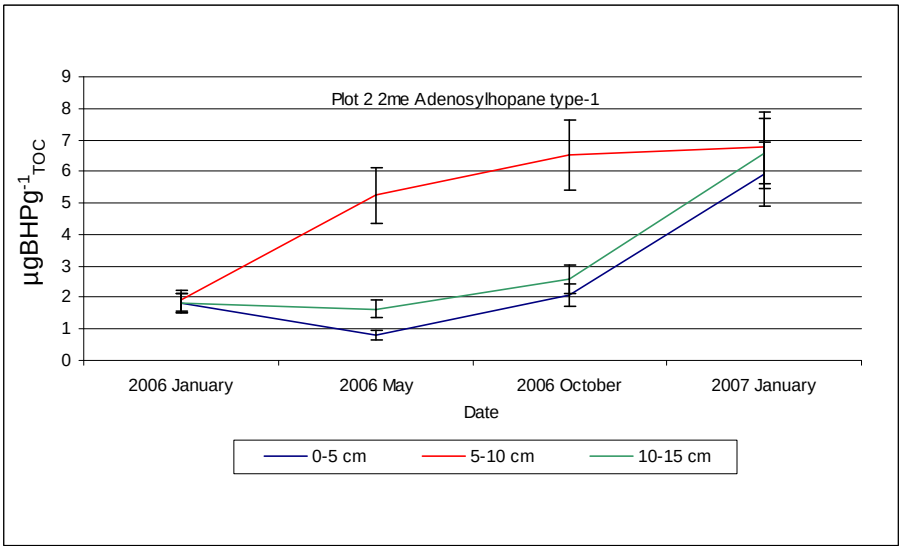


Fig. 3.17 Seasonal variation in 2me Adenosylhopane type-1 (**2u**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 2.

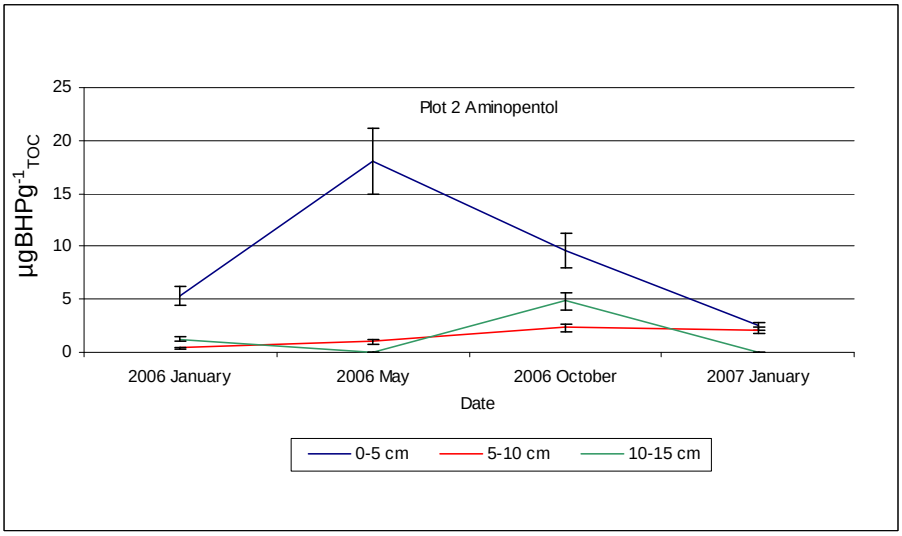


Fig.3.18 Seasonal variation in Aminopentol (**1c**) concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$), plot 2.

The temporal variation in the individual BHP concentrations for plot 6 again mainly mirror the variation in total BHP concentration (Fig. 3.11), 6 BHPs show different variations in concentration with time. BHT (**1a;** Fig 3.19), aminotriol (**1f**) (Fig. 3.20), aminotetrol (**1h;** Fig 3.21) and BHT pentose (**1n;** Fig. 3.22) all show a steep rise in BHP concentration in 0-5 cm layer, May 2006. Unsaturated aminotriol (**5f** or **6f;** Fig. 3.23) has a rise in January 2007 whereas all other BHPs have a drop in BHP concentration in 2007. 2 methyl aminotriol (**2f**) shows a sharp rise in concentration in the 10-15 cm layer, October 2006, with the concentration exceeding that in the upper layers.

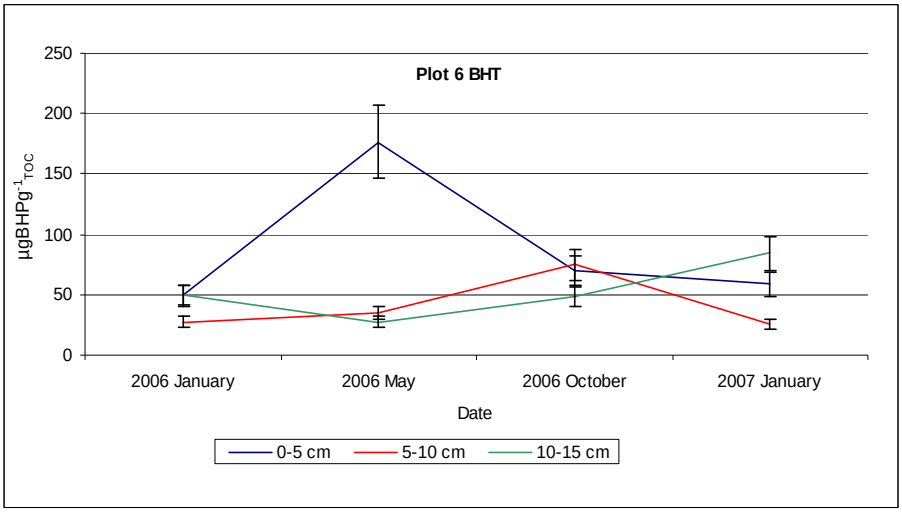


Fig. 3.19 Seasonal variation in BHT (**1a**) concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$), plot 6.

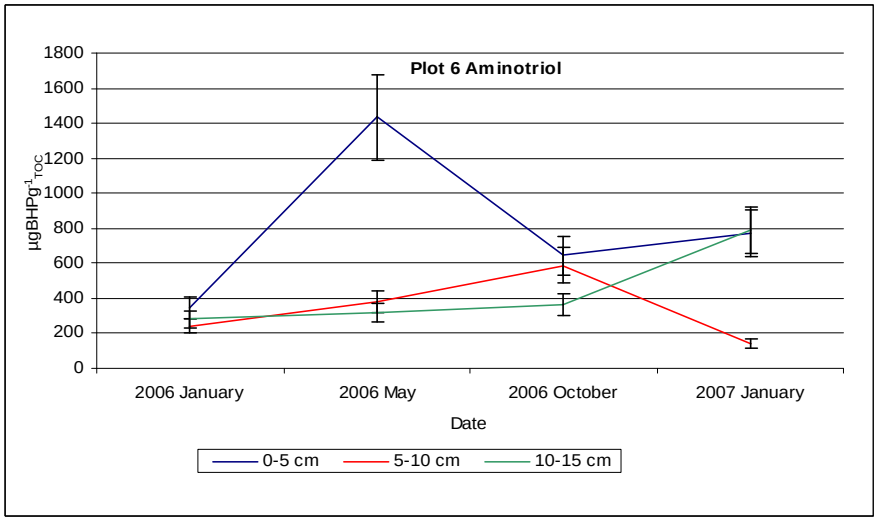


Fig. 3.20 Seasonal variation in Aminotriol (**1f**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 6.

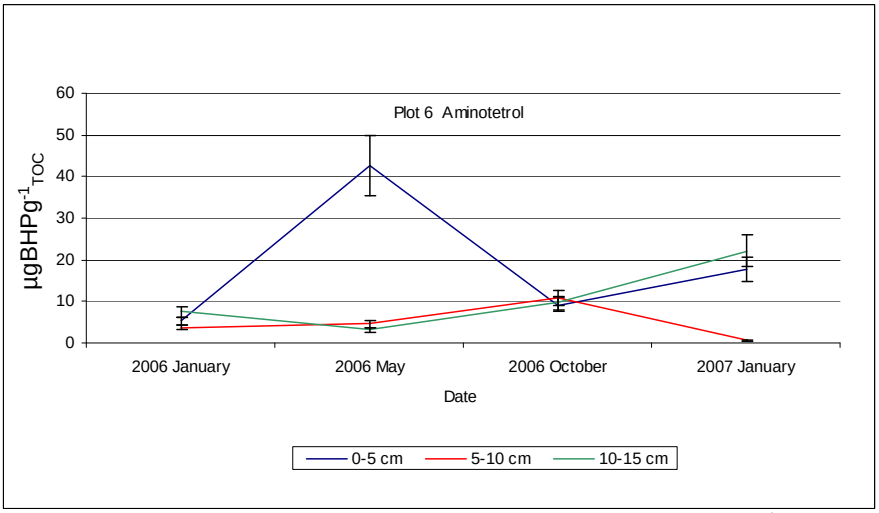


Fig. 3.21 Seasonal variation in Aminotetrol (**1h**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 6.

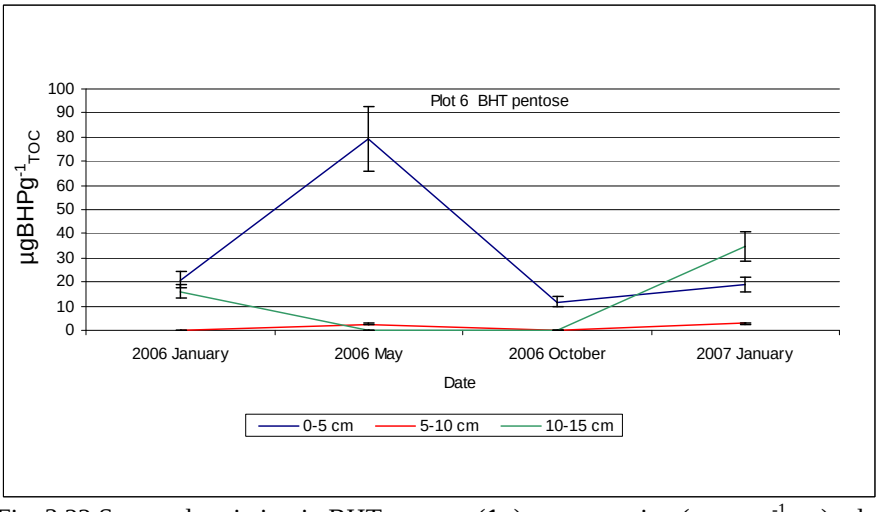


Fig. 3.22 Seasonal variation in BHT pentose (**1n**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 6.

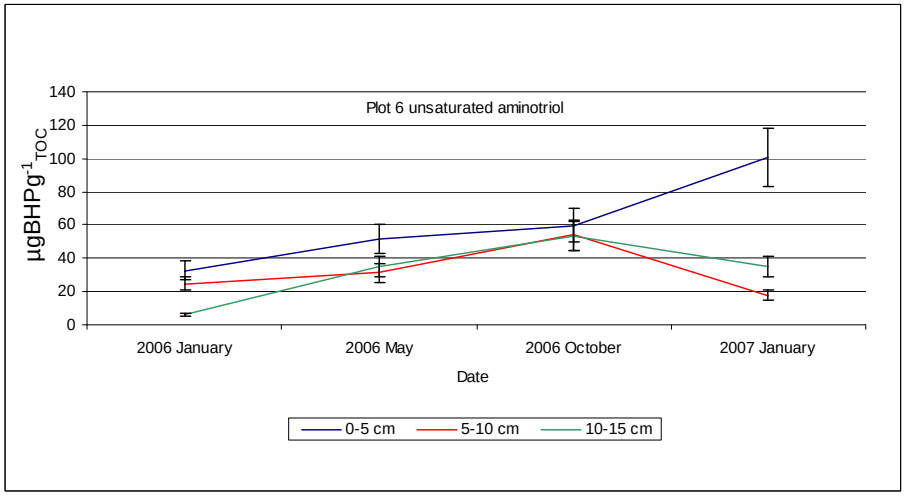


Fig. 3.23 Seasonal variation in Unsaturated Aminotriol (**5f or 6f**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 6.

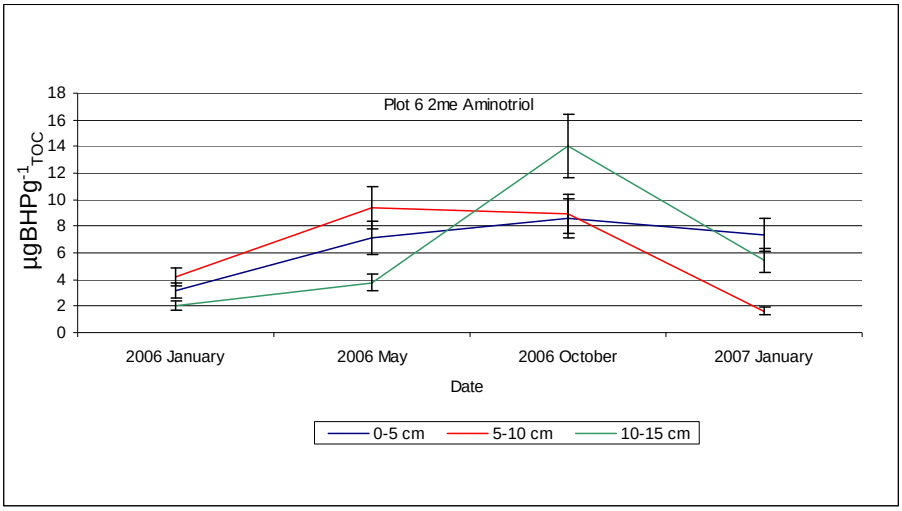


Fig. 3.24 Seasonal variation in 2-Me Aminotriol (**2f**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 6.

The seasonal variations of the concentration of individual BHPs in plot 7 mirror very closely those seen in the total BHP concentration (Fig. 3.11) with the exception of 3 BHPs. Adenosylhopane type-1 (**1u;** Fig. 3.25) and 2-Me adenosylhopane type-1 (**2u;** Fig. 3.26) are both, unusually, absent from the 0-5 cm layer in May and October 2006. BHT cyclitol ether (**1d**) mirrors very closely the total BHP concentration in the 0-5 cm and 5-10 cm layers but the 10-15 cm layer has a dramatic rise in the BHT cyclitol ether (**1d**) concentration in October 2006 to give a concentration higher than the top 2 core layers (Fig. 3.27).

The adenosylhopane (**1e**) concentration rises in May 2006 but the rate of increase is greatest in the 5-10 cm layer, giving this layer the highest concentration (Fig 3.28). Unsaturated aminotriol (**5f or 6f;** Fig. 3.2) has a significant rise in concentration in the

10-15 cm layer during May and October whereas the 0-5 cm layer has a drop in concentration during this time.

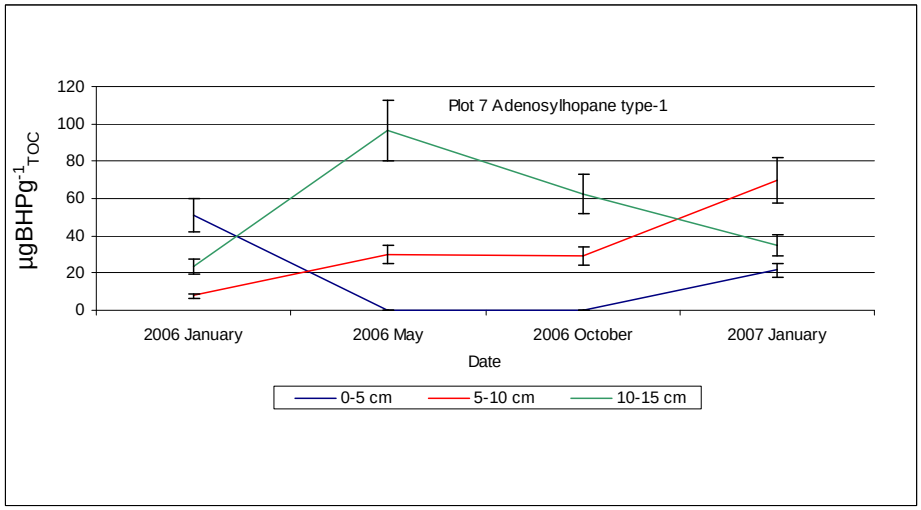


Fig. 3.25 Seasonal variation in Adenosylhopane type-1 (**1u**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 7.

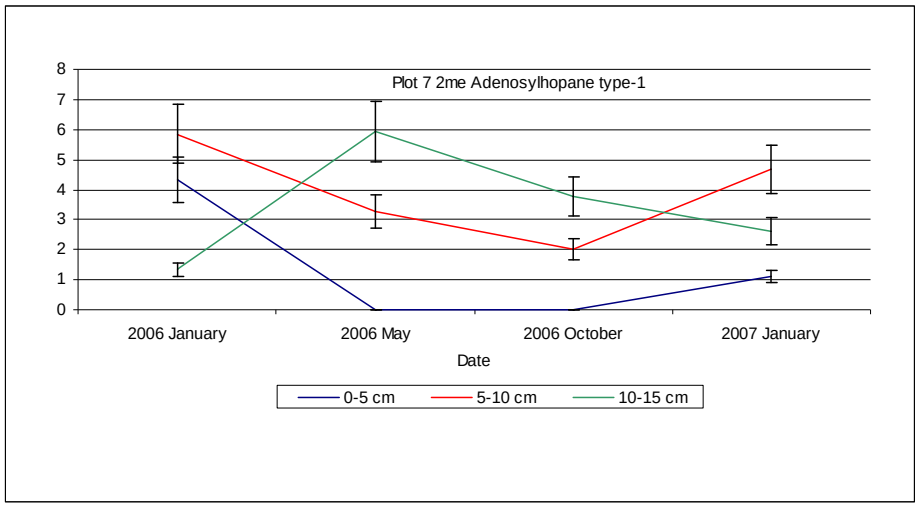


Fig. 3.26 Seasonal variation in 2-me Adenosylhopane type-1 (**2u**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 7.

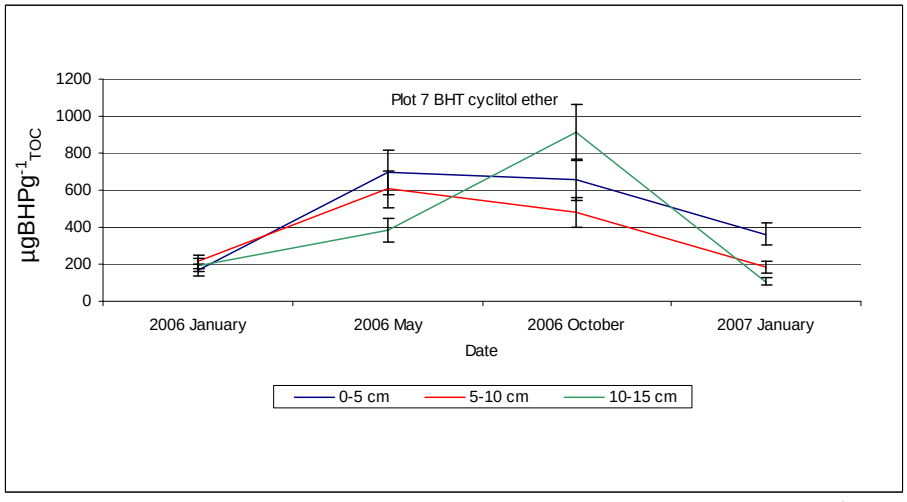


Fig. 3.27 Seasonal variation in BHT cyclitol ether (**1d**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 7.

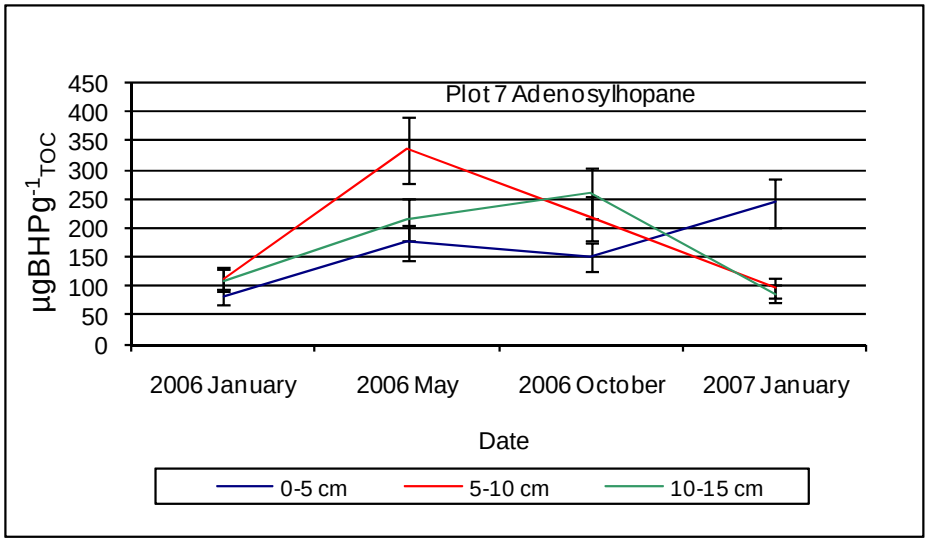


Fig 3.28 Seasonal variation in Adenosylhopane (**1e**) concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$), plot 7.

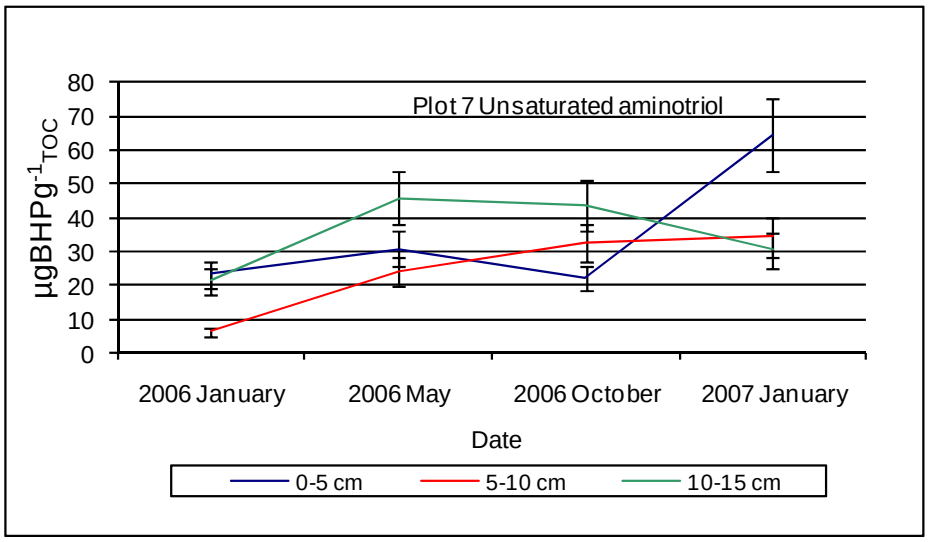


Fig 3.29 Seasonal variation in unsaturated aminotriol (**5f or 6f**) concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$), plot 7.

Analysis of the individual BHP concentrations from plot 8 indicates that all the individual BHP concentrations closely match the concentration changes seen for total BHP concentration changes (Fig. 3.11), except BHhexol cyclitol ether (**1k**) which decreases in May and October 2006 (Fig. 3.30), the opposite of the changes seen with the other BHPs.

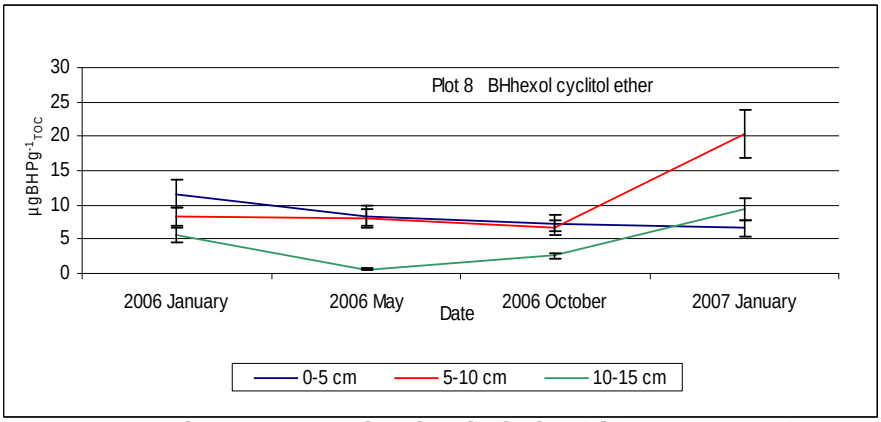


Fig. 3.30 Seasonal variation in BHhexol cyclitol ether (**1k**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 8.

Analysis of the individual BHP concentrations from plot 9 indicate that only 2 of the BHP concentrations do not follow those seen in the total BHP concentration over time (Fig. 3.11). BHP pentose (**1n**) (Fig 3.31) has the highest concentration in the 0-5 cm layer compared to the 5-10 cm layer seen in the other layer.

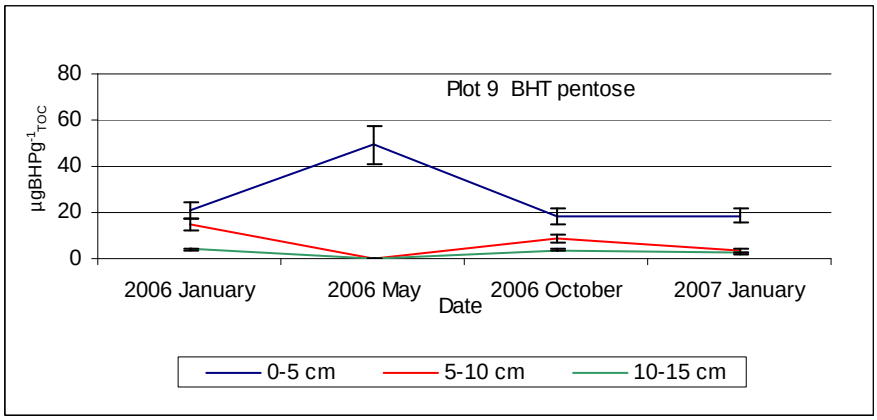


Fig. 3.31 Seasonal variation in BHT pentose (**1n**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 9.

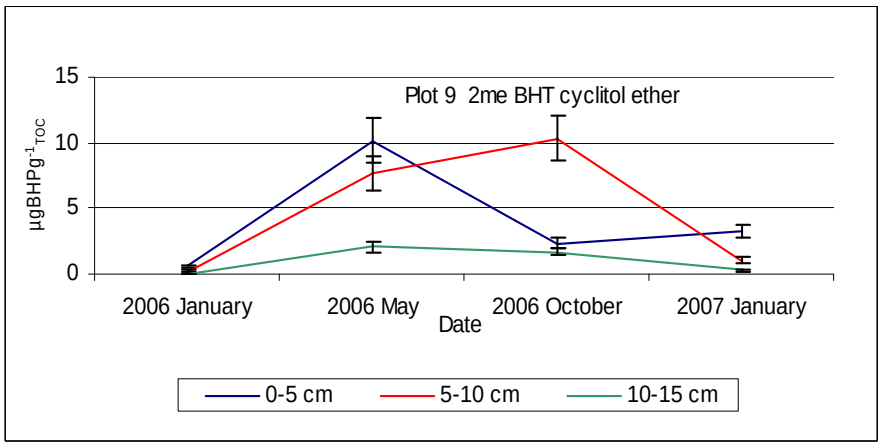


Fig. 3.32 Seasonal variation in 2me BHT cyclitol ether (**2d**) concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$), plot 9.

The concentration changes seen in 2me BHT cyclitol ether (**2d**), plot 9 (Fig. 3.32) over time indicate that the concentration in May 2006 rises, with the 0-5 cm having the highest concentration. In October 2006 the concentration in the 0-5 cm layer drops sharply whereas the 5-10 cm concentration continues to rise.

3.6 Discussion

3.6.1 Variation in BHP Content

Comparison of the differences in the BHP semi-quantative concentrations between cores 1 and 2 for each of the plots indicates that there is consistency within the plots, i.e. the differences between the cores is within the observed experimental error (Appendix and Section 3.5.1). The implication of this is this sampling and extraction technique was able to identify macro scale differences between the different Palace Leas plots, enabling direct comparisons between plots to be made.

A total of 34 different BHPs were identified in the Palace Leas plots, September 2004 (Fig. 3.5), with a further 7 BHPs identified in the other Palace Leas samples (Table 3.2 to 3.5). However the number of individual BHPs identified in each sample varied between 20 and 25 different structures (Fig 3.5).

The distribution pattern of BHPs was largely consistent between the plots with the profile being dominated by 4 or 5 BHPs; BHT (**1a**), aminotetrol (**1h**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**). Differences between the plots can be identified by the relative proportions of these major BHPs, for example, in plot 7 the relative concentration of BHT (**1a**) compared to the other major BHPs is higher than in the other plots (Fig. 3.5).

A further group of minor BHPs were always present in each plot including 2Me BHT (**2a**), unsaturated aminotriol (**5f or 6f**), methyl adenosylhopane (**2e**) and 2 Me cyclitol ether (**2d**). Relative variations in the concentrations of these BHPs can again be used to distinguish between the plots, for example plot 8 has a higher relative concentration of BHpentol cyclitol ether (**1l**) than plot 9 (Fig. 3.5).

Finally a group of BHPs were identified as being present infrequently in the samples at minor levels. It is this last group that accounted for the greatest variation within and between the plots, for example plot 2 contains aminopentol (**1c**) whereas this structure is largely absent from the other plots (Figs 3.5 to 3.10). However in many cases these

differences in BHP concentration between the plots are subtle and difficult to identify and will require statistical analysis to prove differences between the plots based only on BHP distribution and concentration.

The first 2 groups can be said to represent the dominant BHP content and indicate the bacterial population and its response to environmental conditions. The final group represents either minor, transient changes to the bacterial population or BHPs that are only produced in trace quantities within the sample. It is within this group that the majority of novel BHPs were identified. The lack of a clear link between these novel or unusual BHPs and source bacteria makes drawing conclusions regarding the significance of these compounds difficult (Table 2.2).

The concentration of BHPs within each sample indicates the quantity of BHPs produced by the active bacterial population or variations in levels of preservation between different BHPs in the soils, and clearly shows differences between the plots with plots 6 and 7 containing the highest concentrations and plot 2 the lowest as a proportion of TOC. The analysis of glucose induced respiration indicated that plot 2 had the highest bacterial activity and that the bacterial growth conditions on plot 7 were the most stressed (Hopkins and Shiel, 1996). If BHPs were a direct measure of bacterial population then these levels of BHPs would be expected to be reversed in these plots with plot 7 having the lowest BHP concentration and plot 2 the highest concentration. The 0-5 cm layer on plot 7 is characterised by a very high TOC due to the almost peat-like condition of the soil. This difference, due to the lower pH, has dramatically changed the makeup of the bacterial population (Hopkins and Shiel, 1996). It is therefore logical to assume that the stressed, acid conditions have resulted in a few bacteria producing a large number and concentration of different BHPs in response to the stress (e.g. Poralla et al., 2000; Welander, 2009). The low levels of BHPs in plot 2 could be due to a less stressed environment or to a lower percentage of BHP producing bacteria in the soil bacterial population An alternative reason for this high level of BHPs is the high TOC could be acting as a “net” to trap the BHPs within the soil prior to humin formation. This method of interaction between the TOC and the BHPs would be analogous to the first stage of incorporation of organic molecule incorporation into humin by physical sequestration due to van der Waal interactions (Lichtfouse, 1998).

3.6.2 Variation with depth

The most consistent variation with depth for all the Palace Leas plots is the general decrease in the number of observed BHPs structures moving down the soil profile (Figs. 3.6 to 3.10). This decrease may be due to a reduction in the size and variety of the bacterial population. This would be in agreement with the current understanding of the distribution of bacteria in soil.

Soil microbial activity decreases rapidly with depth (Fang and Moncrieff, 2005) due to reduction in the available nutrients and resources moving down the soil column (Feng et al., 2003), reduction in oxygen (Agnelli et al., 2004) and reduction in the effect of climate variations such as dessication (Ekelund et al., 2001).

Analysis of the PLFA content of prairie pasture soils (Allison et al., 2007) indicated that 37% of total microbial biomass inhabits the top 5 cm of the soil with a further 31% inhabiting the 5-15 cm layer, with 89% of total biomass in the top 25 cm. The Palace Leas plots have a hard, dense clay layer at between 15 and 20 cm below the surface indicating that the majority of the soil microbial population must exist between 0 and 15-20 cm and that the distribution of bacteria in the soil is similar to that seen in other soils with the greatest proportion in the near surface.

However the distribution of BHPs in the soil is much more complex and other factors influence the distribution and concentration of BHPs within the soil. These factors are variations in the preferential growth conditions for certain bacteria, different rates of leaching of BHPs (Winkler et al., 2001) and bacteria, incorporation in humin (Farrimond et al., 2003) and diagenesis of the BHPs.

For example the concentration of the cyanobacteria linked BHT pentose (**1n;** Table 2.2), decreases with depth (Figs. 3.7 to 3.10). Cyanobacteria inhabit the surface layers of the soil and become less abundant with depth (White 2006).

BHPs account for an increasing proportion of the TOC with depth for all the plots, except for plot 8, with, for example, the relative proportion of BHPs increasing from 3% to 5.7% of the TOC in plot 7. The other sites have a lower relative proportion of BHPs and a less dramatic increase in the bottom layer. This indicates that the BHPs are relatively more recalcitrant than other soil organic molecules with respect to the solvent extractable organic carbon layer

Plots 2 and 9 show a general increase in BHP concentration in the 5-10 cm layer, followed by a subsequent decrease in the 10-15 cm layer back to levels similar to the

top layer. Plot 6 shows a relative uniform distribution at all depths. These plots indicate that there is a general movement of BHPs down the soil profile due to leaching or translocation of bacteria to depths where they may become inactive. Inactive cells are unable to thrive in the conditions and these cells simply become dormant until the conditions are more favourable. During these conditions it has been shown that bacteria can increase their resistance to stress by producing BHPs to strenghten the cell wall (Berry et al., 1993; Poralla et al., 2000; Bosak et al., 2008; Doughty et al., 2009). It has been shown that some bacteria survive well in fine grained soils such as those found in Palace Leas (Huang and Wang 1998) where the fine grain size protects the bacteria from environmental stress. The extraction technique could cause these cells to lyse and release their BHPs. As the relative rates of free BHP transport due to leaching and bacterial translocation are unknown it is impossible to attribute the relative proportions of each of these sources to the BHP pool. Winkler et al (2001) identified that hopanoids accumulated in forest soil during leaf litter decomposition and humification. The accumulation increased moving down the O horizon (surface zone), reaching a peak in the eluvial zone, the zone of maximum leaching before dropping away. There is a partial recovery in the illuvial zone, where accumulation of recalcitrant molecules would be expected. The increase in the eluvial zone implies that the hopanoids are resistant to leaching either as a result of binding to clay particles which carry a net negative charge or by accumulation into humin. Binding to clay particles would have to be via metal ions that are bound to the clay minerals.

BHPs have been shown to be rapidly incorporated into kerogen in sediments, and therefore by association humin in soils (Farrimond et al., 2003). It is therefore possible that the reduction in BHPs with depth is due to incorporation into humin. BHPs that are fully bound into humin by ether bonds are not extracted from the soil by the extraction techniques used in this investigation. The rapid reduction in BHPs in plot 7, (10-15 cm) can be most readily explained by incorporation into humin in the top 2 layers, where the TOC is high compared to the other plots. This will leave only a small pool of free BHPs in the soil to be leached into the 10-15 cm layer. Prior to incorporation into humin BHPs may be loosely associated with humin by hydrogen bonding (Lichtfouse, 1998). This pool of BHPs may be extracted by solvent extraction and can be included in the BHP profile.

Diagenesis of BHPs in the soil could also explain some of the variation of BHPs with depth. Diagenesis of BHPs to geohopanoids involves the transformation of the BHPs by removal of functional groups giving, for example, C-30, C-31 and C-32 hopanols, and structural rearrangement (e.g. Sinninghe Damste et al., 1995; Innes et al., 1997, 1998). The presence of these structures cannot be identified using HPLC-MS^n.

The presence of AnhydroBHT (**1j**) is an indicator of diagenesis. AnhydroBHT (**1j**) can be formed by acid hydrolysis of BHT (**1a;** Costantino et al., 2001, Schaeffer et al., 2008). It has also been suggested the anhydroBHT (**1j**) is also formed by the diagenesis of adenosylhopane (**1e**) and similar structures (Costantino et al., 2001; Talbot et al., 2005; Cooke et al., 2008a). Analysis of the Palace Leas samples has indicated that anhydroBHT (**1j**) is only a minor component of the BHP profile and cannot be used as a measure of diagenesis in soils.

From the Palace Leas results it is clear that one single process does not drive the distribution of BHPs and that all these different processes are acting simultaneously to affect the distribution of the BHPs in the soil profile producing a complex distribution. However the distribution of bacteria in a soil profile has been linked to the bacterial population in the near surface samples (Allison et al., 2007) and all the drivers for the movement of BHPs down through the soil profile are dependent on the presence of source bacteria in the surface. So it can be stated that BHPs can provide an indication of the bacteria population in the soil but the distribution of the BHPs in the soil cannot provide clear understanding of the location of these bacteria in the soil below the near surface layer.

3.6.3 Seasonal variation

The effect of seasonal variation is poorly understood as many of the studies on this have contradictory results. A rise in soil microbial activity and biomass during summer months was identified by some researchers (Buchanan and King, 1992; Kaiser and Heinemeyer, 1993) but other researchers (Baath and Soderstrom, 1982) identified a highest microbial activity in spring and autumn. Blume et al., (2002) identified that the microbial biomass remained consistent throughout the year in temperate soils but that activity increased by 83% in the summer. Their findings indicated that in the winter months the active layer moved down through the soil to a less hostile environment.

In the Palace Leas soils there is a general increase in the total concentration of BHPs in the spring and summer and a subsequent decrease during the winter months (Fig 3.11). Most individual BHPs mirror the pattern of total BHP concentration (e.g. Figs 3.12 and 3.11). However, certain BHPs do not follow this trend. Plot 2 BHT (**1a;** Fig 3.14) shows a rise in the BHT (**1a**) concentration in the 5-10 cm faction that exceeds the concentration in the 0-5 cm layer in October 2006. This indicates either a rise in production of BHT (**1a**) by bacteria at this level or a selective leaching of BHT (**1a**) down through the soil. This latter suggestion seems unlikely and therefore a rise in BHT (**1a**) production by bacteria active in this level as a result of environmental change it the most probable cause. As BHT (**1a**) is produced by a number of bacteria (Table 2.2) it is impossible to identify a source of this rise. A similar trend is seen in 2-Me BHT (**2a**) and adenosylhopne type-1 (**1u**) although the concentrations remain highest in the 0-5 cm layer and there is a drop in concentration for both in the 5-10 cm layer (Figs. 3.15, 3.16). The summer of 2006 was exceptionally hot and dry and the movement of bacteria down to a cooler, wetter layer of the soil may explain this observation.

The concentration variations for 2-Me adenosylhopane type-1 (**2u**) show a drop in May 2006 before rising in October 2006 and January 2007 in the 0-5 and 10-15 cm layers (Fig. 3.17). The concentration in the 5-10 cm layer has an opposite trend with a rises in May 2006 and October 2006 before dropping in January 2007. This indicates a promotion of growth of 2-Me adenosylhopane type-1 (**2u**) producing bacteria, or production of this BHP, compared to the other levels. However, the source bacteria for this BHP is unknown and so no clear link can be made to the bacterial population.

Aminopentol (**1c**) has been clearly linked to the methanotrophs (Table 2.2) and in plot 2 there is a clear rise in the concentration in May 2006 followed by a fall in October 2006 and January 2007, whereas the concentration in the 2 lower layers remains consistent (Fig. 3.18). This indicates a rise in methanotroph population in the top layer of this soil during the summer months, in agreement with Henckel et al., 2000.

Plot 6 shows 4 different BHPs with sharp rises in concentration in May 2006, in the 0-5 cm layer followed by a fall in October 2006 and a slight rise in January 2007 (BHT, **1a**; Fig 3.19; aminotriol; **1f;**, Fig. 3.20; aminotetrol **1h**; Fig 3.21; BHT pentose, **1n**; Fig. 3.22). The 5-10 cm and 10-15 cm layers for these BHPs show a continual rise in concentration to October 2006 followed by a drop in January 2007. BHT (**1a**) and aminotriol (**1f**) are produced by various bacteria and indicate a general rise in

bacterial population or rise in production of the BHPs. Aminotetrol (**1h**) has been linked to the presence of methanotrophs (Table 2.2) and this change in concentration is similar to that observed in plot 2 for aminopentol (**1c**), indicating that methanotrophs are most active in the summer months. No clear link has been drawn between temperature and methanotroph activity and the relationship appears case dependent (Hanson and Hanson, 1996) although there is a clear link between temperature and methane oxidation rate (Hanson and Hanson, 1996).

BHT pentose (**1n**) has been linked to cyanobacteria (Talbot et al., 2008) and this change in concentration indicates a rise in cyanobacteria activity in the summer in the 0-5 cm layer. 2-Me aminotriol (**2f**) has also been linked to cyanobacteria (Table 2.2) and the concentration changes over time show a gradual rise in this BHP to October 2006 followed by a fall in January 2007 (Fig 3.24). However there is a significant rise in concentration in October 2006 in the 10-15 cm layer, giving concentration higher than in the other layers. The possible reasons for this are that leaching has moved this BHP down the soil profile, but this would have to be at a preferential rate to other BHPs and therefore unlikely, or that 2-me aminotriol (**2f**) can be produced by bacteria other than cyanobacteria. Rashby et al., (2007) have already shown that 2-Me BHPs are produced by *Rhodopseudomonas palustris* and therefore it is possible that this spike in concentration at depth is due to production by an unknown bacteria, or possibly *Rhodopseudomonas palustris*. A third intriguing possibility exists that both BHT pentose (**1n**) and 2-Me aminotriol (**2f**) are produced by cyanobacteria but BHT pentose (**1n**) is produced predominantly when the cyanobacteria are active and that 2-me aminotriol (**2f**) is produced when the cyanobacteria are inactive and under stressed conditions such as would be experienced if the cyanobacteria had been leached to lower depths. This is similar to the observations by Welander et al. (2009) who showed that 2-Me BHPs were produced in response to pH stress in *Rhodopseudomonas palustris*.

The change in concentrations of adenosylhopane type-1 (**1u**) and 2-Me adenosylhopane type-1 (**2u**) in plot 7 shows very similar trends (Figs 3.26, 3.27). In both cases the BHPs are absent from the 0-5 cm layer in May and October 2006, whereas in the 10-15 cm layer the concentrations rise in May 2006 before decreasing. Adenosylhopane type-1 (**1u**) is produced by PNSB (Table 2.2) and it therefore appears that there is no PNSB activity in the 0-5 cm layer of plot 7, 2-Me adensosylhopane type-1 (**2u**) has no known bacterial source but the change in

concentration is so similar to that seen with adenosylhopane type-1 (**1u**) that the 2 BHPs appear to be linked to a common source. Adenosylhopane type-1 (**1u**) is not the only BHP linked to PNSB (Table 2.2) and the concentration changes of adenosylhopane (**1e**; Fig. 3.28) and unsaturated aminotriol (**5f or 6f;** Fig. 3.29) must also be considered. The changes in concentration seen in adenosylhopane (**1e**) differ from those seen in adenosylhopane type-1 (**1u**) and its 2-Me homologue, but adenosylhopane (**1e**) is also believed to be produced by nitrogen fixing and ammonia oxidising bacteria (Table 2.2) and has also been identified as a possible intermediate step in the formation of all BHPs (Seeman et al., 1999). Unsaturated aminotriol (**5f or 6f**) has been identified in the PNSB *Rhodopseudomonas palustris* (Talbot et al., 2007a; 2007b) and the concentration changes seen for unsaturated aminotriol (**5f or 6f;** Fig. 3.29) are similar to those seen in adenosylhopane type-1 (**1u**) and 2-me adenosylhopane type-1 (**2u;** Figs 3.26, 3.27) with a rise in the 10-15 cm layer during the summer with only a slight rise in May 2006 followed by a decrease in October 2006. Although not as dramatic difference as seen with adenosylhopane type-1 (**1u**) and its 2 methyl homologue (**2u**) the trend is similar indicating a decrease in PNSB activity in the surface and an increase at depth. PNSB would be expected to thrive in low oxygen conditions as they would be out competed in more aerobic conditions by other bacteria (Imhoff, 1995).

In plot 8 only 1 BHP, BHhexol cyclitol ether (**1k;** Fig 3.30) has concentration changes that differ from the trend seen in the total BHP concentration (Fig 3.11). Here there is a slight drop in concentration in May and October 2006 before a rise in January 2007. BHhexol cyclitol ether (**1k**) has no known bacterial source (Table 2.2) and it is therefore impossible to identify a possible reason for this difference from the other BHPs found in plot 8.

In plot 9 2 BHPs linked to cyanobacteria (Table 2.2), BHT pentose (**1n**; Fig. 3.31) and 2-me BHT cyclitol ether (**2d**; Fig. 3.32) have changes in concentration that differ from the total BHP concentration (Fig. 3.11) and are similar to the changes seen in plot 6 for BHT pentose (**1n**; Fig. 3.22) and 2-Me aminotriol (**2f;** Fig 3.24). This could indicate similar processes seen in plot 2 with respect to the activity of cyanobacteria.

The increase in BHPs during the spring and summer months indicates that there is an increase in microbial activity during these productive months. The subsequent decrease during the winter months therefore mirrors the reduction in bacterial activity at this time. These results are in agreement with Jaffe et al., 1996 who identified rapid

degradation over a 3 month timescale but contradict the research on the recalcitrance of BHPs. Intact BHPs have been identified in Congo estuary sediments up to 1 million years old (Cooke et al., 2008a) and BHT (**1a**) has been identified in 65 million year old sediment from Tanzania (van Dongen et al., 2006). Other diagenetic routes, such as incorporation into kerogen can take between 0 and 350 years (Farrimond et al., 2003) which is slower than the reduction of BHPs seen in these Palace Leas samples.

The logical cause of the reduction in BHP concentration in winter can therefore only be due to; the adsorption of the BHPs by the soil microbial population where they will provide valuable nutrients, the degradation of the BHPs, accelerated by the presence of oxygen within the soil.

Although only one year of data has been obtained, and further research on this observation is required, this data potentially indicates that there may be an apparent annual cycle to the BHP concentration, as there is no ongoing accumulation of BHPs in the soil. This may enable BHPs to be used to measure seasonal changes in the bacterial population, providing a further use for BHPs as a biomarker.

3.6.4 BHPs as indicators of bacterial community structure

The BHP distribution in the soil can be used to analyse the soil bacterial community for: (1) the overall quantity of BHPs in the sample, as a broad indication of the BHP-producing bacterial population in the sample; (2) the presence or absence of individual marker structures, indicating the presence or absence of specific bacteria and (3) the amount and relative proportions of each structure as an indication of the dominance of the different bacterial groups in the sample.

The current known relationship between BHP production and source bacteria (Table 2.2) enables the identification of 8 broad groups of BHPs and 6 types of bacteria within the soils (Table 3.2). These groups are PNSB, nitrogen and ammonia fixing bacteria, cyanobacteria, methanotrophs, acetic acid bacteria and methylotrophs (Table 3.2). In addition to these 6 broad bacteria groupings 2 additional groups are considered, BHPs from various sources and BHPs of unknown source (Table 3.2). It must be noted that the BHPs from various sources comprise the largest group of BHPs in all the Palace Leas plots (Tables 3.2 to 3.6). In all cases the bacterial population is

based on the average relative percentage of each BHP group from the entire sampling period.

3.6.4.1 Plot 2 Bacterial Community Structure

BHP concentration identified in plot 2 can be attributed to various sources and some of the BHPs identified as being from specific sources are tentative based upon the structural similarity with other BHPs known to be produced by source bacteria, (Table 2.2). The BHP concentrations were then combined using the results from all the plots. As previously discussed there is a general trend of all the BHPs following the same changes in concentration over time (Section 3.6.4) to give the combined results seen in Table 3.6. Overall the relative percentage contribution of the each bacterial group described remains very consistent down the soil profile.

The highest contribution from an identifiable bacterial source is attributed to PNSB and nitrogen fixing bacteria, approximately 35% combined percentage, followed by cyanobacteria, approximately 5% (Table 3.6). The activity and population of cyanobacteria would be expected to decrease with depth (White, 2006), however the concentration and relative percentage contribution of cyanobacterially derived BHPs remains consistent with depth. This indicates that either these BHPs, predominantly 2-methyl BHPs, are not purely produced by cyanobacteria (Rashby et al., 2007) or that translocation of the BHPs or cyanobacteria by leaching is increasing the level of BHPs at lower levels in the soil thus masking the presence of a larger population of cyanbacteria in the top layer of the soil. The cyanobacteria, *Nostoc punctiforme*, produces high concentrations of 2-Me BHPs when forming akinetes, resistant thick walled dormant cells, in response to environmental stress (Doughty et al., 2009), such as that found at depth in soil.

Methanotrophs contribute between 3.5% (0-5 cm) and 1.9% (5-10 and 10-15 cm) of the bacterial composition (Table 3.6). Plot 2 shows considerably higher levels of methanotrophs than the other plots (c.f table 3.6 to Tables 3.7 -3.10). This result is in agreement with Seghers et al. (2003) who identified a significantly higher population of methanotrophs in manure treated soils than in soils treated with chemical fertiliser. It may be the case that aminopentol (1c) is introduced into the soil via the manure and persists in the soil as was observed for C-32ββ hopanoic acid (Bull et al., 1998, 2000).

Although only a C-32ββ hopanoic acid has previously been identified in manure (Bull et al., 1998), this is considered to be a diagentic product of a tetrafunctionalised BHP. The acetic acid bacteria and methylotroph derived BHPs in plot 2 (Table 3.6) present a very minor layer of the soil bacterial community.

Table 3.6. Distribution of BHP producing bacteria in all plot 2 samples, identified by BHPs.

Bacteria	BHPs (*m/z*)	Plot 2 (0-5 cm)	Plot 2 (5 -10 cm)	Plot 2 (10-15 cm)
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)	Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)	Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	285 (58.7)	414 (60.0)	351 (63.2)
Purple non sulphur bacteria	627, 712, 746, 761	131 (26.9)	185 (26.9)	142 (25.5)
[a]without adenosylhopane or hopanelactone		53 (10.8)	85 (12.3)	72 (12.9)
N_2 and NH_3 fixing bacteria	627,746, 760	97 (19.9)	127 (18.5)	89 (16.0)
[a]without adenosylhopane or hopanelactone		41 (8.3)	60 (8.7)	47 (8.4)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	26 (5.4)	38 (5.5)	28 (5.0)
Methanotrophs	3Me 728, 772, 830, 844	17 (3.5)	13 (1.9)	11 (1.9)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	3 (0.5)	3 (0.5)	3 (0.5)
Methylotrophs	1086	1 (0.2)	ND (0)	0.1 (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	9 (1.9)	15 (2.2)	8 (1.5)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

3.6.4.2 Plot 6 Bacterial Community Structure

Plot 6 contains approximately the same relative percentage of nitrogen and ammonia fixing bacteria, 35% to 40%. The relative percentage of methanotrophs is lower than plot 2 and more cyanobacteria as a percentage of total BHPs (Table 3.7). The

contribution of BHPs from various sources is significantly higher than plot 2, as are the BHPs from unknown sources.

The increase in cyanobacterially derived BHPs is due to the relative increase in BHT pentose (**1n**) again remains consistent with depth. The implication of the increase in cyanobacteria is that the addition of manure promotes other bacteria at the expense of cyanobacteria in plot 2.

The decrease in methanotrophs is due to absence of aminopentol (**1c**) from all the plot 6 samples, whereas it was present in all the plot 2 samples, again this can be related to the addition of manure to plot 2.

Table 3.7. Distribution of BHP producing bacteria in all plot 6 samples, identified by BHPs.

Bacteria	BHPs (*m/z*)	Plot 6 (0-5 cm)	Plot 6 (10-15 cm)	Plot 6 (5 -10 cm)
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)	Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)	Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	758 (63.5)	566 (60.3)	949 (68.3)
Purple non sulphur bacteria	627, 712, 746, 761	288 (24.2)	260 (27.7)	270 (19.4)
[a]without adenosylhopane or hopanelactone		156 (13.0)	134 (14.3)	145 (8.9)
N_2 and NH_3 fixing bacteria	627,746, 760	152 (12.7)	145 (15.4)	164 (11.8)
[a]without adenosylhopane or hopanelactone		118 (9.9)	111 (11.9)	87 (6.3)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	81 (6.8)	63 (6.7)	96 (6.9)
Methanotrophs	3Me 728, 772, 830, 844	7 (0.6)	5 (0.5)	15 (1.1)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	8 (0.6)	5 (0.6)	4 (0.3)
Methylotrophs	1086	3 (0.3)	2 (0.2)	0.2 (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	47 (3.6)	29 (3.1)	46 (3.3)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

3.6.4.3 Plot 7 Bacterial Community Structure

The relative percentage of PNSB is lower in plot 7 than in all the other Palace Leas plots (Tables 3.6, 3.7, 3.9, 3.10) and shows a noticeable increase with depth. The nitrogen fixing and ammonium fixing bacteria contribution also increases with depth and could be due to low populations of PNSB and nitrogen / ammonia fixing bacteria in the 0-5 cm layer or the production of different BHPs in this layer in response to stress.

The levels of cyanobacteria are high (9.7% in 0-5 cm) and decrease with depth (7.8% in 10-15 cm). There is also an accompanying decrease in the concentration of these BHPS (Table 3.8). This appears to match the expected distribution of the cyanobacteria population (White et al., 2006).

Both acetic acid and methylotrophs have very low levels indicating a very minor contribution from these bacteria.

The relatively high methanotroph results for plot 7 differ from those observed by Seghers et al., (2003) and Hutsch (2001) who showed that the addition of nitrogen fertiliser inhibited methane oxidation. The concentration and relative contribution of methanotrophs to plot 7 decrease significantly with depth and may indicate that in plot 7 these bacteria are inhabit the near surface layer.

The relatively low levels of nitrogen and ammonia fixing bacteria in plot 7 do not agree with the results from other studies (Hutsch 1998; Seghers et al., 2003), where the addition of ammonium fertiliser causes these bacteria to thrive. This is unusual and may indicate that adenosylhopane (**1e**) is not a significant BHP in ammonia oxidisers, ammonium oxidisers do not produce BHPs in significant quantities or that other factors in the soil may be influencing the growth of ammonia oxidising bacteria such as limiting concentrations of potassium or phosphate, as plots 2, 8 and 9 contain the highest numbers of these bacteria. It must also be reiterated that adenosylhopane (**1e**) has been linked to the formation of the BHP side chain and may not be specific to PNSB or ammonia oxidising bacteria but may be an intermediate in the formation of all BHPs (Seeman et al., 1999).

Table 3.8. Distribution of BHP producing bacteria in all plot 7 samples, identified by BHPs

Bacteria	BHPs	Plot 7 (0-5 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)	Plot 7 (5 -10 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)	Plot 7 (10-15 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	1150 (73.6)	876 (68.2)	623 (64.4)
Purple non sulphur bacteria [a]without adenosylhopane or hopanelactone	627, 712, 746, 761	179 (11.4) 41 (2.6)	226 (17.6) 48 (3.8)	239 (22.6) 80 (7.6)
N_2 and NH_3 fixing bacteria [a]without adenosylhopane or hopanelactone	627,746, 760	155 (9.9) 17 (1.1)	204 (15.8) 30 (2.3)	190 (17.9) 56 (5.3)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	152 (9.7)	106 (8.3)	83 (7.8)
Methanotrophs	3Me 728, 772, 830, 844	21 (1.3)	13 (1.0)	7 (0.6)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	2 (0.2)	2 (0.2)	3 (0.3)
Methylotrophs	1086	ND (0)	ND (0)	0.4 (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	53 (3.4)	41 (3.2)	20 (1.9)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

3.6.4.4 Plot 8 Bacterial Community Structure

The distribution of bacteria, as identified by BHPs, in plot 8 (Table 3.9) is very similar to that observed in plot 6 with approximately 60% of the BHPs being identified as from various sources. PNSB account for 37 to 41% of BHP total. Cyanobacteria account for 6% of the total, methanotrophs 1% and acetic acid bacteria less than 0.5%.

There is very little relative change with depth indicating that the distribution is possibly dominated by leaching down the soil profile.

Table 3.9 Distribution of BHP producing bacteria in all plot 8 samples, identified by BHPs.

Bacteria	BHPs	Plot 8 (0-5 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)	Plot 8 (5 -10 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)	Plot 8 (10-15 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	467 (60.9)	478 (60.2)	354 (56.0)
Purple non sulphur bacteria	627, 712, 746, 761	208 (27.2)	217 (27.3)	196 (31.1)
[a]without adenosylhopane or hopanelactone		105 (13.8)	119 (15.0)	106 (16.7)
N_2 and NH_3 fixing bacteria	627,746, 760	131 (17.0)	130 (16.4)	117 (18.5)
[a]without adenosylhopane or hopanelactone		77 (10.0)	80 (10.0)	79 (12.5)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	46 (6.0)	50 (6.3)	39 (6.2)
Methanotrophs	3Me 728, 772, 830, 844	7 (1.0)	8 (1.1)	5 (0.8)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	5 (0.6)	5 (0.6)	2 (0.3)
Methylotrophs	1086	0.6 (0.1)	0.4 (0)	0.4 (0.1)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	13 (1.7)	13 (1.7)	15 (2.4)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid.

3.6.4.5 Plot 9 Bacterial Community Structure

The BHP distribution in plot 9 (Table 3.10) is similar to that for the other sites and is very similar to that for plot 8 with a relatively high percentage of PNSB (Table 3.9), approximately 37% to 41%. The level of cyanobacterially derived BHPs decreases

with depth. The level of methanotrophs is also consistent with depth and similar to the levels identified in plots 6 and 8.

Table 3.10. Distribution of BHP producing bacteria in all plot 9 samples, identified by presence of BHPs.

Bacteria	BHPs	Plot 9 (0-5 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)	Plot 9 (5 -10 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)	Plot 9 (10-15 cm) Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	562 (62.0)	691 (59.4)	404 (59.9)
Purple non sulphur bacteria [a]without adenosylhopane or hopanelactone	627, 712, 746, 761	223 (24.7) 103 (11.3)	328 (28.2) 164 (14.1)	198 (29.3) 116 (17.2)
N_2 and NH_3 fixing bacteria [a]without adenosylhopane or hopanelactone	627,746, 760	133 (14.7) 79 (8.7)	176 (15.2) 129 (11.1)	13.6 (8.7) 87 (12.9)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	71 (7.8)	81 (7.0)	4.1 (6.1)
Methanotrophs	3Me 728, 772, 786, 830, 844	7 (0.8)	9 (0.7)	7 (1.0)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	3 (0.3)	6 (0.5)	2 (0.2)
Methylotrophs	1086	0.2 (0)	0.3 (0)	0.2 (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	34 (3.8)	46 (4.0)	17 (2.5)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid.

3.7 Statistical Analysis

Further statistical analysis, using principle component analysis (PCA) was carried out to identify more subtle differences in the plots. BHPs identified only once during investigation were excluded from analysis.

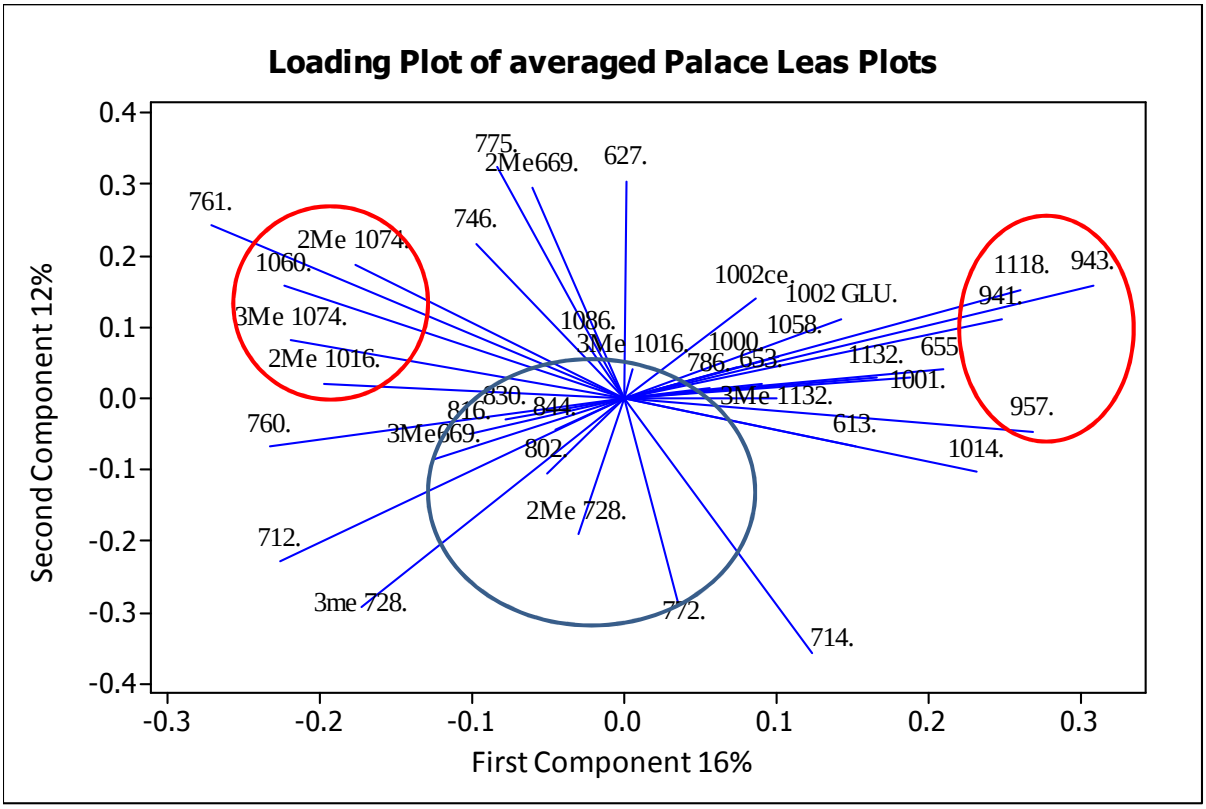


Fig. 3.33 PCA Loadings plot for all Palace Leas Plots using all BHP concentrations. Circles indicate grouping of bacterially related BHPs

The level of significance of the 2 components measured is low, 16% and 12% respectively. This implies that many minor differences account for the differences between the plots and not a few significant BHPs. Further analysis, using other components up to the 4th component, did not improve the separation of the plots.

The loading plot (Fig. 3.33) shows a significant clustering of BHPs around the centre with a positive correlation for cyanobacteria related BHPs; unsaturated BHT pentose (**5n or 6n**; 941), BHT pentose (**1n**; 943) and 2Me BHT pentose (**2n**; 957). However there is also a negative correlation, with respect to the 1st component for another set of cyanobacteria related BHPs, 2-Me BHT cyclitol ether (**2d**) and 2-Me BHpentol cyclitol ether (**2l**). There is a negative correlation with respect to the 2nd component

for methanotrophs indicated by the relationship between 3-Me aminotriol (**3f**; 3me 728), aminotetrol (**1h**; 772), aminopentol (**1c**; 830) and 2-Me aminopentol (**2c**; 844). Overall, however, the loadings plot gives a confusing picture that makes resolution of significant trends with respect to bacterial source difficult to resolve.

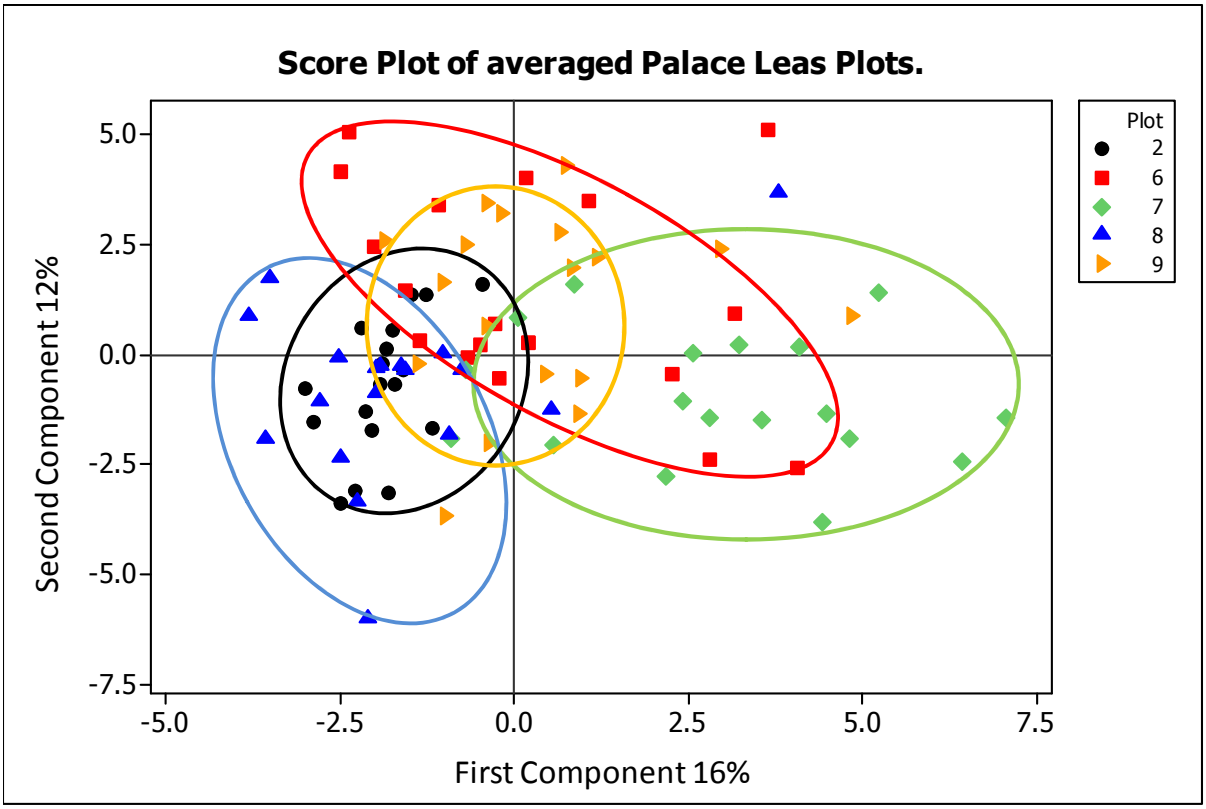


Fig. 3.34 PCA Scores plot for Palace Leas plots using all BHP concentrations

Analysis of the grouping of the plots using all the BHPs (Fig. 3.34) clearly demonstrates that plots 7 and 8 differ, with plot 7 being positively correlated indicating the importance of BHT pentose (*m/z* 943; **1n**) related structures in this sample. Plot 8 is negatively correlated with respect to the first component implying the lack of importance of the BHT pentoses (**1n**). A variety of BHPs appear to be significant in plot 8 compared to plot 7 including unsaturated aminotriol (**5f or 6f**), BHpentol cyclitol ether (**1l**), methylated BHpentol cyclitol ether (**2l**) and 3Me aminotriol (**3f**). Plots 2, 6 and 9 are all approximately overlapping near the centre of the of the loadings plot indicating that this analysis does not distinguish clearly between all the plots based upon BHP distribution.

The separation of the plots analysed by grouping the BHPs in the various bacterial groups is described in section 3.6.4. The BHPs relating to various and unknown

sources were removed from the analysis and 3 distinct loadings were identified (Fig. 3.35), cyanobacteria; PNSB and nitrogen fixing bacteria (linked by presence of adenosylhopane (**1e**) in both); and methylotrophs, acetic acid bacteria and methanotrophs, all C-1 utilising bacteria. The significance of the components is relatively high at 31% and 19%. The scores plot (Fig 3.36) indicates that plot 7 contained the most diverse set of results, whereas the other 4 plots were quite tightly grouped indicating a degree of difference between the bacterial populations. Plot 2 was most significantly influenced by the presence of methane oxidising bacteria, specifically due to the presence of aminopentol (**1c**). The use of PCA clearly shows some differences between the various plots but there is still significant overlap between the plots. The separation of the plots was further improved by using the average bacterial population results for each site (Figs 3.37 and 3.38).

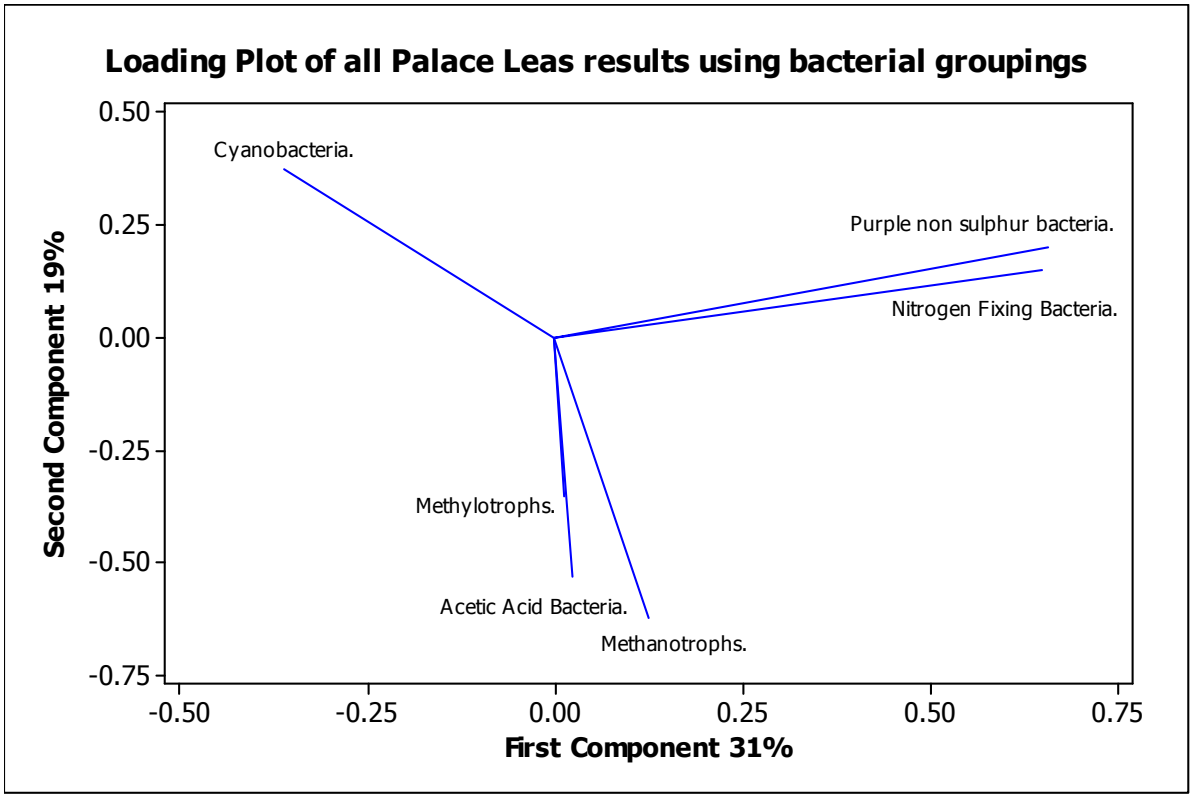


Fig. 3.35 Loading plot of all Palace Leas results using bacterial groupings

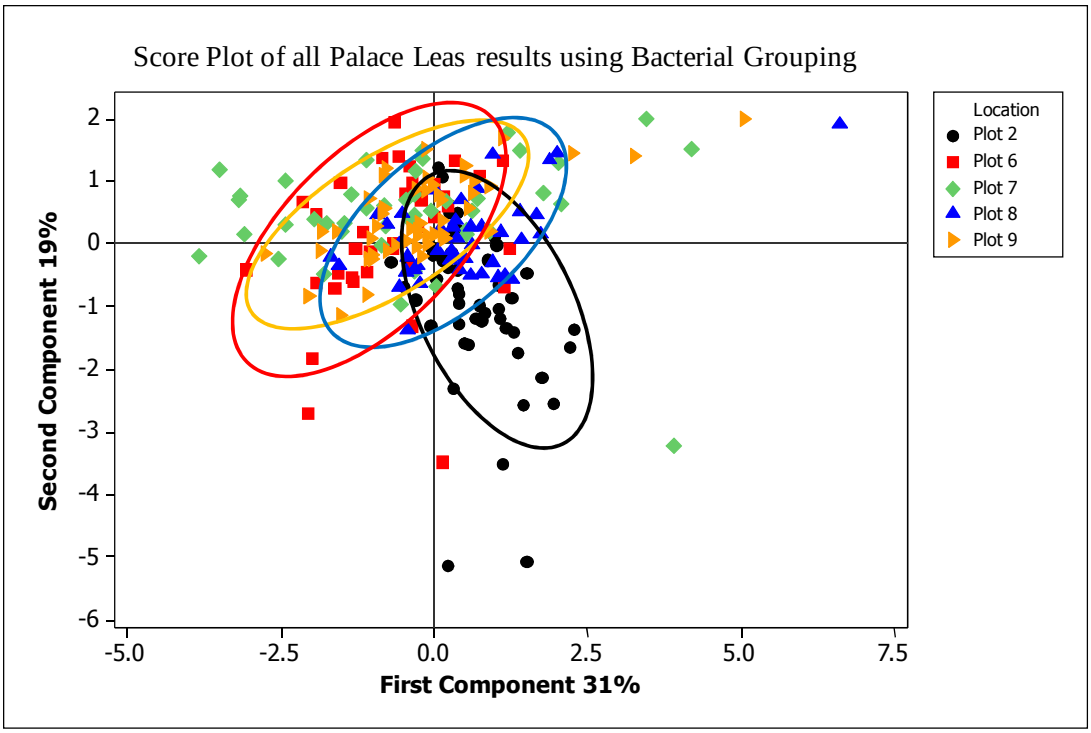


Fig. 3.36 Score plot of all Palace Leas results using bacterial groupings.

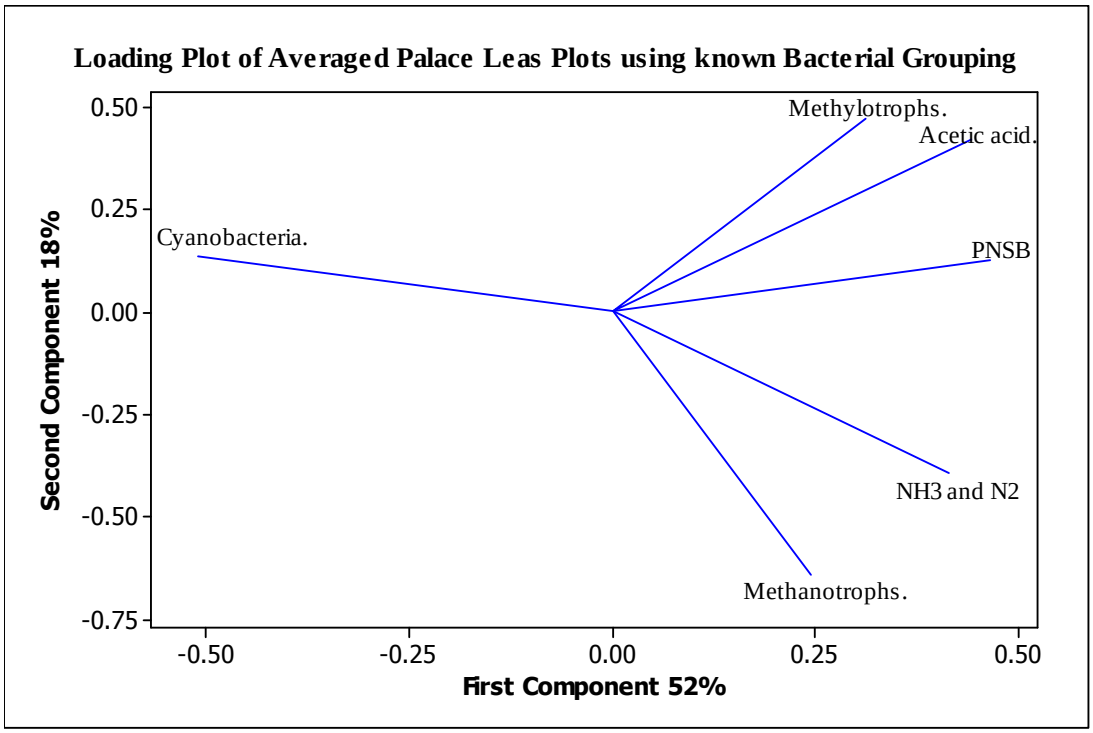


Fig 3.37 Loading Plot of average Palace Leas results using bacterial groupings of BHPs.

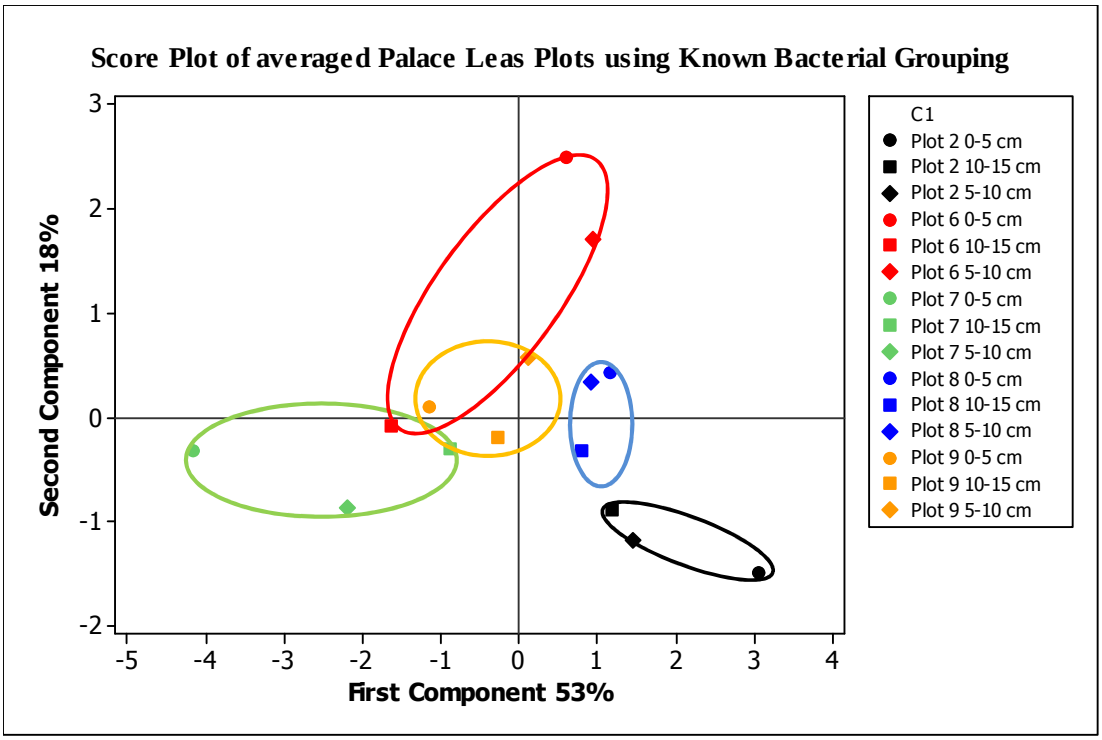


Fig 3.38 Scores plot of average Palace Leas results using bacterial groupings of BHPs. Points are summary % distributions of all samples from each plot at each different depth

The scores plot using average values for each plot (Fig 3.38) clearly separates the plots based upon the bacterial populations identified in each as a result of the BHP analysis. Both components are significant (PC1 = 53% and PC 2 = 18%). Plot 2 tends towards a score dominated by methanotrophs, as would be expected by the presence of aminopentol (**1c**). Plot 7 trends towards a score dominated by cyanobacteria and methanotrophs, again as would be expected from the BHP results. Plot 6 trends to scores dominated by cyanobacteria, acetic acid bacteria and methylotrophs with some influence from PNSB, again indicated by the absence of methanotrophs. In the case of plot 8 there a trend towards PNSB and nitrogen and ammonia fixing bacteria. Plot 9 is located in the centre of the plot and indicates no significant influence by any individual group of bacteria.

Using PCA it is clear that the BHP profile of each plot is different and since the only influence on the plots is the fertiliser regime it can be clearly stated that the fertiliser has an effect on the bacterial population.

3.8 Summary

Goal 1

Identify the BHP profile of a soil from different plots.

Based upon the linkages between specific BHPs and bacteria identified by other researchers (Table 2.2), 6 different bacterial groups can be identified in the Palace Leas soils using BHP analysis; methanotrophs, cyanobacteria, purple non-sulphur bacteria, nitrogen fixers and ammonia oxidisers, acetic acid bacteria and methylotrophs. This work has built upon the use of BHPs as a taxonomic marker and has added to the weight of evidence supporting this use of BHPs as a marker for bacteria.

The tentative identification of many new BHPs, not previously identified in cultured organisms (Table 2.2), has opened up the possibility that further bacteria can be linked to BHPs but it is outside the scope of this research to be able to identify these new links.

Goal 2.

Identify any differences in soil BHP profile due to differences in fertilizer regime.

Using BHP profile analysis differences between the Palace Leas plots as a result of fertiliser regimes can be ascertained. The differences can be simple (such as the presence of significantly more aminopentol (**1c**) in plot 2 manure treatment) or more complex and require the use of PCA to separate the plot characteristics (Fig. 3.37). Using the bacterial source groupings of BHPs the separation between the plots is clear (Fig 3.38) the differences between the plots are variable with plots 2 and 7 being the most changed when compared to the control, plot 6. This indicates that the different fertilisers have different effects on the soil BHP profile. In some cases the effects are small, (plots 8 and 9), and indicates that the soil make up could be a significant force in determining the BHP profile, and therefore the bacterial population. These results are in agreement with the evidence that soil type is a dominant factor in the

composition of the soil microbial community (e.g. Gelsomino et al., 1999) due to the physiochemical properties that dominate such as pH, aeration, mineral content and carbon availability (Hu et al., 1999)

The relationship between individual BHPs and source bacteria is still tentative under natural environmental conditions, due to the lack of knowledge on the role of BHPs within the cell and the production of BHPs by bacteria in the soil. However, there is agreement with other long term fertiliser projects with respect to methanotroph activity (Seghers et al., 2003), indicating that aminopentol (**1c**) is a robust marker for methanotroph activity. This implies that all that is needed to accurately identify which BHP producing bacteria are active in the soil using BHP analysis is further insight into the link between BHPs and bacteria.

The effect of nitrogen fertiliser on plot 7 is a more complex picture as there would be an expected drop in methanotroph activity and a rise in ammonia oxidising and nitrogen fixing bacteria (in accordance with Hutsch, 2001; Seghers et al., 2003 and Chu et al., 2007) but plot 7 and plot 2 are the only plots to show any presence of the methanotroph indicator aminopentol (**1c**) and the concentration of BHPs related to ammonia oxidising bacteria and nitrogen fixing bacteria is the lowest of all the plots (Table 3.8).The reason for this is unclear and may relate to adenosylhopane (**1e**) being a possible precursor to all BHPs (Seeman et al.,1999)

The distribution of BHPs in the soil appears to be mainly a result of leaching of the BHPs, or translocation of the bacteria, down the soil column. Some evidence was obtained that the BHPs are being actively degraded in the soil, with the number of identifiable structures tending to decrease with depth, although very few degradation products were identified. This is particularly highlighted by the decrease in the occurrence of cyanobacterially related BHT pentose (**1n**) and its homologues (**2n; 5n or 6n**) with depth.

Goal 3.

Identify differences in BHP profile between stressed and non-stressed plots.

Previous work has identified that plot 2 has the highest bacterial population and plot 7 had a smaller but more stressed bacterial population (Hopkins and Shiel, 1996). The BHP concentrations of these plots showed that plot 7 had the largest BHP

concentration and plot 2 the lowest. The implication of these results is that bacteria produce BHPs in response to environmental stress and cannot be directly correlated to the size of the bacterial population.

Goal 4.

Identify any temporal differences in the BHP profile.

The analysis of the BHP distribution from January 2006 to January 2007 showed no significant variation in the BHP distribution within the Palace Leas plots. The implication of this is that the types of BHP producing bacteria in the bacterial population do not change significantly. The Palace Leas plots are a mature set of experimental plots and it is likely that the bacterial population has adapted to become a stable population.

Within a year there is, however, a significant change in the total concentration of BHPs in the plots with rises in the summer and autumn months. This implies that there is either an increase in the total population of BHP producing bacteria in the soil or that the bacteria are producing more BHPs in response to environmental change. Logic suggests that it is the former as during the colder wetter months a reduced bacterial population will be less active.

This potential annual cycling indicates that intact BHPs are are possibly short lived in the soil and are rapidly removed from the solvent extractable population either bydegradation or incorporation into humin, enabling levels of BHPs in soil to be used as an indicator of bacterial activity. The recalcitrance of the BHPs in the soil needs further investigation to identify the rate and mechanism for degradation.

3.9 Conclusions

The investigation in the Palace Leas plots has identified a consistent distribution of BHPs in the plots than identifies the different BHP producing bacteria populations within the plots. The fertiliser regime on the different plots has caused variations in the bacterial population that can be identified using the BHP profile. The distribution of BHPs is complicated by leaching of BHPs and translocation of bacteria down the

soil column. There appears to be an overall annual cycle to the BHP concentration in the soil that implies that in aerobic conditions intact BHPs are not recalcitrant.

The Palace Leas soils contained a wide variety of BHPs with concentrations up to 2500 $\mu g BHP g^{-1}_{TOC}$. A soil sample can contain up to 25 different BHPs that can be identified at levels down to <0.5 ($\mu g_{BHP}\ g^{-1}_{TOC}$). In total 44 different BHPs were identified in the Palace Leas soils. Distribution of the BHPs throughout the soil appears to be predominantly driven by leaching of BHPs and bacteria throughout the soil profile. There is a relative increase in BHPs as a percentage of the soil total lipid extract (TLE) moving down the soil profile indicating that BHPs are relatively more recalcitrant than other organic structures. Coupled with this relative increase as a proportion of soil TLE there is a decrease in the number of observed structures indicating that some degradation of BHPs is occurring.

Analysis of the BHP profiles from the Palace Leas plots clearly showed differences between the plots as a result of fertiliser treatment, e.g. Plot 2 contains significant levels of aminopentol (**1c**) when compared to other plots as a result of the application of manure. This is in agreement with previous research (Seghers et al., 2007; Hutsch 1998) who found that mineral fertilisers inhibited methanotrophs and promoted ammonium oxidising bacteria.

Comparison of the Palace Leas results to other long term fertiliser projects clearly shows that the addition of manure to plot 2 has resulted in the promotion of methanotroph activity. The addition of nitrogen fertiliser to plot 7 has not resulted in the absence of methanotrophs. Several factors could have influenced this difference; the Palace Leas plots are pasture and the addition of manure from grazing animals may have promoted methanotrophs in this plot; the Palace Leas plots have been operating for more than 100 years whereas the other sites are all less than 20 years. This longer time frame has resulted in the concentration of organic matter in the top 5 cm and peat like conditions may enable methanotrophs to grow in this layer.

In many cases the differences in BHP profile are small and require principle component analysis and selection of specific BHP groups to highlight the differences. Using PCA with BHPs grouped according to known source organisms there is significant separation of the plots indicating that the different plots can be distinguished to some extent based purely on their BHP distribution. However, even with PCA there can be significant overlap between the BHP profiles of the plots

implying that that the soil may be the most important factor in determining the bacterial population.

The relationship between bacterial population and BHP concentration is not clear as the most productive site, plot 2, produced the lowest level of BHPs and the least productive site, plot 7, the highest quantity and diversity of BHPs. These results are in agreement with other research that suggests that BHPs are produced in response to stress (Welander et al., 2009).

Seasonal variations can be readily identified by changes in the total BHP concentration. There is an overall increase in the total BHP concentration at all depths during the summer months followed by a drop during winter. This implies that there is increased bacterial activity during the summer months. This change in concentration also indicates that the measured BHPs are indicators of the current bacterial population and not recalcitrant relics of much older bacterial activity. This observation contradicts previous research (Cooke et al., 2008b, van Dongen et al., 2006) and research within this thesis (Chapter 5), that shows that at least a proportion of the total BHP production can survive for up to 65 million years. It appears that intact BHPs are not extremely recalcitrant in soil, although more recalcitrant than other lipids, and may be quickly degraded in the presence of oxygen or absorbed into the humic mass in the soil where they are inaccessible to the analytical technique used here. It would be expected that in aerobic conditions the functional groups would be readily consumed by microorganisms whereas the triterpenoid ring system would remain relatively intact. This observation would explain the extreme recalcitrance of the ring system in the environment as well as the apparent rapid turnover of BHPs during a year.

4. Hack Hall Farm Case Study

4.1 Introduction

The Hack Hall Farm case study investigates the effect of flora on the underlying bacterial population to determine if the soil BHP profile can be used to identify any differences in bacterial populations and processes due to variation in flora. The Hack Hall Farm site is a small (approximately 2 hectares) woodland planted in 1986 on an area of poorly drained land and has remained untreated by any fertiliser or other human activity since that date. Therefore the only influence on the bacterial population is the soil, climate and the overlying vegetation making it an ideal location to assess the effect of the flora on the soil BHP producing bacterial population.

Numerous researchers have studied the effect of different plant species on the soil bacterial population. For example, Fang and Moncreiff (2005) investigated soil respiration rates under birch (*Betula pendula*), Sitka spruce (*Picea sitchensis*), Scots pine (*Pinus sylvestris*) and pasture and found that in all cases 50% of microbial activity occurred in the top 0-8 cm. In addition the respiration rate under Scots pine was at least 50% higher than in the other locations. Liang et al (2008) measured the differences in microbial population under sugar maple (*Acer saccharum*), basswood (*Tilia americana*) and eastern hemlock (*Tsuga canadensis*) using phopholipid fatty acid (PLFA) analysis and found no significant differences between the soil bacterial populations. However using amino sugars, a more recalcitrant structure in soils, clear differences in the accumulation of biomass was identified, with basswood having the greatest accumulation and eastern hemlock the smallest. This indicates that there are differences in the accumulation rates of amino sugars due either to lower microbial metabolism rates or faster decomposition rates under eastern hemlock when compared with Basswood.

Research by Menyailo et al (2002, 2003) at the Siberian artificial afforestation experiment was carried out on variations in methane consumption by soils taken from beneath different tree species. It was found that soils under hardwood species such as aspen and birch consumed methane at a higher rate than the soils under coniferous trees and grassland. The implication of this research is that the exudates from different species or the decay leaf litter specific to each tree type promotes different bacterial species in the rhizosphere. Menyailo et al. (2003) also investigated the effect of soil

moisture on methane consumption and discovered that changes in moisture content had a dramatic effect on methane consumption that was also closely related to plant species. An increase in soil moisture increased methane consumption under spruce trees but reduced it under Scots pine and larch. The effect under other species was negligible. The implication of this is that the dominant methanotrophs under spruce trees are resistant to changes in moisture content and therefore osmotic pressure. The dominant methanotrophs under Scots pine and larch are conversely susceptible to changes in osmotic pressure. Other species, where no change is observed, would contain a wider range of methanotroph species and moisture changes will promote some species and retard others.

Studies of the effect on tree species on methane oxidation in 2 German forests showed that the rate of methane uptake by the soil associated with a beech forest was 2-3 times greater than that in an adjacent pine / spruce plantation (Borken et al, 2003), indicating that the tree species has a direct effect on the methane consumption rate, with oxidation occurring in the top 5 cm of the soil.

Henckel et al (2000) investigated changes in active methane oxidation in forest soils due to seasonal variation, and identified that in the summer months methane oxidation occurred throughout the soil (0 – 26 cm) whereas in the winter active methane oxidation was limited to a well defined 6-14 cm sub surface layer. This change in distribution of methane oxidation is linked to the level of water saturation of the soil which reduces the oxygenation of the soil in the winter and limits the potential of methane oxidation.

Lau et al (2007) identified that methane oxidation in forests was predominantly carried out by methanotrophic bacteria of the families the type II methanotrophs *Methylocystaceae* (whose abundance was an order of magnitude greater than other methanotrophs) and the acidophilic nitrogen fixing bacteria *Beijerinckiaceae*, which produce aminotriol (**1f**), BHT (**1a**), BHT cyclitol ether (**1d**) and BHpentol cyclitol ether (**1l**; Vilcheze et al., 1994).

Wilkinson and Anderson (2001) investigated the spatial distribution of bacterial populations on a spruce plantation using PLFA analysis. They discovered that the different moisture properties of the soil between 0 m and 2 m away from the trunk resulted in different bacterial populations with different tolerance to moisture stress. As the membrane will be of primary importance in moisture stress then there would be expected to be a similar variation in BHP composition with higher concentrations

and more polar BHPs, with greater membrane condensation properties, in areas of greatest moisture stress (Kleeman, 1994, Poralla et al., 2000).

An investigation of forest soil profiles will therefore identify changes in BHP distribution associated with changes in BHP producing bacteria activity resulting from differences in overlying flora and seasonal changes as indicated in the research previously identified.

4.2 Site Background Information

Hack Hall farm is a privately owned, mixed use farm, Dinnington, Newcastle upon Tyne (Figs 4.1 and 4.2). The woodland site was an area of poorly drained land that was planted with Scots pine (*Pinus sylvestris*), common fir (*Abies alba*) and sycamore (*Acer pseudoplatanus*) in 1986. Since that time the area has been untreated and left to grow naturally. The trees are planted approximately 3 m apart and are up to 7 m tall. The resulting canopy is complete and has resulted in sparse grass cover between the trees. The sycamores are sited in one corner of the site and here the grass cover is more complete due to the greater exposure to sunlight in this area. The Scots pine and common fir are dispersed more randomly throughout the woodland.

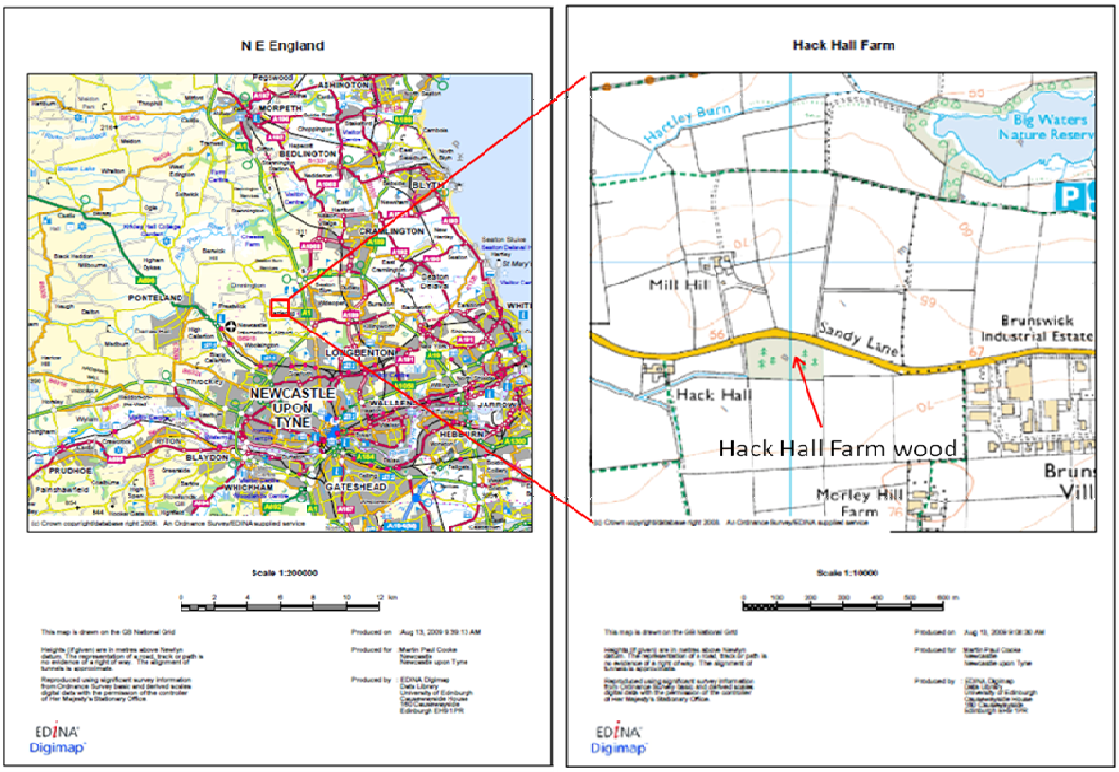


Fig 4.1 Hack Hall Farm location maps (Edina.co.uk)

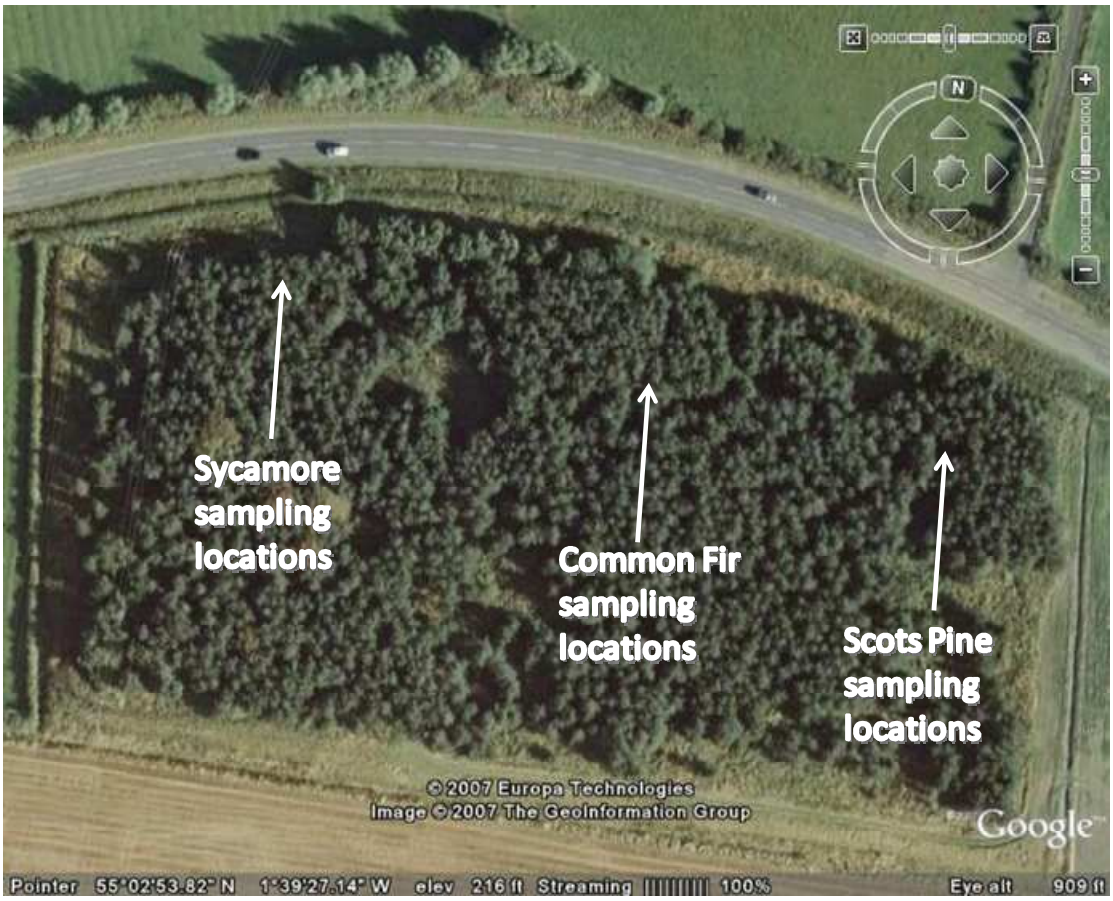


Fig. 4.2. Hack Hall Farm wood, aerial view and sampling locations. (Google Earth)

4.3 Goals

The goal of the analysis of Hack Hall Farm wood was to test hypothesis 1

The BHP fingerprint of a soil indicates the current bacterial population

This is to done by achieving 2 goals

Goal 5

Identify the BHP profile in the soil under different tree species

For this goal to be achieved, discernable differences must be observed in the BHP concentrations under different tree species. The BHPs will enable the tentative

identification of source bacteria (Table 2.2) and the differences observed will enable the effect of the overlying tree species on the bacterial population to be identified.
The proximity to the tree species will also have an effect, with the variations in soil PLFA distribution due to moisture variation resulting from distance from overlying trees (Wilkinson and Anderson, 2001) will be repeated in the BHP distribution with an increase in concentration proportional to distance from the nearest tree. This increase in BHPs will condense the membrane protecting the cell from environmental stress caused by variation in soil moisture (Kleeman, 1994, Poralla et al., 2000).

Goal 4

Identify any temporal differences in the BHP profile.

This is a repeat of Goal 4 set in Chapter 3. Analysis of variations in BHP profile over a 12 month sampling period will indicate changes in bacterial population and BHP production during this time. This is fundamental to linking the BHP profile to the current bacterial population.

4.4 Sampling Methodology

Samples were taken approximately every 3 months in May, August and December 2006, and March 2007 in accordance with the methodology described in 2.1. Samples were collected from 6 different locations. The “near” samples were taken from approximately the same position on each occasion within 30 cm of the base of each individual tree type. The “between” samples were taken from the centre of a cluster of identical tree species and approximately 2 m from the trees (Fig. 4.2). In each case the locations were selected in a group of trees of the same species to reduce the influence of different species on the soil BHP profile. A single sample was taken from each location. The soil was black in colour, showed no variation with depth and at all times during sampling was soft indicating high moisture content. The error bars used in the results indicate the standard experimental error of +/- 17%.

4.5. Results

4.5.1 Scots Pine soil BHP profiles.

4.5.1.1 Near Scots Pine

The near Scots pine soil has a relatively high TOC when compared to the Palace Leas plots (8% in 0-5cm decreasing to 3.7% in 10-15cm, for May 2006; Appendix) and has a poorly aerated appearance. In the near Scots pine results (Fig. 4.3) the concentration of BHPs was low, less than 400 $\mu gBHPg^{-1}_{TOC}$, in all 4 seasonal samples, except for August 2006 10-15 cm (809 $\mu gBHPg^{-1}_{TOC}$), when compared to the results from the Palace Leas plots (Chapter 3). In total 24 different BHPs were identified, 23 in 0-5cm, 16 in 10-15 cm layers, and 20 in 5-10 cm.

A total of 19 different BHPs were identified in the May 2006 samples, 17 in 0-5 cm layer, 13 in 5-10 cm and 14 in 10-15 cm, with similar reductions with depth in the other near Scots pine samples. The profile is dominated by BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**). These 4 BHPs account for between 69.6% (May 2006, 5-10 cm) and 79.4% (March 2007, 5-10 cm) of the BHP total. Three other BHPs contribute a significant proportion of the BHP total, 2-Me BHT (**1a**), Adenosylhopane type-1 (**1u**) and BHpentol cyclitol ether (**1l**), accounting for approximately 15% of the total BHP concentration. The other BHPs account for approximately 10% of the BHP total. A total of 6 BHPs were identified as being infrequent and minor contributors to the BHP total; AnhydroBHT (**1j**), hopanelactone (**1t**), unsaturated BHT (**5a** or **6a**), unsaturated BHT pentose (**5n** or **6n**), BHT glucosamine (**1g**) and 2-Me BHpentol cyclitol ether (**2l**). These structures were also among those identified in the Palace Leas samples as being infrequent and minor contributors to the BHP profile (Chapter 3).

In May 2006 the concentration of BHPs with depth remains constant or shows a slight decrease (Fig. 4.3). In August 2006 the maximum BHP concentration is again in the 10-15 cm layer and the minimum concentration is in the 5-10 cm layer. In December 2006 the maximum BHP concentration is in the 0-5 cm layer (Fig. 4.3) and there is a general decrease in concentration with depth. In March 2007 there is a general

increase in concentration with depth although the aminotriol (**1f**) concentration is at a maximum in the 5-10 cm layer (Fig. 4.3).

Aminopentol (**1c**) is identified in all the samples (Fig 4.3) and reaches a maximum concentration in the 5-10 cm layer in May and August 2006 and in the 10-15 cm layer in December 2006 and March 2007.

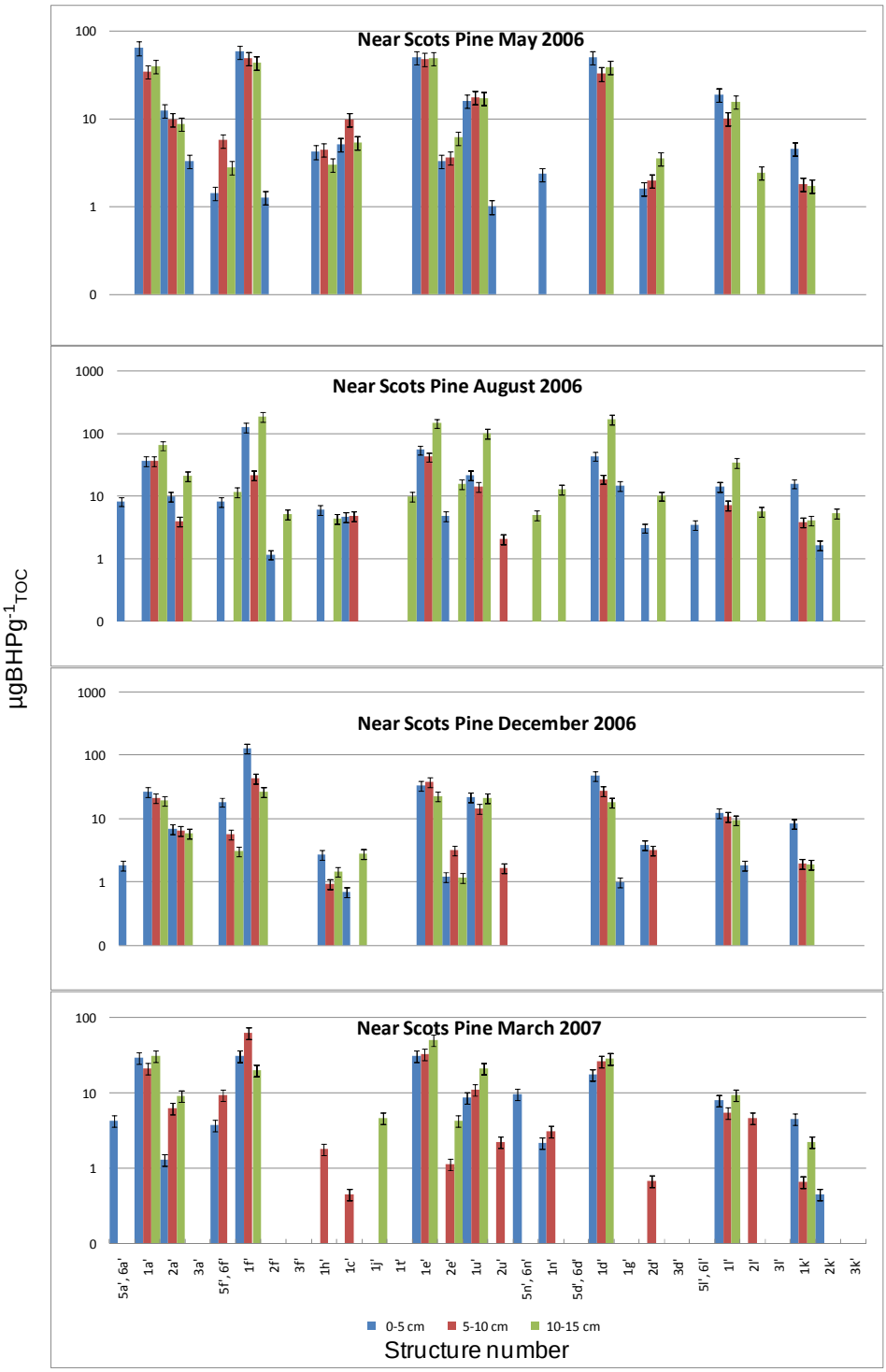


Fig.4.3 Log distribution of semi-quantitative BHP concentration ($\mu gBHPg^{-1}_{TOC}$) for Hack Hall, near Scots pine.

4.5.1.2 Between Scots Pines

The between Scots pine samples (Fig. 4.4) have a lower TOC than that observed in the samples collected near to the Scots pine, 4.8% in 0-5 cm decreasing to 3.5% in 10-15 cm layer for the May 2006 samples. Whilst the May 2006, 0-5 cm layer is significantly lower in the between Scots pine samples, the other samples from this site have similar TOC values to the near Scots pine samples at approximately 6.5% (Appendix). The concentration of BHPs is much higher than the near Scots pine samples with the totals for May 2006 being 848 $\mu gBHPg^{-1}_{TOC}$ in 0-5 cm, 481 $\mu gBHPg^{-1}_{TOC}$ in 5-10 cm and 1250 $\mu gBHPg^{-1}_{TOC}$ in 10-15 cm. In total 25 different BHPs were identified but there is no significant decrease in the number of BHPs with depth as seen in the near Scots pine samples (Fig. 4.3) with the 0-5 cm layers containing 22 different BHPs, 20 in the 5-10 cm layers and 21 in the 10-15 cm layers.
The BHP profile is dominated by 3 BHPs; aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**), accounting for between 49.4% (March 2007, 5-10 cm) and 70.3% (May 2006, 5-10cm) of the total BHP concentration, with BHT (**1a**) being relatively reduced compared to the near Scots pine sample (Fig 4.3).
Unsaturated BHT (**1a**) is absent from all the between Scots pine samples (Fig. 4.4) but is found in the near Scots pine samples at every sampling period. The other minor BHPs are similar to those seen in the near Scots pine samples and the Palace Leas samples.
Aminopentol (**1c**) is again present in almost all samples and the concentration increases down the soil profile reaching a maximum in the 10-15 cm layer for all time periods (Fig.4.4).
There is a pattern of decrease in concentration in the 5-10 cm followed by an increase to a maximum concentration in 10-15 cm which is repeated for all the between Scots pine samples except March 2007 where there is a decrease with depth (Fig. 4.4). The May 2006 samples have a very high concentration of aminotriol (**1f**; 424 $\mu gBHPg^{-1}_{TOC}$) in the 10-15 cm layer, more than twice the 0-5 cm concentration (193 $\mu gBHPg^{-1}_{TOC}$). The December 2006 sample has an increase in the concentrations of all BHPs in the 10-15 cm layer compared to the other depths at this time frame with the concentration of BHT cyclitol ether (**1d**; 313 $\mu gBHPg^{-1}_{TOC}$) being more than 10 times the concentration at the other 2 depths.

Whilst these BHPs dominate the total BHP concentration there is an increase in the concentration of all BHPs in the between Scots pine samples (Fig. 4.4) when compared to the near Scots pine samples (Fig. 4.3).

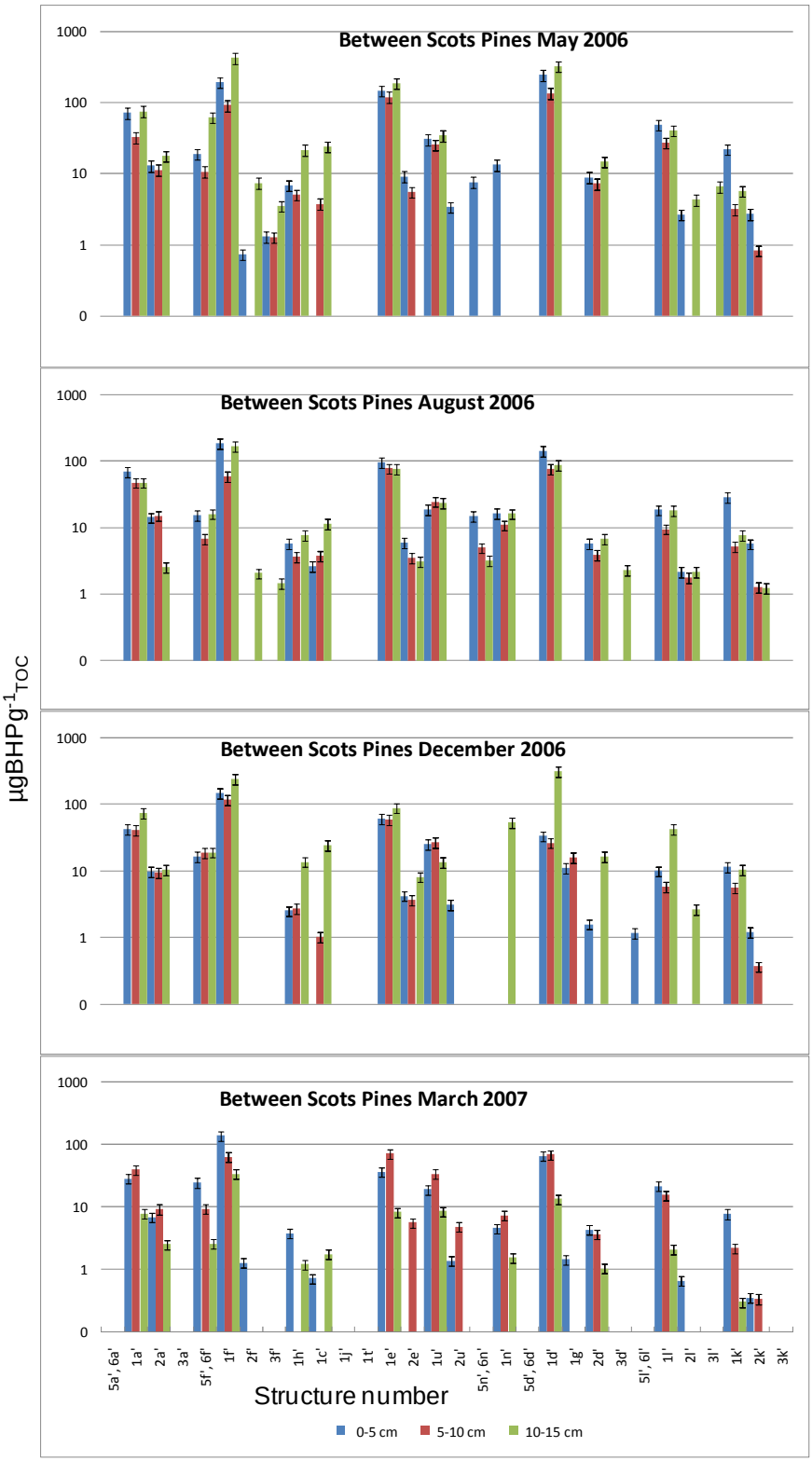


Fig. 4.4 Log distribution of semi-quantitative BHP concentration (µgBHPg$^{-1}_{TOC}$) for Hack Hall, between Scots pine.

4.5.2 Common Fir soil BHP profiles

4.5.2.1 Near Common Fir

The TOC levels in the near common fir samples decrease with depth except for the May 2006 samples which increases with depth from 5.1% in 0-5 cm to 6.9% in 10-15cm (Appendix). The samples from Ma 2006 contained 25 different BHPs with 23 in the 0-5 cm layer, 18 in the 5-10 cm layer and 19 in the 10-15 cm layer.

The BHP profile is dominated by BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**), accounting for between 69.5% (March 2007, 5-10 cm) and 79.0% (August 2006, 10-15 cm) of the BHPs (Fig 4.5).

The concentration of BHPs in the near common fir samples is very variable with the May 2006 samples showing a decrease in total BHPs from 869 $\mu gBHPg^{-1}_{TOC}$ in 0-5 cm down to 504 $\mu gBHPg^{-1}_{TOC}$ in the 10-15 cm sample. However, the August 2006 results show a decrease in total BHPs from 1507 $\mu gBHPg^{-1}_{TOC}$ in 0-5 cm to 1108 $\mu gBHPg^{-1}_{TOC}$ in 10-15 cm (Note that the sample for the 5-10 cm layer was lost). The December 2006 and March 2007 results are much lower and show an increase with depth (291 $\mu gBHPg^{-1}_{TOC}$ in 0-5 cm to 552 $\mu gBHPg^{-1}_{TOC}$ in 10-15 cm for March 2007).

The BHP profile shows a consistent decrease in the concentration each of each of the BHPs with depth (Fig. 4.5) for May and August 2006 whereas the BHP concentrations for December 2006 and March 2007 show the opposite trend with an increase in BHP concentration with depth.

In all samples there is an increase in the concentration of adenosylhopane type-1 (**1u**) with depth, with a maximum concentration of 86 $\mu gBHPg^{-1}_{TOC}$ in the 10-15 cm layer of August 2006 (Fig. 4.5). The concentration of aminopentol (**1c**) similarly increases with depth but at much lower concentrations, maximum of 12 $\mu gBHPg^{-1}_{TOC}$, in August 2006.

Only 3 BHPs were identified in 1 near common fir sample; unsaturated BHT (5**a** or **6a**; March 2007, 0-5 cm), 3-Me aminotriol (**3f**; May 2007, 10-15 cm) and unsaturated BHpentol cyclitol ether (**5l** or **6l;** August 2006, 0-5 cm), (Fig 4.5).

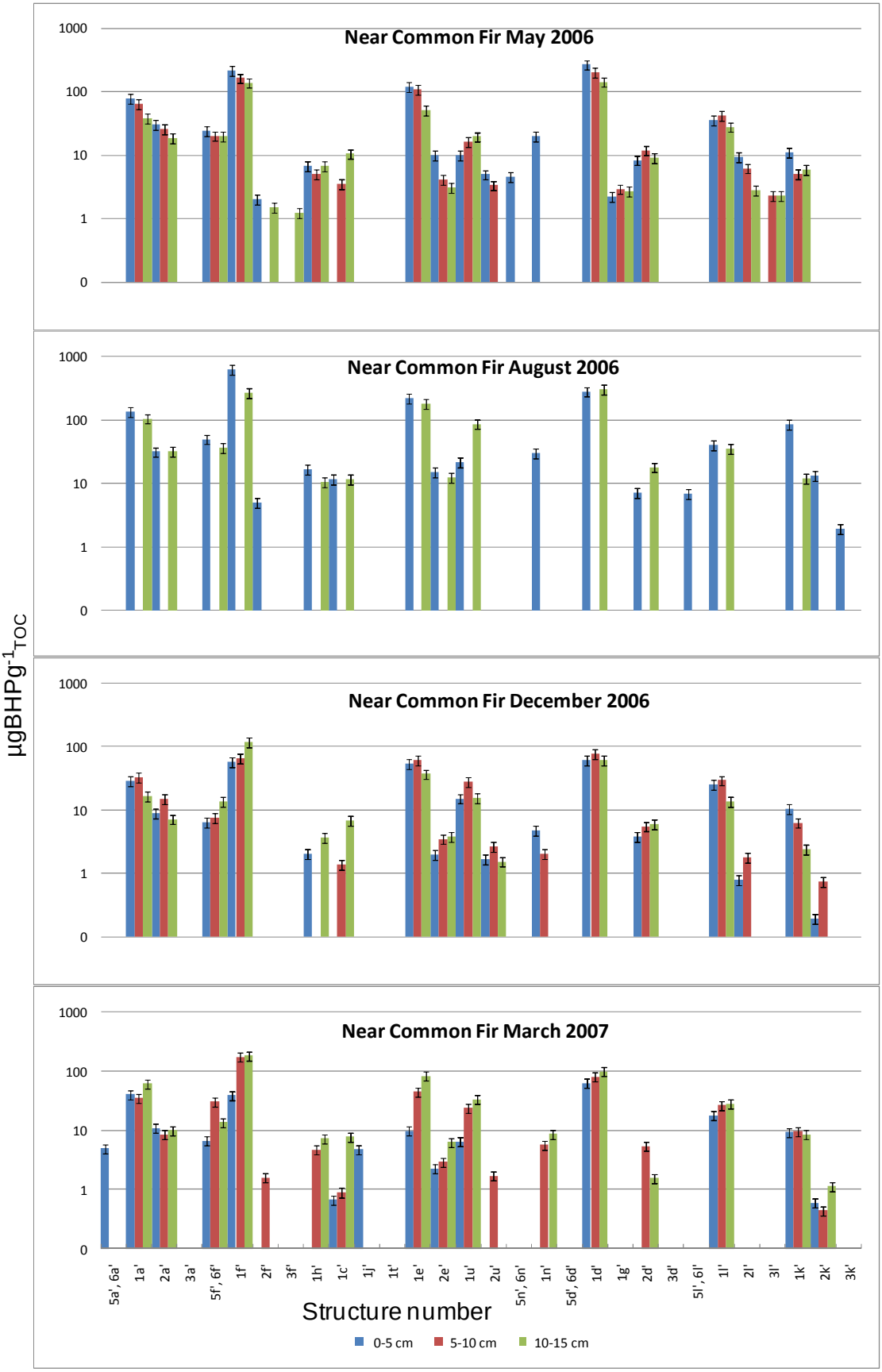


Fig. 4.5 Log distribution of semi-quantitative BHP concentration for Hack Hall (µgBHPg$^{-1}_{TOC}$), Near Common Fir.

4.5.2.2 Between Common Firs

The TOC for the between common fir samples varies from 6.7% (December 2006, 0.5 cm) down to 2.8% (March 2007, 10-15 cm) and decreases with depth for all cores. A total of 27 different BHPs were identified (Fig 4.5), with 24 BHPs in 0-5 cm, 22 BHPs in 5-10 cm and 20 BHPs in 10-15 cm layers.

All the 3 depth layers are dominated by 4 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**), accounting for between 60.0% (March 2007, 5-10 cm) and 80.6% (May 2006, 0-5 cm) of the BHP total. The other locations have similar distributions although May 2006, August 2006 and March 2007 do have an accumulation of adenosylhopane (**1e**) in the 5-10 cm layer.

The distribution of the minor BHPs is far less consistent than the other locations with 6 different BHPs being identified in 1 sample only; anhydroBHT (**1j**; March 2007, 10-15 cm), unsaturated BHT (**5a** or **6a;** December 2006, 0-5 cm), 3 methyl aminotriol (**3f**; May 2006, 5-10 cm), unsaturated BHT pentose (**5n** or **6n**; March 2007, 0-5 cm), unsaturated BHT cyclitol ether (**5d** or **6d**: December 2006, 0-5 cm), 3 methyl BHT cyclitol ether (**3d**; March 2007, 10-15 cm) and unsaturated BHpentol cyclitol ether (**5l** or **6l**; May 2006, 0-5 cm).

There is no clear trend in the distribution of the BHPs with depth. In May 2006 the 0-5 cm and 10-15 cm layers have approximately the same concentration of BHPs but the 5-10 cm layer has a much higher concentration (3526 $\mu gBHPg^{-1}_{TOC}$ in 10-15 cm compared to 1015 $\mu gBHPg^{-1}_{TOC}$ in 0-5 cm and 1226 $\mu gBHPg^{-1}_{TOC}$ in 10-15 cm). In August 2006 and December 2006 there is a general decrease in BHP concentration with depth except for adenosylhopane type-1 (**1u**) in both samples which increases with depth.

The concentration of BHPs in March 2007 is much more variable with BHT (**1a**) having a maximum concentration in the 5-10 cm layer whereas aminotriol (**1f**) has the lowest concentration in this layer.

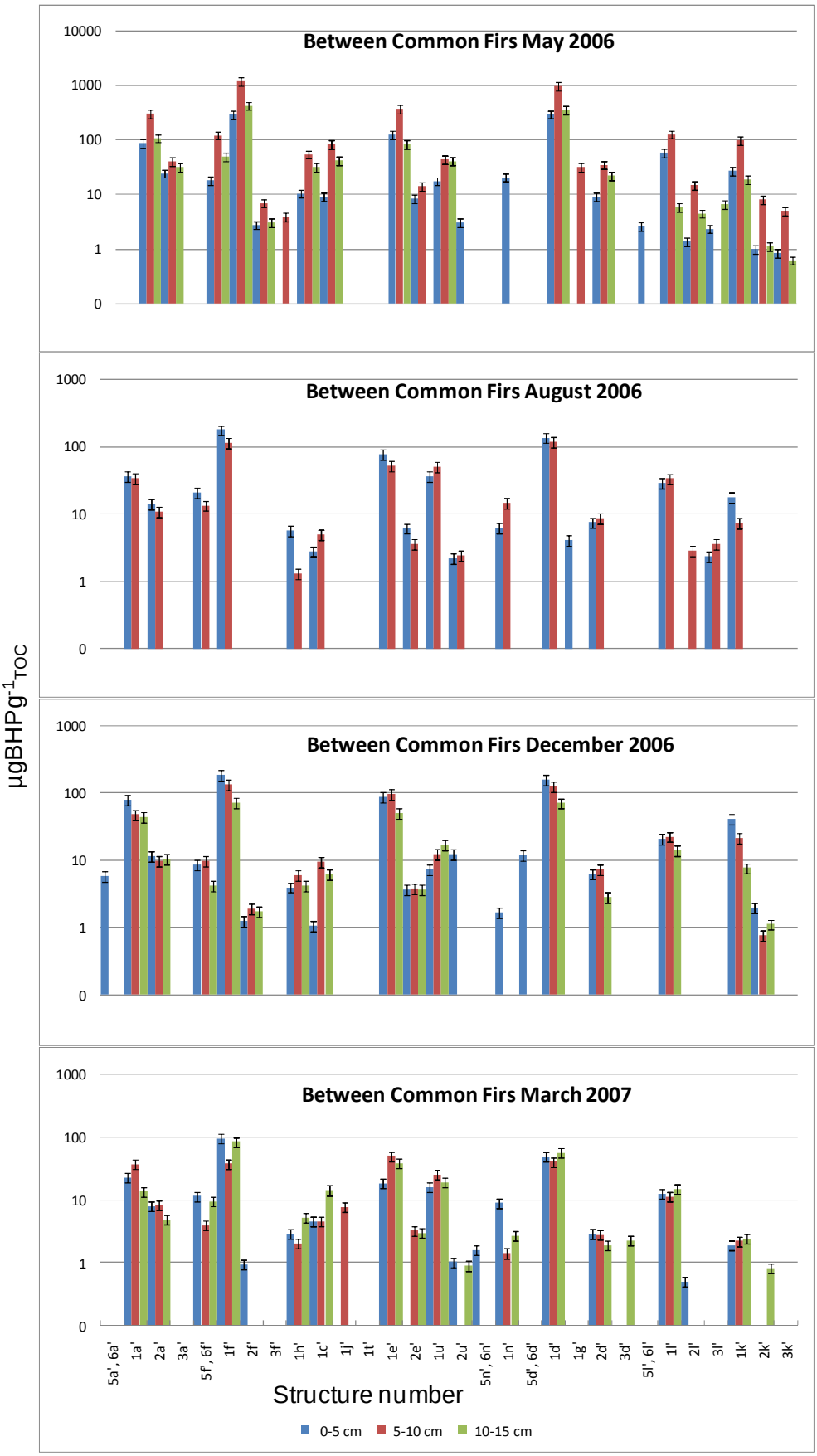


Fig. 4.6 Log distribution of semi-quantitative BHP concentration for Hack Hall, Between Common Firs.

4.5.3 Sycamore soil BHP profiles.

4.5.3.1 Near Sycamore

A total of 24 different BHPs were identified in the Near Sycamore samples (Fig 4.7) with 22 in the 0-5 cm layers, 20 in 5-10 cm layers and 22 in the 10-15 cm layers. There is a gradual decrease in TOC with depth from 7.55% in 0-5 cm to 4.94% in 10-15 cm layers, which is mirrored in all the near Sycamore samples (Appendix).

The total BHPs shows a slight increase in the 5-10 cm layer to 1395 $\mu gBHPg^{-1}_{TOC}$ from 1298 $\mu gBHPg^{-1}_{TOC}$ in the 0-5 cm layer before dropping to 771 $\mu gBHPg^{-1}_{TOC}$ in the 10-15 cm layer, May 2006. This is similar to the August 2006 sample but the remaining 2 samples show a slight increase in the 10-15 cm layer, although the levels in these 2 winter samples is lower than in the 2 summer samples (Appendix). The sample is dominated by 3 BHPs; aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**), accounting for between 50.3% (May 2006, 0-5 cm) and 75.2% (Aug 206, 0-5 cm). BHT (**1a**) is the next largest component, as seen in the other Hack Hall samples, accounting for 6 – 8% of the total. The remaining 21 BHPs account for 20 – 25% of the BHPs. For the first time in the Hack Hall samples 3-Me BHT (**3a**) has been identified, although only in the 0-5 cm layer of the May 2006 sample, 3-Me aminotriol(**3f**) was also identified once in the December 2006, 10-15 cm layer.

There is no clear trend of BHP distribution with depth with some BHPs; unsaturated aminotriol (**5f** or **6f**), aminotriol (**1f**) and BHpentol cyclitol ether (**1l**) and BHhexol cyclitol ether (**1k**) decreasing with depth whereas other structures; BHT (**1a**), adenosylhopane (**1e**), BHT cyclitol ether (**1d**) and BHT pentose (**1n**) all show a rise in the 5-10cm layer and a decrease in the 10-15cm layer. Further structures; 2-Me BHT (**2a**), 2-Me adenosylhopane (**2e**) and adenosylhopane type-1 (**1u**) all increase with depth.

The distribution of the BHPs in these samples was variable with the May 2006 and August 2006 results containing significantly higher levels of BHPs than the December 2006 and March 2007 results. The August 2006 results showed an increase in the 5-10cm layer followed by a significant drop in the 10-15cm layer. This sample also showed a significant drop in the relative levels of aminotriol (**1f**) and BHT cyclitol ether (**1d**) in the 10-15cm layer which was mirrored by a significant increase in the levels of BHT (**1a**) and adenosylhopane type-1 (**1u**). This increase with depth for

adenosylhopane type-1 (**1u**) was also seen in the December and March samples, whereas the changes in aminotriol (**1f**) were much less significant.

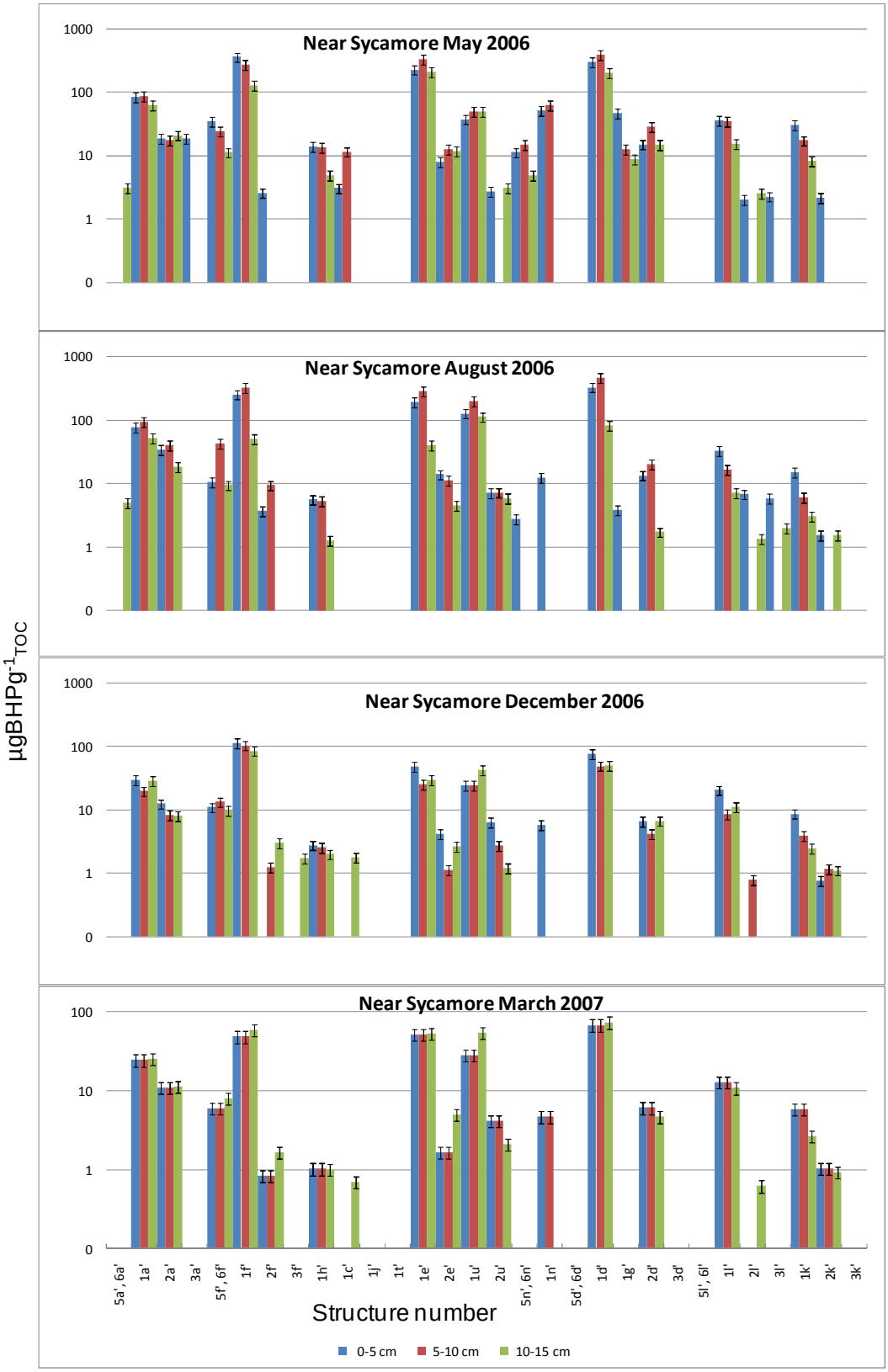


Fig. 4.7 Log distribution of semi-quantitative BHP concentration for Hack Hall, Near Sycamore.

4.5.3.2 Between Sycamores

The levels of TOC in the between sycamore samples are comparable with the other Hack Hall samples and show a decrease with depth (Appendix).

A total of 24 BHPs were identified in the between Sycamores samples (Fig. 4.8), with 21 BHPs in the 0-5 cm layers, 22 in the 5-10 cm layers and 21 in the 10-15cm layers. The BHP distribution was dominated 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**) accounting for between 77.9% (August 2006, 0-5 cm) and 87.5% (March 2007, 0-5 cm) of the total BHP concentration.

There is a decrease in the individual BHP concentrations moving down the soil profile in the 2006 samples except for adenosylhopane type-1 (**1u**) which increases with depth from 26 to 56 $\mu gBHPg^{-1}_{TOC}$ in the May 2006 samples and aminotriol (**1f**) which in the December 2006 samples increases to a maximum in the 5-10 cm layer (Fig 4.8). The March 2007 samples have a much greater variation in BHP concentration with most BHPs having a maximum concentration in the 5-10 cm layer except May 2006 and the 0-5 and 10-15 cm layers having very similar concentrations for BHT (**1a**), unsaturated aminotriol (**5f** or **6f**) and aminotriol (**1f**) all increasing with depth.

Similarly to the near sycamore samples the variation in BHP structures in the between sycamore samples is low with only 2 BHPs being identified in only one sample; hopanelactone (**1t**; March 2007, 10-15 cm) and 3-Me BHpentol cyclitol ether (**1l**; May 2006, 10-15 cm).

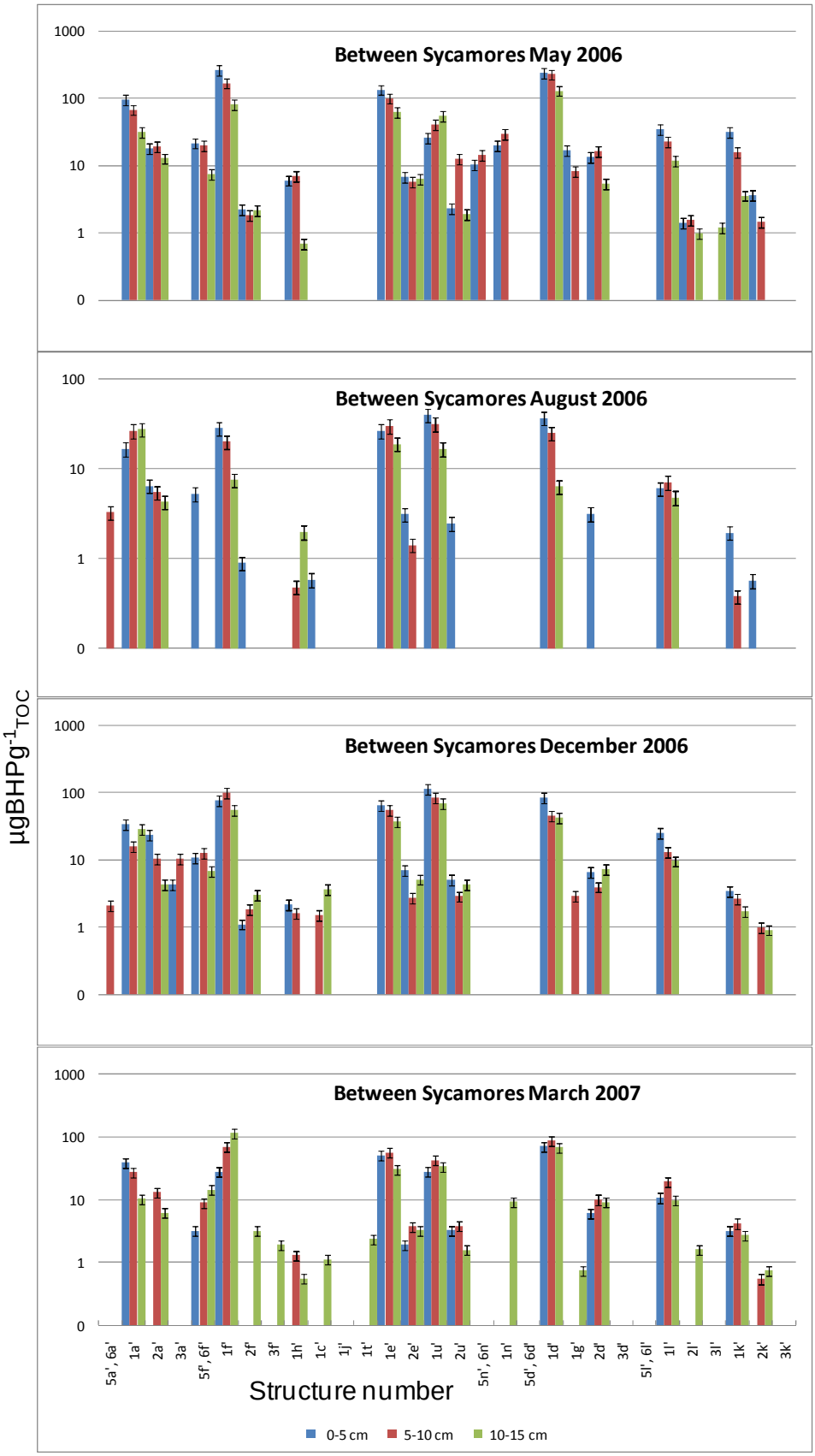


Fig. 4.8 Log dfistribution of semi-quantitative BHP concentration for Hack Hall, Between sycamores.

4.5.4 Seasonal Variation in BHP levels

The overall trend in the levels of BHPs was that there was as general reduction in the BHP concentrations from May 2006 for all the between species samples at all depths (Fig. 4.9).

The near species samples BHP concentration increased in May 2006 and decreased subsequently, however, there was significant variation between months and samples making clear conclusions difficult. The near Scots pine samples total BHP concentration in August 2006 increased dramatically in the 10-15 cm layer to 809 $\mu gBHPg^{-1}_{TOC}$ (Fig. 4.9) but the concentration in 0-5 cm layer only had a small rise and the 5-10 cm layer a slight fall. The 10-15 cm layer then falls in August 2006 and levels off in March 2007. The 0-5 cm layer total concentration falls for the rest of the year and the 5-10 cm layer remains constant. The total concentrations for near common fir at all 3 depths mirror each other at all depths throughout the year (note that the 5-10 cm sample for August 2006 is missing) with a rise in August 2006 followed by a decrease in December 2006 and a small rise in March 2007 (Fig. 4.7). The near sycamore samples have a drop in total concentration in the 0-5 cm and 10-15 cm layers throughout the year but the 5-10 cm layer has a rise in total BHP concentration in August 2006 before falling to levels similar to the other 2 layers in March 2007 (Fig. 4.7).

The between species samples all had similar changes in total BHP concentration (Fig. 4.9) with maximum in May 2006, a fall in August 2006 followed by a small rise in December 2006 and a small fall in March 2007. The general trend is for decreasing BHP concentration with depth with the exception of between Scots pine which has the highest total concentration in the 10-15 cm layer in May 2006, fall in August 2006 and a significant increase in the 10-15 cm layer in December 2006, where it is again the highest total concentration.

The total concentrations for the near species samples are approximately half that observed in the related between species samples with the exception of the sycamore related samples which show the opposite relationship.

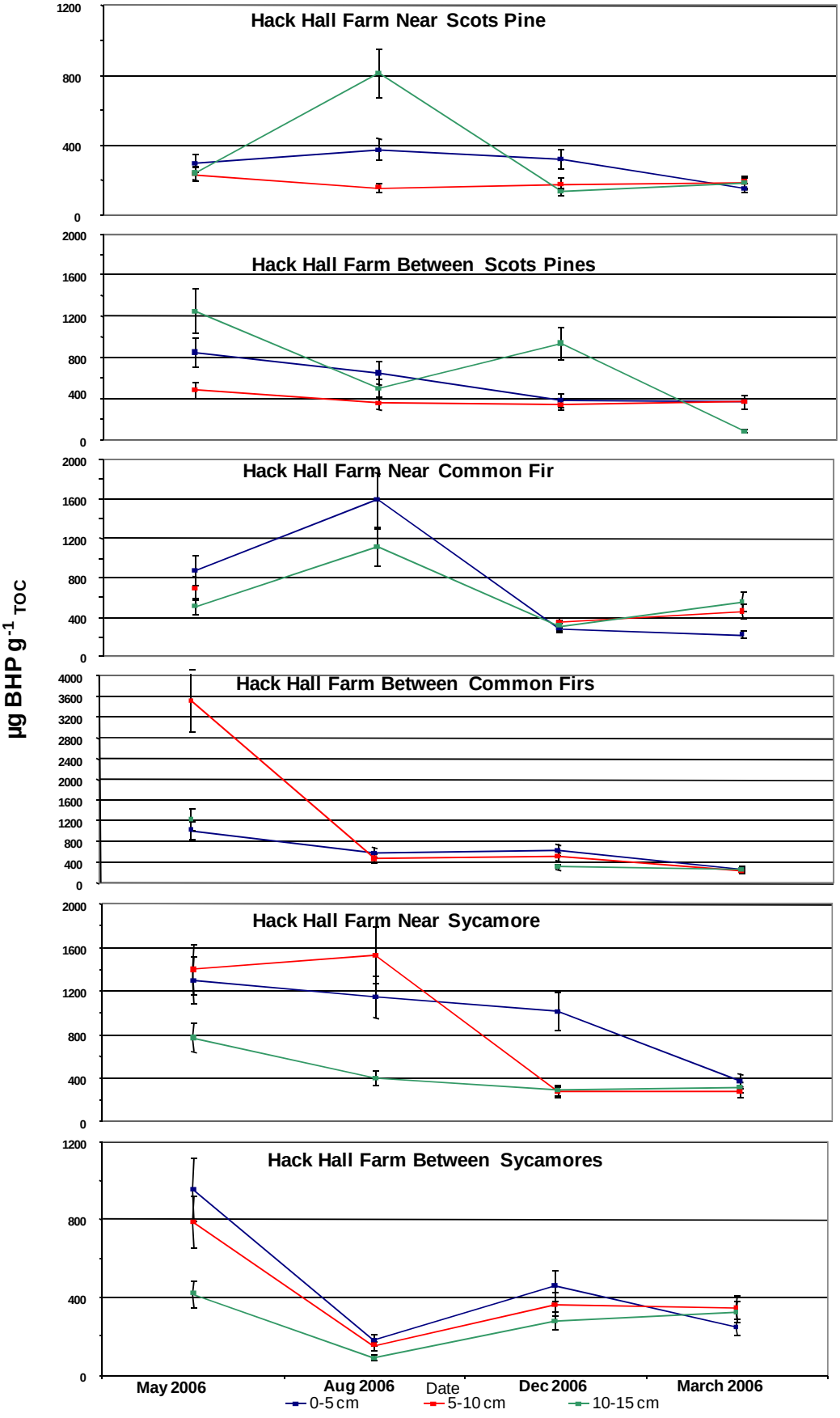


Fig 4.9 Seasonal Variation in BHP total concentration ($\mu gBHPg^{-1}_{TOC}$) for Hack Hall Farm.

The individual BHPs, similarly to the Palace Leas samples, tended to mirror the pattern seen in the total BHP concentration especially in the common fir samples, where all the individual BHPs followed the changes in concentration seen in the total BHP concentration (Fig. 4.9).

In the other locations some BHPs differed from the seasonal changes observed in the total BHP concentration. The near Scots pine samples contained 3 different BHPs whose concentration changes through the year differed from the total BHP concentration. Aminopentol (**1c**) concentration (Fig. 4.10) dropped throughout the year down to trace levels in December 2006 and March 2007 except for December 2006 were the concentration in the 10-15 cm layer increase, but the concentration remained very low.

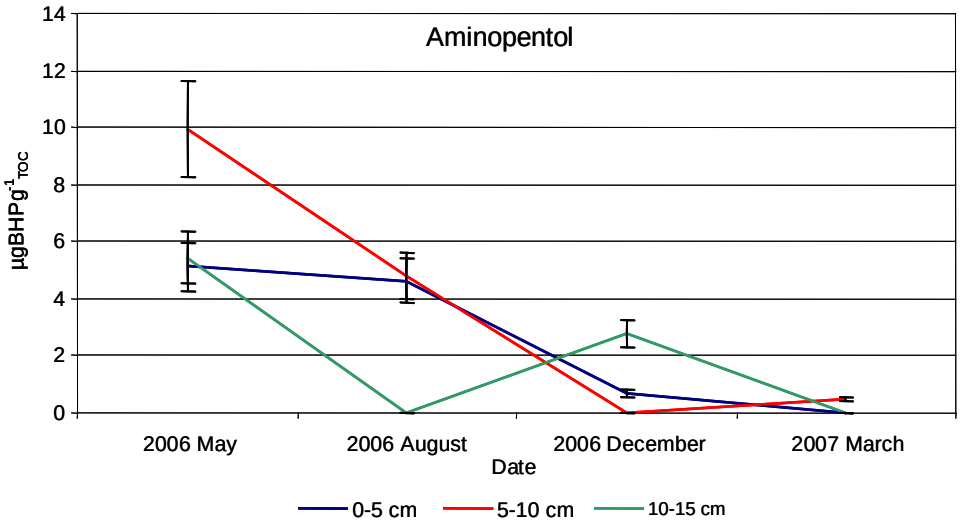


Fig. 4.10 Aminopentol (**1c**) concentration ($\mu gBHPg^{-1}_{TOC}$), near Scots pine

The changes in concentration observed for unsaturated aminotriol (**5f** or **6f**) were an increase in the 5-10 cm layer from May 2006 to December 2006 before a drop to the approximate starting concentration. In the 5-10 cm layer unsaturated aminotriol (**5f** or **6f**) was not detected in August 2006 and then rose in both December 2006 and March 2007 (Fig. 4.11). The 10-15 cm layer had a rise in concentration in August 2006 before a drop in December 2006 and in March 2007 unsaturated aminotriol (**5f** or **6f**) was not detected.

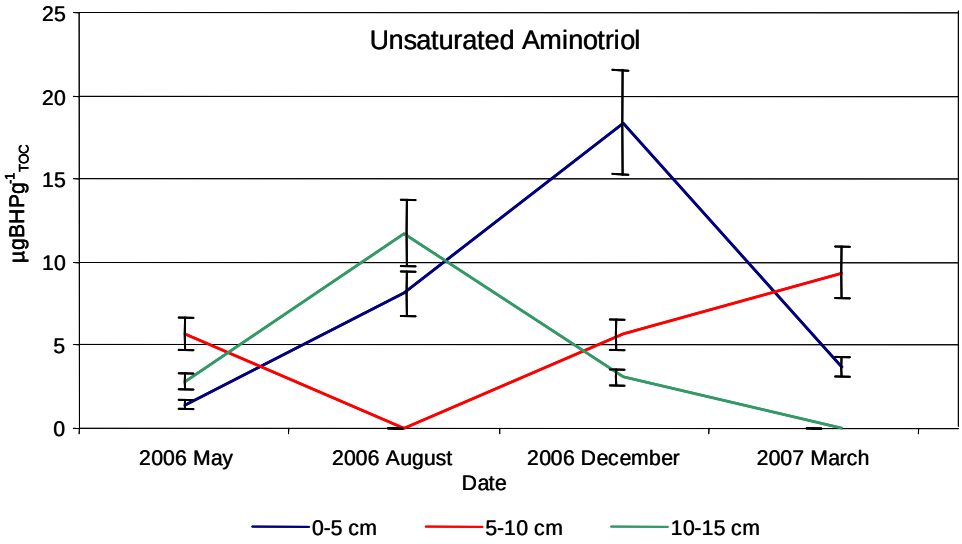


Fig. 4.11 Unsaturated aminotriol (**5f** or **6f**) concentration (µgBHPg$^{-1}_{TOC}$), near Scots pine

The concentration of BHhexol cyclitol ether (**1k**) in all 3 depth layers follow the same pattern with an increase in August followed by decreases in December and March where the concentration returns to the level observed in May 2006 (Fig. 4.12).

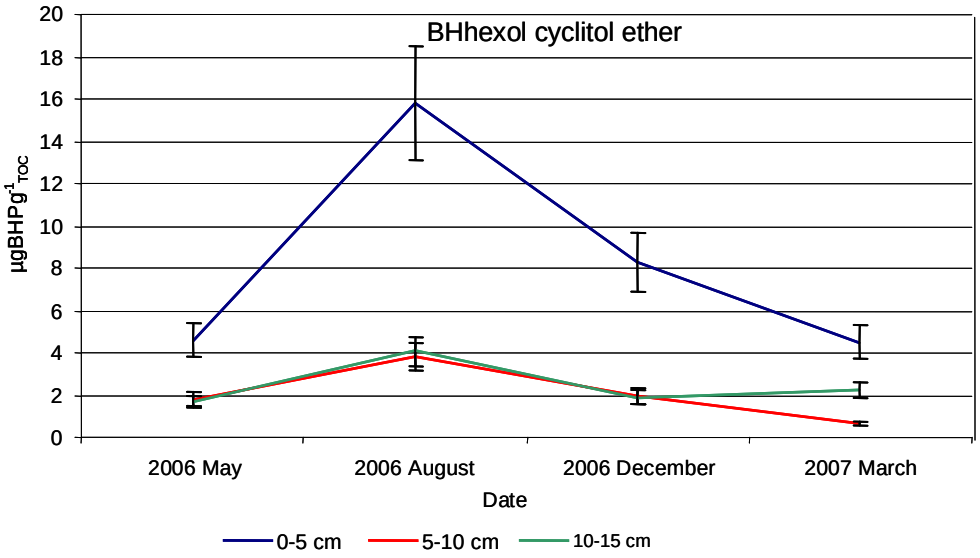


Fig. 4.12 BHhexol cyclitol ether (**1k**) concentration (µgBHPg$^{-1}_{TOC}$), near Scots pine

The between Scots pine samples contained only one BHP whose concentration variation during the year differed significantly from that observed in the total BHP concentration (Fig. 4.7), adenosylhopane type-1 (**1u**; Fig 4.13). The 0-5 cm layer had a slight decrease in concentration over the year punctuated by s slight rise in August.

The 5-10 cm remained constant before a slight rise in concentration in March 2007. The concentration in the 10-15 cm layer was the highest of any depth layer in May 2006 but dropped continually during the year to be the lowest concentration in March 2007.

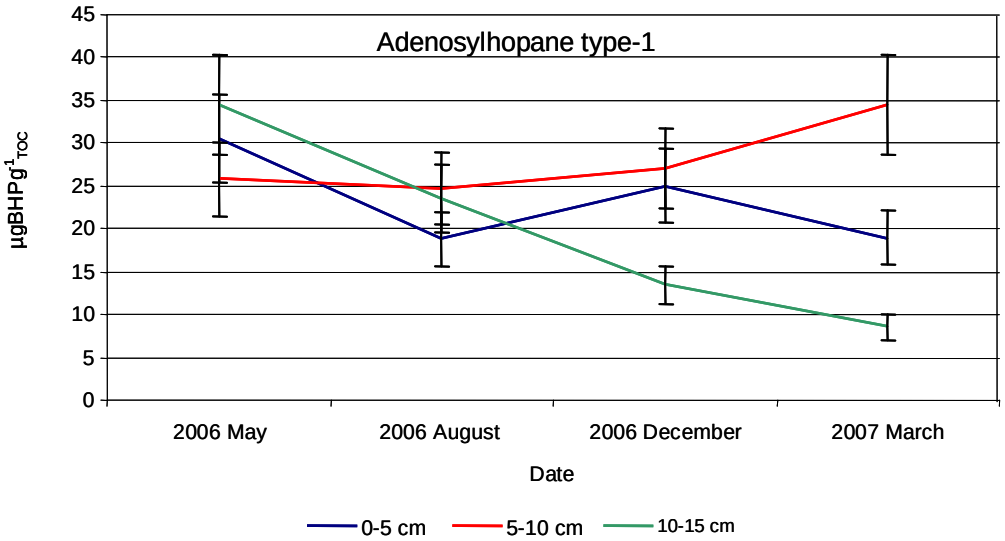


Fig. 4.13 Adenosylhopane type-1 (**1u**) concentration ($\mu gBHPg^{-1}_{TOC}$), between Scots pine

The variation in the aminotetrol (**1h**) concentration in the near sycamore samples (Fig. 4.14) follows the same pattern at all depths, a continual decrease throughout the year with concentration decreasing with depth. This pattern is also repeated for BHhexol cyclitol ether (**1k**; Fig. 4.15)

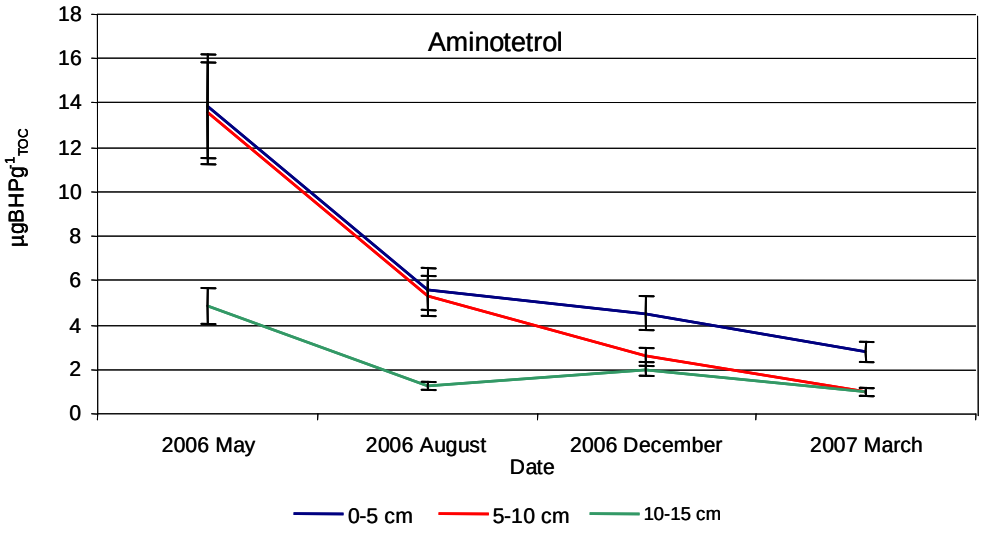


Fig 4.14 Aminotetrol (**1h**) concentration ($\mu gBHPg^{-1}_{TOC}$), near sycamore

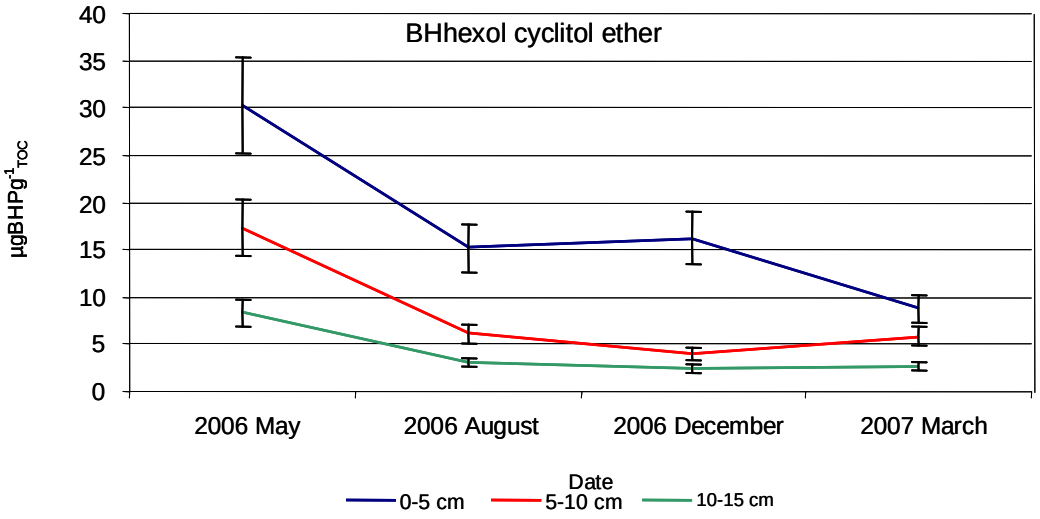


Fig 4.15 BHhexol cyclitol ether (**1k**; concentration (µgBHPg$^{-1}_{TOC}$), near sycamore

BHpentol cyclitol ether (**1l**) concentration in the near sycamore samples (Fig. 4.16) has a similar decrease with depth however the 0-5 cm layer has a concentration increase in December 2006 followed by a sharp drop in March 2007. The 5-10 cm layer has a continual drop to December 2007 before a slight rise in March 2007. The 10-15 cm layer remains relatively constant throughout the year although there is a slight decrease.

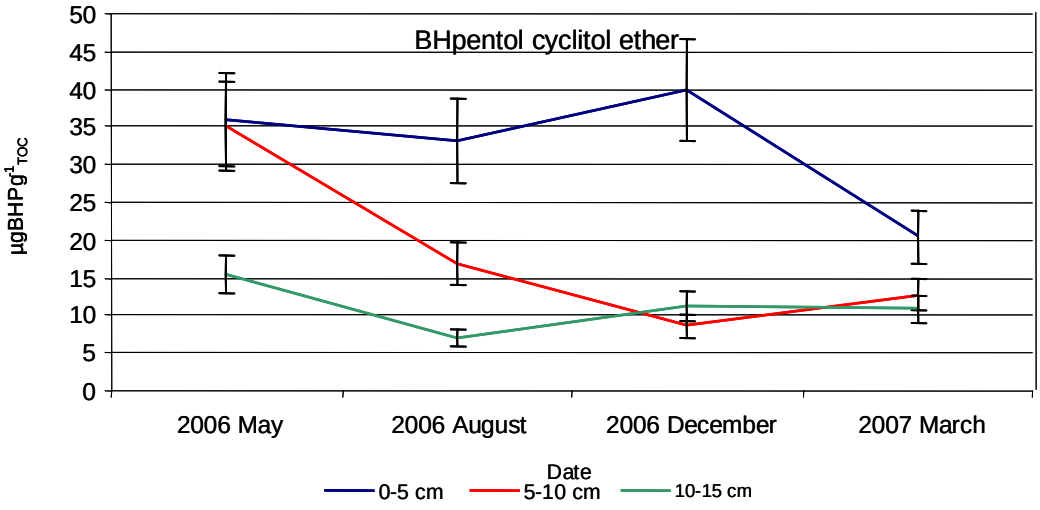


Fig 4.16 BHpentol cyclitol ether (**1l**) concentration (µgBHPg$^{-1}_{TOC}$), near sycamore

4.6 Discussion

4.6.1 Variation in BHP content

The BHP profiles for Hack Hall Farm samples are, like the Palace Leas samples, dominated by 4 or 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**) with up to 20 other BHPs only being minor contributors to the BHP profile (Figs 4.3-4.8). BHT (**1a**) is a more significant contributor to the total BHP contribution in the Hack Hall Farm samples (9% average in the between common fir samples to 12 % average in the near Scots pine samples, Figs 4.6 and 4.3, respectively) compared to the Palace Leas samples (4.4% average in plots 6 and 8 to 6.7% in plot 7, Figs. 3.7, 3.9 and 3.8, respectively). A smaller increase in percentage contribution of adenosylhopane (**1e**) is also observed in the Hack Hall Farm samples (14.7% average in between sycamore samples to 19.5% average in near common fir samples, Figs. 4.7 and 4.5 respectively) compared to Palace Leas (12.5% average in plot 6 and 15.1% average in plot 2, Figs 3.7 and 3.6 respectively). This increase in BHT (**1a**) is mirrored by a decrease in the percentage contribution of aminotriol (**1f**; 22.8% average in between sycamore samples to 28.8% average in near Scots pine samples, Figs. 4.8 and 4.3) relative to the Palace leas samples (28.0% in plot 2 and 34.6% average in plot 7, Figs 3.6 and 3.8).

There is a general reduction in the number of BHPs with depth (Figs.4.3-4.8) but the variation in BHP concentration is much less clear with some sites showing a decrease in depth (e.g. near common fir May 2006, Fig. 4.5), others an increase in BHP concentration with depth (e.g. near common fir March 2007, Fig. 4.5) and further locations a mixed picture with individual BHPs increasing or decreasing with depth (e.g. near Scots pine December 2006, Fig. 4.3).

4.6.2 Seasonal Variations

The analysis of the total BHP content of each location throughout the year is shown in Fig. 4.15 – 4.20. The Palace Leas results (Chapter 3) appeared to indicate an annual cycle of increasing BHP levels in the summer months with a decrease in the winter months. With the Hack hall Farm results the picture is much less clear. None of the samples show a consistent trend over the 12 month sampling period with relative

concentrations of BHPs varying dramatically from quarter to quarter and within the soil profile. The between sycamore samples (Fig 4.20) shows a consistent trend, but this is counterintuitive, with a decrease in the August samples followed by an increase in the December samples. There is an inversion in the levels of BHPs for the March samples that may imply an increase in the lower soil layer due to leaching. All plots show the lowest variation in BHP concentration in the March samples, this convergence of BHP concentrations appears to indicate that leaching has evened out the distribution of the BHPs throughout the soil during the winter months.

It appears that the levels of BHPs identified in the soil are highly influenced by leaching down through the soil and accumulation at the various depths. Any influence due to changes in bacterial population must be tempered with the knowledge that 3 of the 4 dominant BHPs identified in the soil are found in numerous different bacteria. There may be substantial changes in the soil conditions over the year that result in the bacteria modifying the production of specific BHPs but the data does not support any conclusions as to the origin of the potential changes in population, BHP production rates or bacterial activity.

The overall trend in the levels of BHPs was that the December and March samples had lower levels of BHPs than the May and August samples implying an increase in activity and BHP production during the summer months when compared to the winter early spring. There is also an accumulation of BHPs in the lower layers during the winter and early spring indicating a reduction in degradation, probably due to more waterlogged conditions.

In the winter and spring samples there is a relative increase in aminotriol (**1f**) moving down the soil profile indicating an increased relative recalcitrance of this structure compared to other BHPs, or production by bacteria operating at these depths. This trend of increase with depth is also seen in aminopentol (**1c**) where it is believed that the increase is due to production by methanotrophs in the soil.

The August 2006 samples closely resemble the May 2006 sample, although there is a significant increase in the level of BHT cyclitol ether (**1d**) with depth.

However there are no clear trends in the results. The differences could to due to variations in the floral cover or changes in the BHP producing bacteria population, or as a result of the production of different BHPs by the same bacteria in response to changes in the environmental conditions. Without other supporting evidence the

complexity of the differences in the results makes interpretation of the data mere speculation.

4.6.4 BHPs as indicators of bacterial community structure

4.6.4.1 Scots Pine

Using the groups of BHPs identified in Table 2.2 and the same groupings as the Palace Leas plots the distribution of BHP producing bacteria in the near Scots pine is described in Table 4.1. PNSB and nitrogen fixing bacteria dominate the bacterial population, similar to the Palace Leas plots. The contribution of cyanobacteria is low when compared to Palace Leas, probably due to the tree canopy cover.

The methanotroph contribution is between 1.8% and 2.8% which is comparable to that observed in Palace Leas plots 2 and 7 and higher than the other plots, due to the presence of aminopentol (**1c**) in the samples. The peak of methanotroph activity is in the 5-10 cm layer but in plots 2 and 7 of Palace Leas (Tables 3.6 and 3.8) the methanotroph related BHP concentration decreases with depth but for the near Scots pine samples the difference in distribution is either due to preservation and accumulation in the 5-10 layer, differences in the soil properties resulting in this depth being the optimum for aerobic methane oxidation or the influence of the overlying Scots pine on the activity of methanotrophs. The level of aminopentol (**1c**) decreased at all depths during the sampling period (Fig. 4.8) indicating that local changes in the water level of the soil were not affecting the depth of activity of methanotrophs.

The acetic acid bacteria contribution is absent below the top section and methylotroph markers are absent.

The lack of variation with depth for cyanobacterial markers indicates leaching of BHPs down the soil profile. Only acetic acid bacteria and PNSB show any significant variation with depth, with PNSB increasing significantly with depth.

Table 4.1 Distribution of BHP producing bacteria in all near Scots pine samples, identified by presence of BHPs

Bacteria	BHPs	(0-5 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(5 -10 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(10-15 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	183 (63.5)	107 (57.0)	188 (54.5)
Purple non sulphur bacteria (without adenosylhopane and hopanelactone)	627, 712, 746, 761	67 (24.3)	60 (32.0)	114 (34.0)
		25 (4.8)	20 (4.5)	47 (5.8)
N_2 and NH_3 fixing bacteria (without adenosylhopane and hopanelactone)	627,746, 760	45 (16.4)	42 (22.6)	76 (22.8)
		2 (0.7)	2 (1.0)	9 (1.9)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	15 (5.6)	10 (5.2)	24 (5.7)
Methanotrophs	3Me 728, 772, 786, 830, 844	7 (1.8)	6 (2.8)	4 (1.8)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	5 (1.9)	ND (0)	ND (0)
Methylotrophs	1086	ND (0)	ND (0)	ND (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	9 (3.1)	4 (2.0)	4 (2.1)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

The bacterial population and distribution of the between Scots pine samples shows a significant decrease in the levels of PNSB when compared to the near Scots pine samples (Table 4.2). The other bacterial groups are very similar to that seen in the near Scots pine samples.

Table 4.2 Distribution of BHP producing bacteria in all between Scots pine samples, identified by presence of BHPs.

Bacteria	BHPs	(0-5 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(5 -10 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(10-15 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	368 (66.0)	217 (60.7)	481 (68.3)
Purple non sulphur bacteria	627, 712, 746, 761	128 (23.0)	121 (29.8)	135 (20.4)
(without adenosylhopane and hopanelactone)		42 (4.3)	43 (3.4)	45 (3.4)
N_2 and NH_3 fixing bacteria	627,746, 760	90 (15.4)	83 (19.9)	93 (12.7)
(without adenosylhopane and hopanelactone)		5 (0.8)	5 (1.1)	3 (0.4)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	35 (5.4)	20 (5.3)	42 (6.2)
Methanotrophs	3Me 728, 772, 786, 830, 844	6 (1.0)	5 (1.9)	27 (3.9)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	1 (0.1)	1 (0.1)	2 (0.1)
Methylotrophs	1086	ND (0)	ND (0)	ND (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	22 (3.9)	6 (1.3)	6 (1.0)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

The general trend is for the BHPs to reduce in the 5-10 cm layer before a slight increase in the 10-15 cm layer. The soil has a relatively high TOC when compared to the Palace Leas plots and has a poorly aerated appearance. This indicates a low level of oxygen penetration which will result in preservation of BHPs with depth, as seen in the soil profile.

There are 3 major differences between the 2 sets of Scots Pine related samples. The between Scots pine samples have a lower TOC and contain a much higher level of BHPs than the near Scots pine samples. The between Scots pine samples also have a lower level of BHT (**1a**) compared to the near samples and a higher level of aminotriol (**1f**). There is a minor difference in the levels of methanotrophs with the between tree samples showing an increase with depth whereas the near tree samples peak in the 5-10 cm layer. As the main process for distribution of BHPs appears to be

leaching the variations in distribution could be due to differences in the TOC levels or soil physical properties. In both cases these could be affected by proximity to the tree where root exudates and accumulation of leaf litter will differ from those conditions further away from the tree.

Principle Component Analysis of the 2 sets of summary results for Scots pine using the bacterial groupings (Figs 4.17 and 4.18) shows clear distinction in the BHP distribution with depth. Variations in the samples are primarily influenced by levels of methanotroph, PNSB and nitrogen fixing bacteria derived BHPs. There is a significant influence due to levels of BHPs from unknown sources and BHPs with various bacterial origins. The influence of cyanobacteria derived BHPs are minor and confirm the previously noted lack of importance of these BHPs in the BHP profile. Acetic acid bacteria are also important in the 0-5 cm layer only.

There is a degree of overlap between the 2 locations with the 0-5 cm and 5-10 cm layers of both locations clearly overlapping indicating that there is no significant difference between the 2 locations in terms of BHP distribution at these depths. The 10-15 cm layers differ significantly with the near tree samples being significantly more influenced by PNSB and nitrogen fixing bacteria than the between tree samples. This difference could be as a result of Scots pine induced differences or differences in preservation at depth. Overall this analysis indicates that there is little difference in the BHP distribution between the 2 locations and the effect of distance from Scots Pines is an increase in the quantity of BHPs produced moving away from the base of the tree.

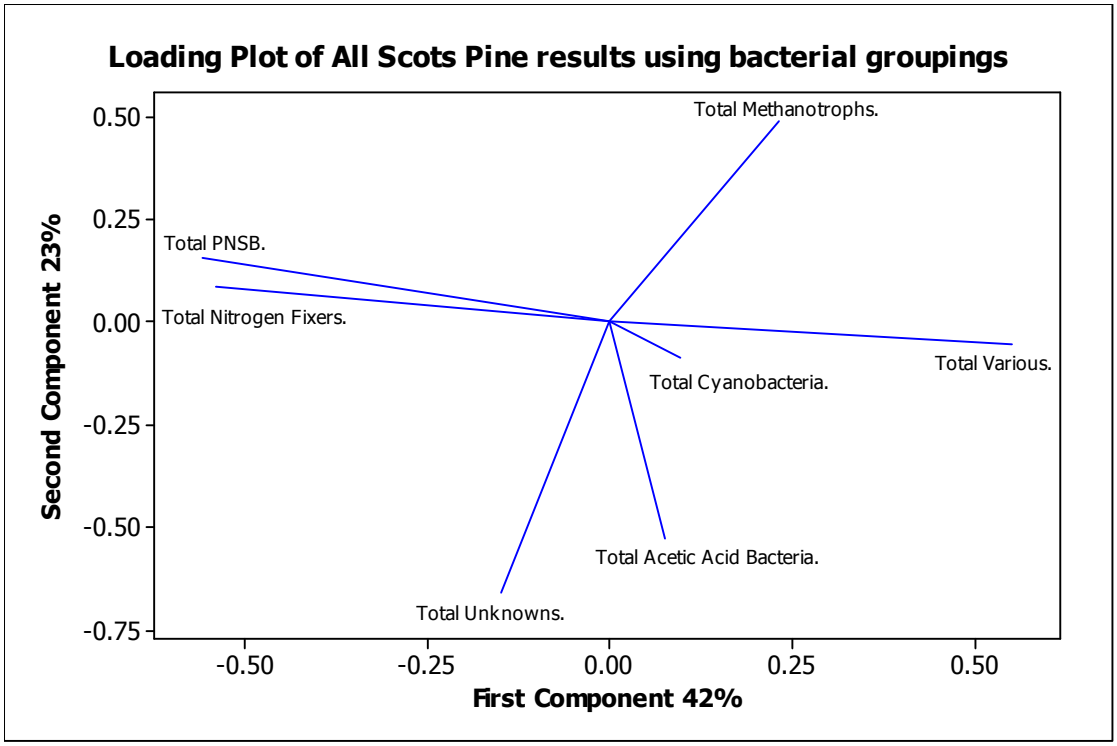


Fig. 4.17. Loading plot of Scots Pine results using bacterial groupings

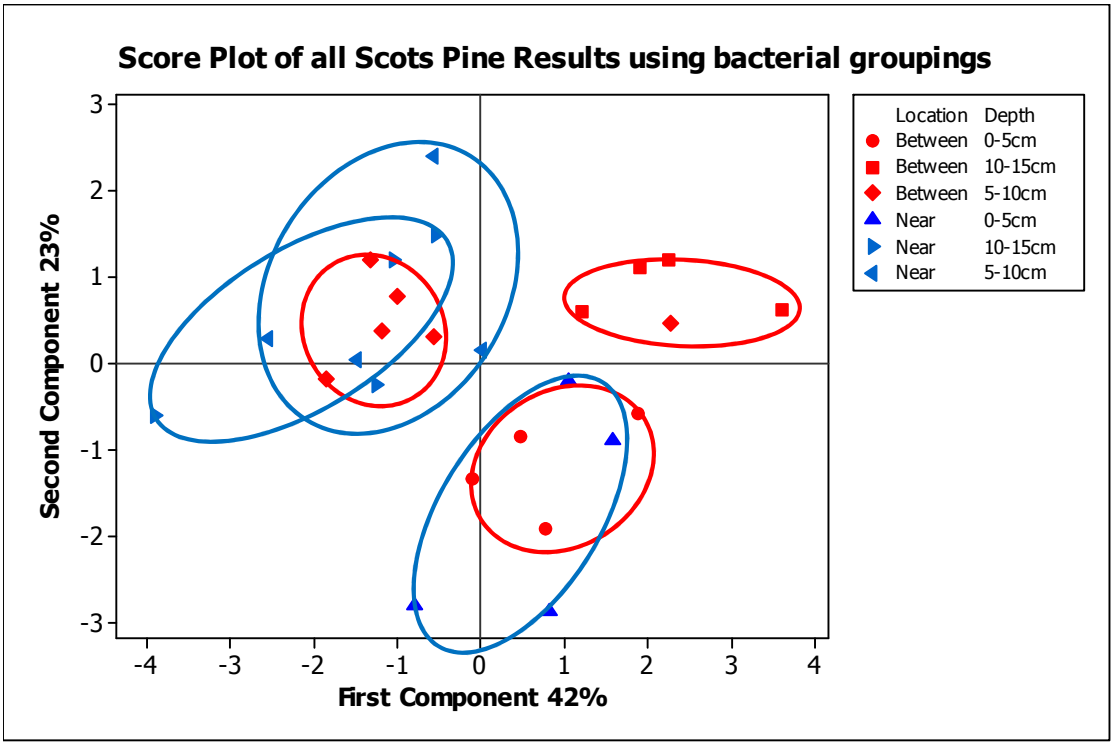


Fig. 4.18 Score Plot of all Scots Pine results using bacterial groupings

4.6.4.2 Common Fir

Analysis of the results using the previously discussed bacterial groupings (Table 4.3) indicates that PNSB and nitrogen fixing bacteria again dominate the soil, as indicated by the presence of adenosylhopane (**1e**). The level of cyanobacteria decreases slightly with depth and is at a similar level to that for the Scots pine samples. There is a significant decrease with depth for acetic acid bacteria. Methanotrophs show an increase to 3% of BHP total in the 10-15 cm layer. These variations indicate that leaching is less significant in these samples than in the Scots pine samples. Therefore the levels of BHPs at each depth may be more indicative of the bacterial activity with depth. However it must be noted that leaching will still be a significant factor in the BHP distribution and any conclusions drawn about the spatial distribution of the bacterial population must be treated with caution.

Analysis of the bacterial groupings (Table 4.4) indicates that the bacterial population is dominated by PNSB and nitrogen fixing bacteria. The levels of cyanobacteria are similar to those found in the near common fir samples and similarly decrease with depth. The biggest difference is in the level of methanotrophs with the between common fir samples containing approximately twice the levels found in the near common fir samples also increasing with depth.

Table 4.3. Distribution of BHP producing bacteria in all near to common fir samples, identified by presence of BHPs.

Bacteria	BHPs	(0-5 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(5 -10 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(10-15 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	446 (66.2)	332 (66.2)	411 (67.4)
Purple non sulphur bacteria	627, 712, 746, 761	130 (20.3)	114 (23.7)	147 (22.6)
(without adenosylhopane and hopanelactone)		34 (6.1)	42 (6.9)	60 (4.6)
N_2 and NH_3 fixing bacteria	627,746, 760	102 (15.2)	75 (15.3)	94 (14.4)
(without adenosylhopane and hopanelactone)		7 (1.0)	4 (0.8)	6 (1.0)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	44 (6.5)	30 (6.1)	29 (4.7)
Methanotrophs	3Me 728, 772, 786, 830, 844	8 (0.9)	5 (0.9)	16 (3.0)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	2 (0.5)	1 (0.1)	1 (0.1)
Methylotrophs	1086	ND (0)	ND (0)	ND (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	30 (4.7)	10 (2.2)	8 (1.3)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

Table 4.4. Distribution of BHP producing bacteria in all between common fir samples, identified by presence of BHPs.

Bacteria	BHPs	(0-5 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(5 -10 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(10-15 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	438 (69.3)	874 (64.0)	423 (66.7)
Purple non sulphur bacteria	627, 712, 746, 761	112 (18.3)	203 (24.2)	104 (20.6)
(without adenosylhopane and hopanelactone)		34 (4.7)	71 (5.0)	47 (3.5)
N_2 and NH_3 fixing bacteria	627,746, 760	82 (12.3)	149 (16.2)	59 (13.1)
(without adenosylhopane and hopanelactone)		5 (0.6)	6 (0.8)	2 (0.8)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	33 (6.1)	44 (4.9)	30 (4.4)
Methanotrophs	3Me 728, 772, 786, 830, 844	10 (1.8)	42 (2.8)	34 (5.5)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	3 (0.5)	1 (0.2)	3 (0.2)
Methylotrophs	1086	ND (0)	ND (0)	ND (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	28 (4.1)	36 (3.4)	11 (2.3)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

Analysis of the common fir results using PCA (Figs 4.19 and 4.20) indicates a clear difference between the different layers in the near and between common fir samples driven by the levels of methanotrophs and cyanobacteria. Other bacterially driven differences are not clear indicating that the samples are very similar with respect to other BHP producing bacteria.

The loadings plot clearly shows that methanotrophs and cyanobacteria have almost opposite influences on the PCA and this is due to their location within the soil depth profile, with a decrease with depth of cyanobacteria and increase with depth of methanotrophs. These results would be expected if leaching was not a significant influence on the distribution of the BHPs. As previously stated, leaching appears to be less significant in this plot than in others, e.g. Scots pine samples. However it cannot

be ignored as the common fir and Scots pine samples were taken less than 20m apart and the soil was visually identical.

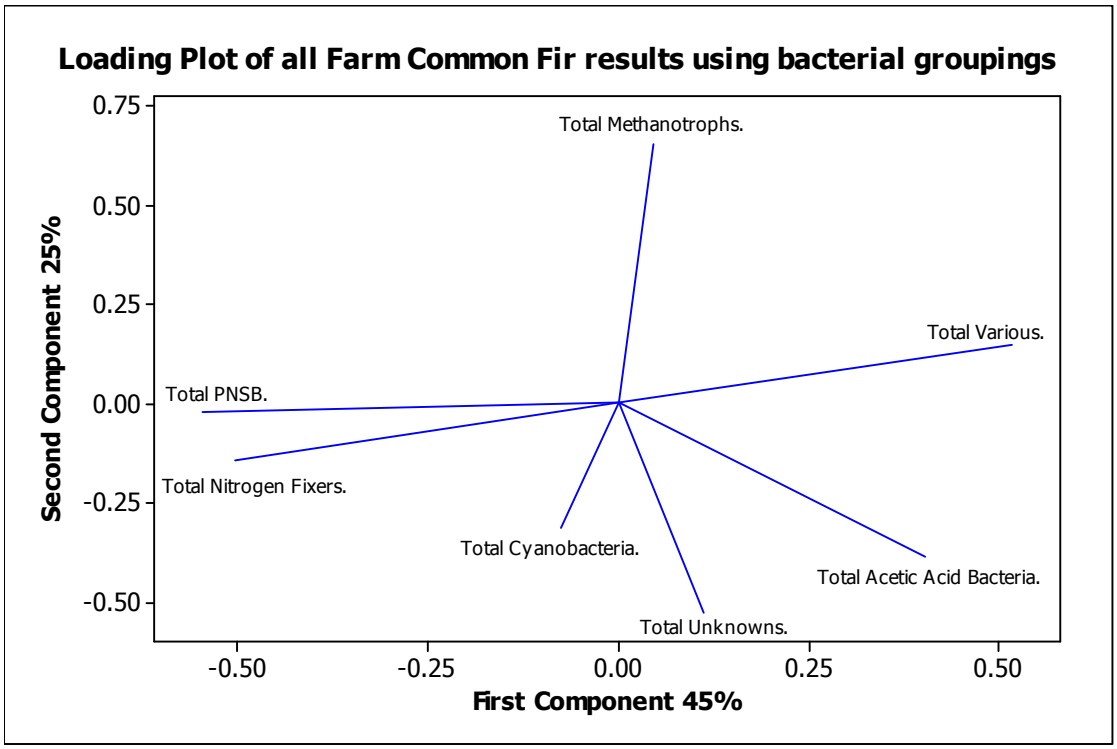


Fig 4.19 Principle Component Analysis Loadings plot for all common fir results using bacterial groupings.

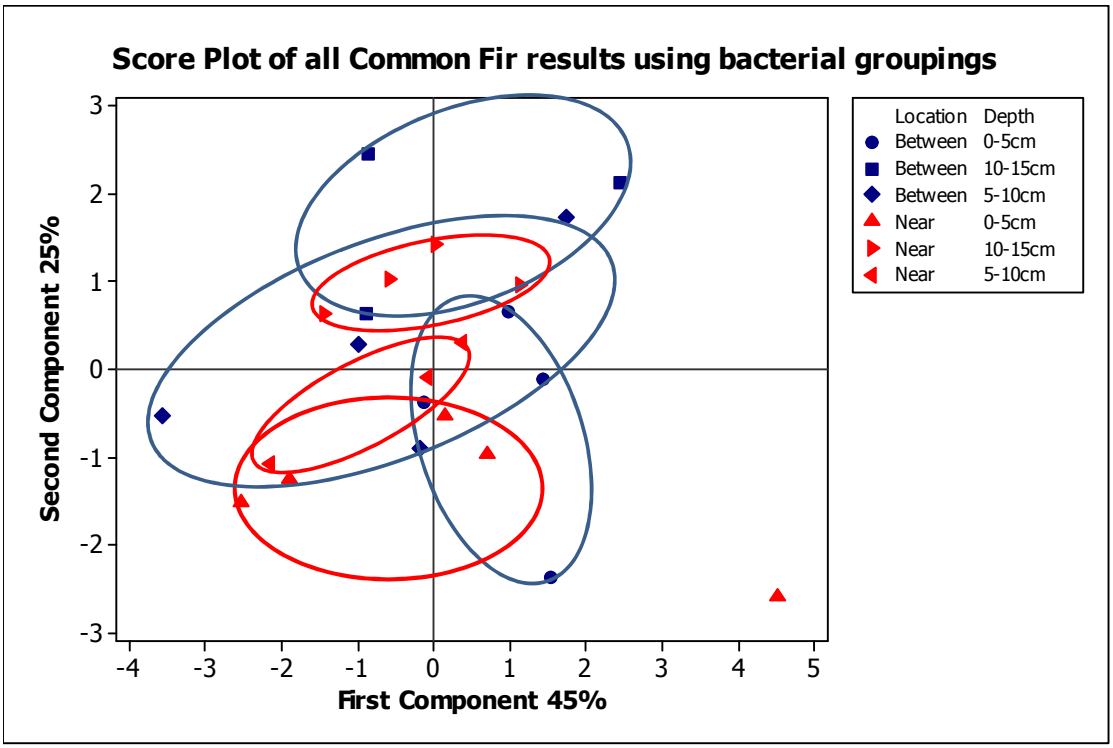


Fig 4.20 Principle Component Analysis Scores plot of all common fir results using bacterial groupings.

4.6.4.3. Sycamore

Analysis of the relationship between the BHPs and source bacteria (Table 4.5) for the near sycamore samples indicates a significant increase in the levels of PNSB derived BHPs with depth, due to the increase in levels of adenosylhopane type-1 (**1u**). There is a slight decrease in the levels of cyanobacteria with depth and the levels in the near sycamore samples are higher than those observed in the Scots pine and common fir samples. The sycamore samples were collected from the edge of the wood where there is much less canopy cover and more direct sunlight. The BHPs results appear indicate this and therefore confirm that these BHPs do relate to cyanobacteria.

The levels of methanotroph derived BHPs are low and remain relatively consistent with depth indicating that methanotroph activity is low in this area of the wood and less important than in the Scots pine and common fir related samples. Acetic acid bacteria activity is low and comparable to the other sites.

The between sycamore samples (Table 4.6) indicate that again the soil is dominated by PNSB and nitrogen / ammonia fixing bacteria, accounting for approximately 35% of the total BHPs. The distribution is uniform with depth indicating that leaching may be a dominant driver for distribution within the soil profile. Cyanobacterially derived BHPs account for approximately 6% of the total and again are uniform throughout the profile. The level of methanotroph source BHPs is low and reaches a maximum in the 10-15 cm layer indicating that methanotrophs are of little importance in this soil but are most abundant in the bottom of the soil profile. Acetic acid bacteria are present in the soil but at low levels. Unusually they are at a maximum in the 5-10 cm layer where they account for 1.4% of the total. Methylotrophs are absent from the samples.

Table 4.5. Distribution of BHP producing bacteria in all near Sycamore samples, identified by presence of BHPs.

Bacteria	BHPs	(0-5 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(5 -10 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(10-15 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	602 (63.0)	509 (59.8)	240 (54.6)
Purple non sulphur bacteria	627, 712, 746, 761	248 (25.5)	272 (30.0)	158 (35.3)
(without adenosylhopane and hopanelactone)		93 (3.8)	97 (2.9)	74 (2.8)
N_2 and NH_3 fixing bacteria	627,746, 760	164 (16.6)	181 (18.4)	90 (17.5)
(without adenosylhopane and hopanelactone)		9 (1.0)	7 (0.7)	6 (1.3)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	69 (7.0)	58 (6.8)	26 (5.8)
Methanotrophs	3Me 728, 772, 786, 830, 844	12 (0.7)	9 (0.9)	3 (0.9)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	8 (0.6)	ND (0)	3 (0.5)
Methylotrophs	1086	ND (0)	ND (0)	ND (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	24 (2.8)	12 (2.3)	8 (1.9)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

Table 4.6. Distribution of BHP producing bacteria in all between sycamore samples, identified by presence of BHPs.

Bacteria	BHPs	(0-5 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(5 -10 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)	(10-15 cm) Ave [BHP] ($\mu gBHPg^{-1}_{TOC}$) (% ave BHP contribution)
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	278 (56.6)	260 (55.7)	161 (56.3)
Purple non sulphur bacteria	627, 712, 746, 761	131 (33.5)	121 (33.7)	89 (34.0)
(without adenosylhopane and hopanelactone)		62 (4.2)	60 (4.2)	52 (3.7)
N_2 and NH_3 fixing bacteria	627,746, 760	74 (17.2)	64 (16.9)	42 (16.0)
(without adenosylhopane and hopanelactone)		5 (1.2)	3 (0.9)	4 (1.1)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	29 (5.5)	33 (6.4)	18 (6.1)
Methanotrophs	3Me 728, 772, 786, 830, 844	3 (0.4)	6 (0.6)	2 (1.2)
Acetic Acid Bacteria	653, 3Me 669, 1058, 3Me 1016, 3Me 1074	1 (0.2)	4 (1.4)	1 (0.1)
Methylotrophs	1086	ND (0)	ND (0)	ND (0)
Unknown	775, 802, 816, 818, 1001, 1014ce, 1014 on sugar, 1016 on sugar, 1118, 2Me 1132, 3Me 1132, 1132 on sugar	14 (2.8)	11 (2.1)	4 (1.5)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

Analysis of the BHP profiles using PCA (Figs 4.21 and 4.22) indicates that there is little difference between the near and between sycamore samples at any depth. The scores plot (Fig. 4.22) shows significant overlap between the various locations making clear delineation between the plots and depth layers impossible. This indicates that Sycamore has no significant influence on the BHP profile with respect to distance from the tree and that leaching has resulted in a relatively uniform distribution of the BHPs. Where previously variations on cyanobacteria and methanotrophs have driven separation of the depth layers, with sycamore these bacteria are less significant, especially methanotrophs, resulting in other more uniformly distributed bacteria, such as PNSB, having a dominant role in defining the bacterial population.

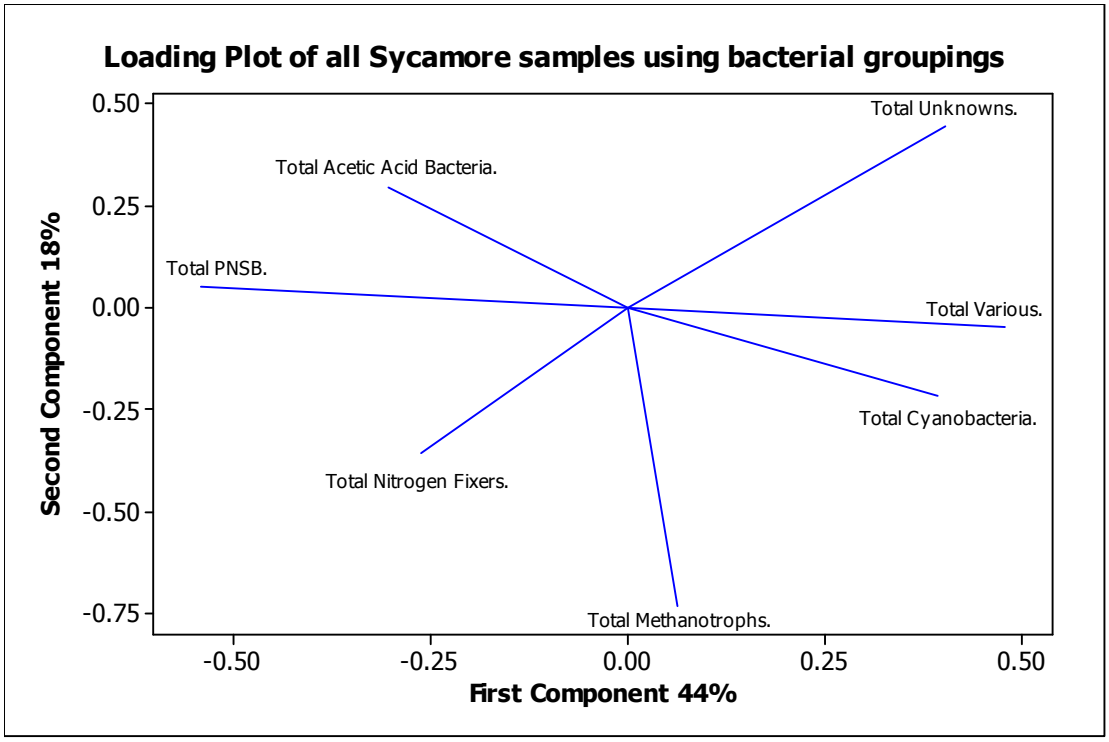


Fig. 4.21 Loading plot of all sycamore samples using bacterial groupings

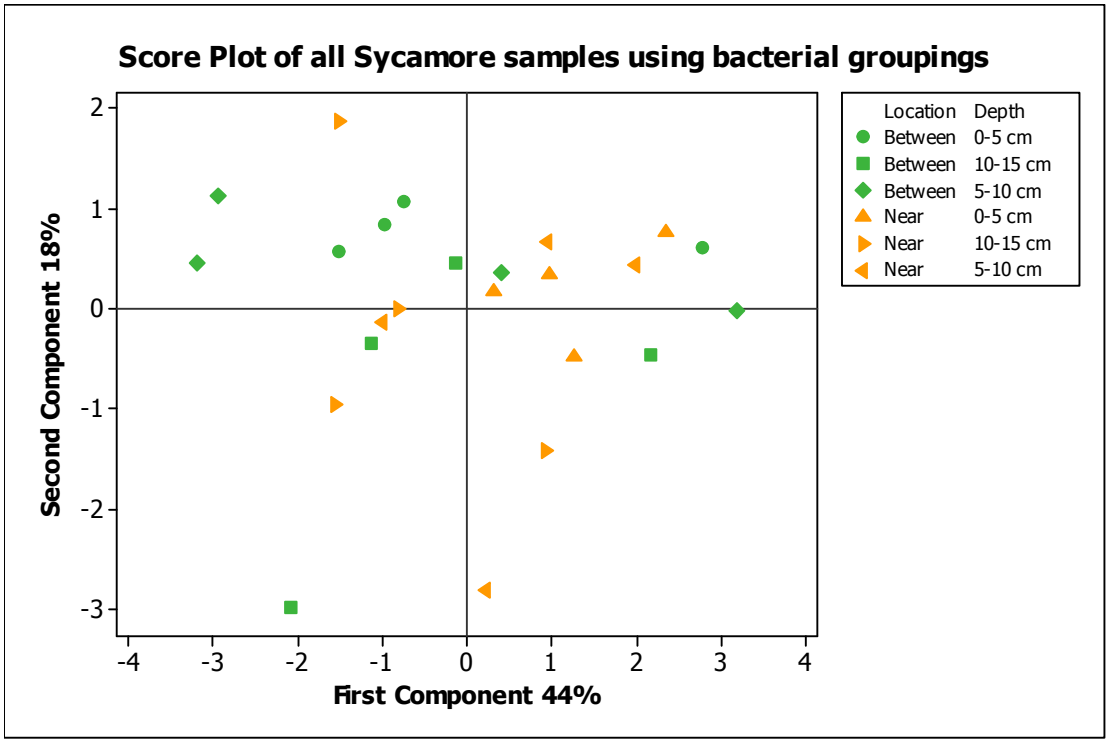


Fig. 4.22. Score plot of all sycamore samples using bacterial groupings.

4.6.4.4 Comparison of all Hack Hall Farm results

All the results obtained from each plot were analysed using PCA to identify any key trends that can distinguish the different locations with Hack Hall and therefore identify any effects caused by the influence of the different tree species.

The loading plot (Fig. 4.23) identified 4 directional influences on the scores plot. Methanotrophs are positive with respect to the first and second components, PNSB and nitrogen and ammonia fixing bacteria are negative with respect to the first component and slightly positive with respect to the second component (both are closely related due to the presence of adenosylhopane (**1e**) which is common to both), cyanobacteria, acetic acid bacteria and unknown source bacteria are all negative with respect to the second component and close to zero for the first component (all are closely related as they are all more common in the 0-5 cm layer than at other depths), and finally BHPs from various sources are positive in the first component and close to zero for the second component. The most obvious trend in the PCA loading plot (Fig. 4.23) is the opposite influences of methanotrophs and cyanobacteria which would be expected to inhabit different depths in the soil, with the cyanobacteria in the near surface and the methanotrophs at a deeper level (White, 2006). The implication of this is that the BHP profile either reflects the current BHP producing bacterial population or there is little translocation of cyanobacterially derived BHPs down the soil profile or that the BHPs are rapidly consumed within the soil.

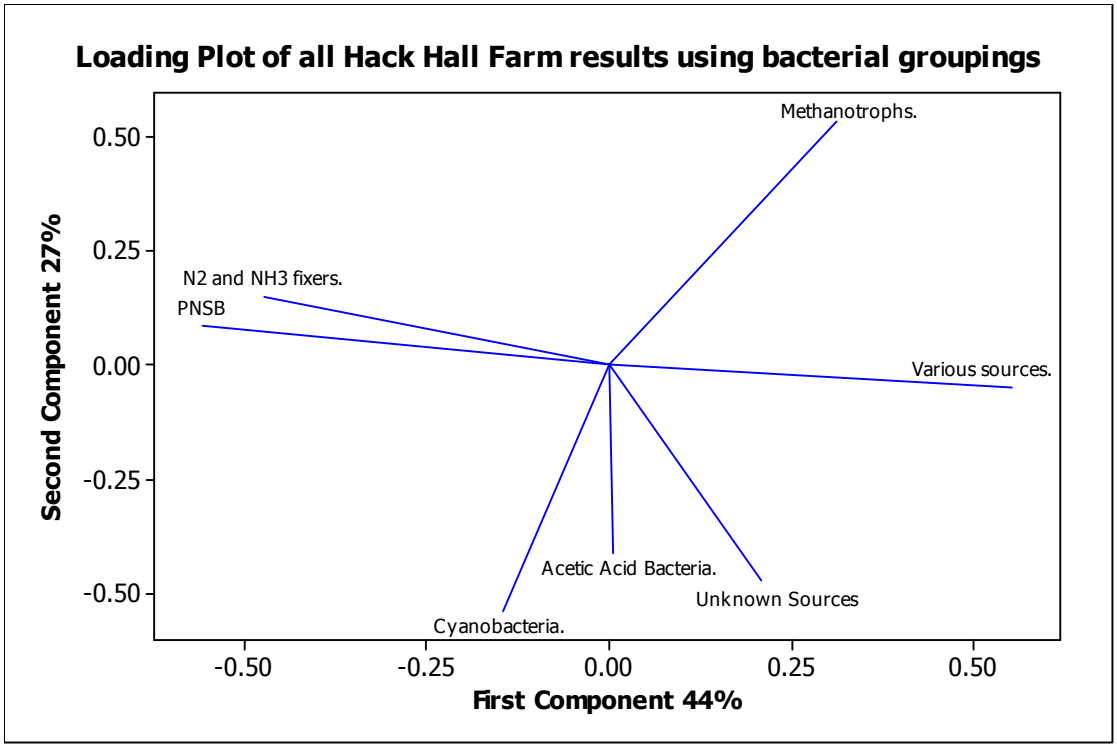


Fig 4.23. Loading plot of all Hack Hall Farm results using bacterial groupings

The scores plot (Fig. 4.24) shows a degree of separation between the 6 sites with the sycamore samples the most negative with respect to the first component, and therefore most influenced by the level of PNSB and nitrogen / ammonia fixing bacteria. The common fir sites are the most positive with respect to the first component and therefore the least influenced by PNSB and nitrogen / ammonia fixing bacteria. The Scots pine samples occupy the middle region. There is considerable overlap between the Scots pine and the sycamore and common fir but no overlap between sycamore and common fir. This separation can only be due to the influence the different tree species or the different position of the samples within the wood.

The sycamore samples are located at the edge of the wood and are a stand of deciduous trees. Therefore this location will receive the most sunlight. This is observed in the thicker grass cover in this area and can be seen by the position of the sycamore samples in the PCA scores plot (Fig. 4.22) which implies a degree of influence from cyanobacterially derived BHPs.

Both the Scots pine and the common fir samples were taken from the centre of the wood where the canopy is complete all year round and has much thinner grass cover. Again the lack of influence of cyanobacteria in these samples can be seen by their locations on Fig 4.24.

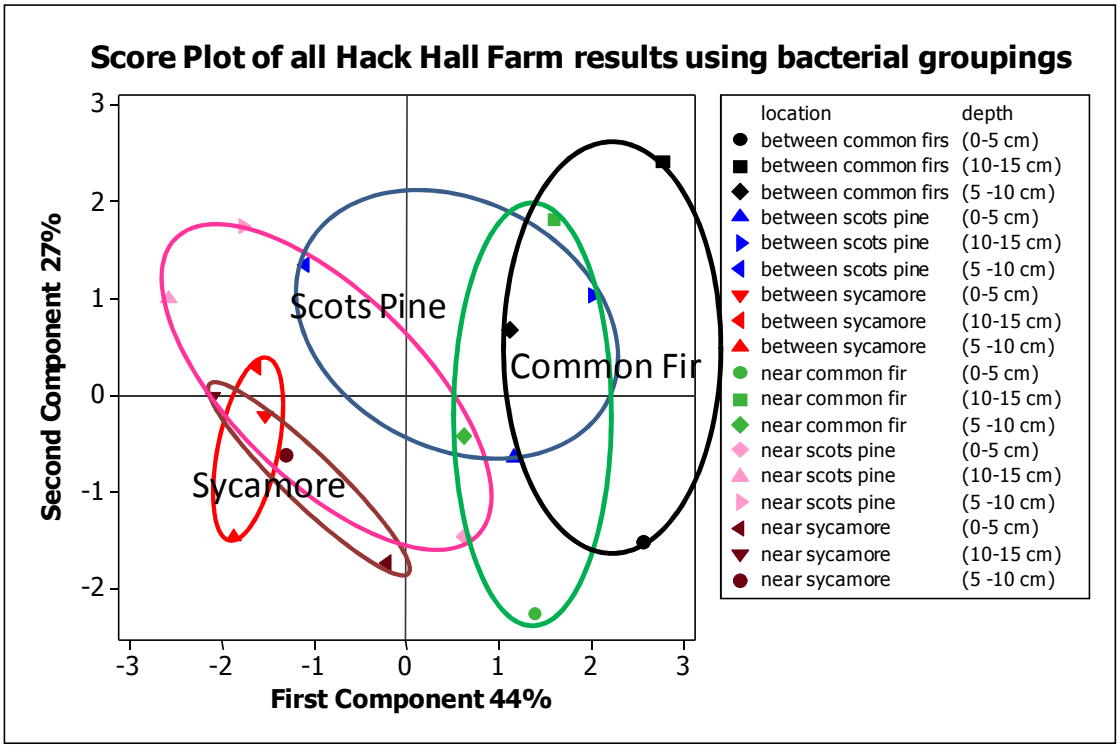


Fig. 4.24 Scores Plot for all Hack Hall Farm results using bacterial groupings.

However the differences in the BHPs results cannot simply be explained by the presence of all year round canopy as there is a clear difference in the BHP profiles between the near and between tree samples. In the case of both Scots pine and common fir the near samples are both more influenced by PNSB and nitrogen / ammonia fixing bacteria than the between samples, which themselves are more influenced by methanotroph signals (Fig. 4.23). This clearly indicates that distance from the tree is having an effect on the bacterial population of the surrounding soil.

With respect to the depth of the sample there is a clear trend that the deeper 10-15cm layers are clearly influenced by the presence of methanotroph derived BHPs (Fig 4.23) for both the Scots pine and common fir samples in both the near and between samples. The implication of this is that the bacterial processes at depth include significant methanotroph activity. With the common fir samples there is also an obvious cyanobacterial influence in the 0-5cm layer.

4.7 Summary

Goal 5

Identify the BHP profile in the soil under different tree species

Using the PCA analysis it is clear that there are differences in the soil BHP producing bacterial (Fig. 4.23) due to the type of associated tree species. These differences are driven primarily by the proportion of methanotrophs and cyanobacteria (Fig. 4.24). The sycamore soil contains a higher proportion of cyanobacteria than the other soils. This is likely to be due to the position of the sycamores on the perimeter of the wood with greater accessibility to direct sunlight. The common fir and Scots pine samples were taken from the centre of the wood where there is complete canopy cover all year round.

The common fir samples contained the highest level of methanotroph related BHPs, with the highest proportion being identified in the 10-15 cm layer. The implication of this is that common fir may promote the activity of methanotrophs in its associated soil when compared with other trees. These results are contrary to the evidence that hardwood species, e.g. sycamore, consumed more methane, and therefore have a higher methanotrophs population, that softwood species, e.g. Scots pine and common fir (Menyailo, 2002, 2003). The implication of this is that the soil conditions and degree of canopy cover may be more significant than tree type when defining the microbial population.

Both the between Scots pine and between common fir samples showed a significant increase in the level of BHPs detected when compared with the near species samples. This indicates that either there is more bacterial activity away from the base of the tree or a greater degree of preservation.

There is a general trend of increasing proportion of methanotroph derived BHPs with depth and a general decrease with cyanobacterial BHPs with depth, in agreement with the expected distribution of methanotrophs and cyanobacteria within the soil. Overall no clear distinction could be drawn in the BHP distribution between any of the near and between tree samples for each species (Figs.4.10, 4.12 and 4.14) with only the Scots pine samples showing a difference with depth (Fig 4.10) for both 0-5 cm sets of results for Scots pine which were significantly different to the other Scots pine

samples indicating that in addition to changes in bacterial population, such as the decrease in cyanobacteria and the increase in methanotrophs, leaching and preservation act to homogenise the BHP distribution in the soil.

The analysis of the distribution of BHPs between the near and between tree samples (Figs.4.3 – 4.8) shows no clear trend in the variation in total BHP concentration, number of BHP structures or in the relative proportions of tetra, penta and hexafunctionalised BHPs. Using PCA (Fig. 4.16) there is a general trend of the near tree samples being slightly more influenced by PNSB and N_2 and NH_3 fixing bacteria but this trend is subtle and may not be significant.

Overall from this data it is clear that there is an influence on the BHP producing bacterial population by the overlying floral species either directly or indirectly by variations in canopy cover for example.This relationship is masked to a degree by the influence of soil properties, leaching and BHP preservation.

Goal 4

Identify any temporal differences in the BHP profile.

The seasonal variations indicate that there are greater levels of BHPs during the summer months than the winter months, mirroring the expected bacterial activity. The separation of methanotroph and cyanobacteria derived BHPs (Fig. 4.15) appears to indicate that the BHP profile reflects the current BHP producing bacterial activity as significant leaching and preservation of these BHPs would reduce the separation of these groups of BHPs on the PCA. However, the results are not clear and require further investigation.

4.8 Conclusions

The general BHP profile is very similar to that seen in Palace Leas (Chapter 3) with similar total BHP concentrations, approximately 20 different BHPs being identified in each sample and the BHP profile being dominated by 4 or 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**), although BHT (**1a**) has a relatively higher concentration in the Hack Hall

samples than in the Palace Leas samples (Figs 4.3 to 4.8). There is a decrease in the number of BHP structures observed with depth but there is no clear trend in the BHP concentration with depth (Figs 4.3 to 4.8).

The seasonal variation in BHP concentrations (Fig. 4.9) indicates a general decrease in the December and March with an increase in the BHP concentration at the lower depths during these months indicating preservation and a reduction in the bacterial activity in the upper layers during winter. There are significant variations in the concentrations depending on the distance from the tree. All the between tree samples show a fall in BHP concentration in August 2006 and a further decrease in the subsequent winter months whereas the near tree samples show a great variation in BHP concentration in the August 2006 samples followed by falls in the following months (Fig. 4.9). This could be related to changes in moisture content in the soils further from the tree during the hot summer months but there were no observed differences in the soil which was generally black and moist at all sample locations and during all time points.

The PCA using bacterial groupings (Figs. 4.15 and 4.16) shows that the 0-5 cm layers of all samples are more strongly influenced by cyanobacteria than the 10-15 cm layers which are more strongly influenced by the presence of methanotrophs. This is in agreement with the expected distribution of these bacteria in the soil and it appears to confirm that the relationship between source bacteria and specific BHPs is robust. The sycamore samples are more strongly influenced by cyanobacteria than the other samples as would be expected from the increased sunlight that these samples will receive. There are differences between the BHP profiles that enable a separation to be observed between the different tree species with the common fir samples being most strongly influenced by methanotrophs and the sycamore samples influenced by cyanobacteria (Fig. 4.24).

5. Landfill Cover Soils

5.1 Introduction

Landfills are an important method of solid waste disposal and a specific set of chemical and biological processes occur to breakdown the putrescible waste (e.g. Christensen et al., 1989). The degradation of land filled waste occurs in 4 distinct phases; (1) aerobic hydrolysis where microorganisms convert some carbohydrates to simple sugars, such as glucose, and to carbon dioxide and water, (2) hydrolysis and fermentation where lipids, carbohydrates and proteins are broken down and fermented yielding volatile acids, acetate, carbon dioxide, hydrogen and inorganic salts, (3) acetogenesis where bacteria convert long chain soluble acids to carbon dioxide and hydrogen before subsequent transformation with carbon dioxide into acetic acid and (4) methanogenesis where bacteria convert acetic acid to methane and carbon dioxide. This final process, if uncontrolled, can release large quantities of methane into the atmosphere with landfills accounting for up to 10% of global anthropogenic methane production (IPCC Climate Change 2001: the scientific basis). To control the release of contaminants and gases from a landfill site a compacted soil cover is placed over the landfill. These covers consist of a simple soil cover in older sites or clay cap and soil cover up to 2m thick placed over the landfill. In more modern landfills a geotextile such a high density polyethylene (HDPE) is also used to improve the quality of the cover. The generated CH_4 gas is collected and either burnt in situ or collected and used to produce electricity.

5.2 Site Background Information

Four different sites were investigated to assess the BHP content of their cover soils. Three of these sites were inactive and had been for more than 30 years, but were being actively monitored for gas production by North Tyneside Council. These sites are all located to the north and east of Newcastle, (Chicken Road, West Allotment and Octavia Court) (Fig 5.1).

The site at Octavia Court (grid reference: NZ293676) was a landfill until 1968 (Desk Study Report Q655B; 2003, North Tyneside Council) after which time it was covered

over and monitoring wells subsequently installed. Gas monitoring in 2003 recorded CH_4 concentrations of between 12.5% and 40.0% v/v and CO_2 concentrations of 20% v/v (Report Q655 G2; 2004, North Tyneside Council). The site is an area of flat ground covered with grass and surrounded by housing (Fig. 5.3).

The site at West Allotment (grid reference NZ310701) was a landfill until 1980 (Desk Study Report 3047/1; 2002, Bellway Homes North East Ltd) and is a raised area of steep sided land covered with grass. There are houses to the immediate North (Fig. 5.2). Gas monitoring in 2004 indicated elevated CH_4 levels of up to 11% (v/v) and CO_2 concentrations up to 12% v/v (Report Q655G2; 2004, North Tyneside Council).

The site at Chicken Road (grid reference: NZ314675) was abandoned before 1970 but the exact date is unknown (Report Q655G2; 2004, North Tyneside Council). The area is grass covered and slopes to the North. The site is flanked by houses to the North, East and West (Fig. 5.2). Gas monitoring indicated trace levels of CH_4 but the CO_2 levels are up to 5.5% (v/v) (Report Q655G2; 2004, North Tyneside Council).

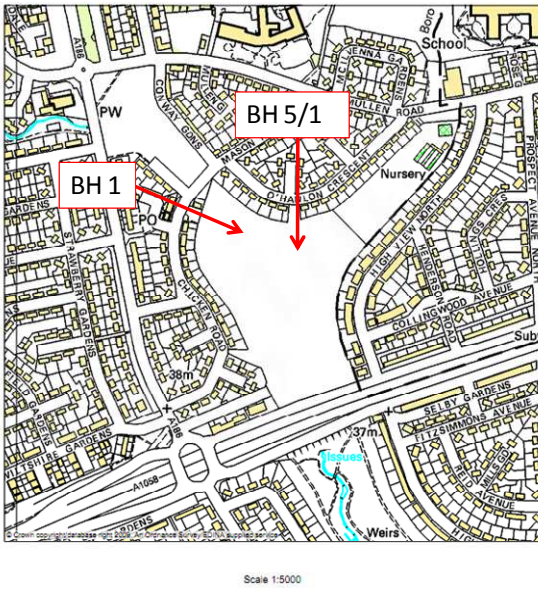
The fourth site was an active landfill site, managed by Sita UK, at Seghill, near Newcastle (grid reference: NZ294743) (Figs 5.2 and 5.4). The Seghill site has been in use since the late 1950s and it is currently used as a disposal point for approximately 26% of Northumberland municipal waste including residual household waste (www.sita.co.uk). Household waste only accounts for a small proportion of the overall waste input into the Seghill landfill site with the majority of the waste being commercial and industrial waste from across the region. No specific gas data was obtained from the site but there is active production of CH_4 and CO_2. The landfill is capped with clay and a HDPE liner above which there is approximately 1 m of cover soil, with extensive grass growth in the sampling areas (Fig. 5.4).

Landfill Sampling Sites

Scale 1:10,000

Fig. 5.1 Location Map for landfill sampling sites

Chicken Road



West Allotment

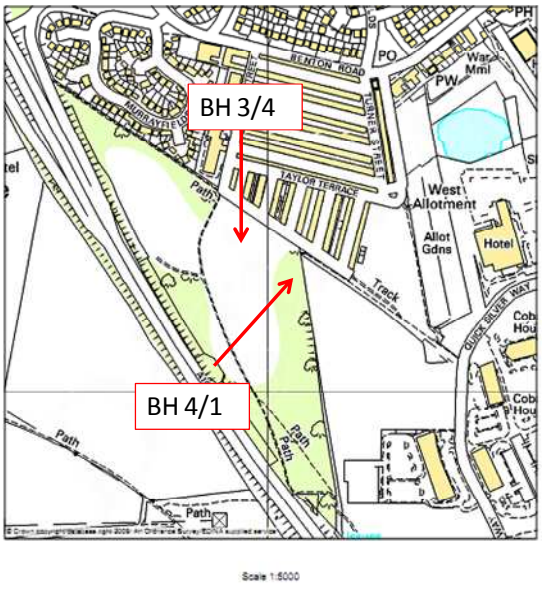


Fig 5.2 Sampling locations for Chicken Road and West allotment landfill sites

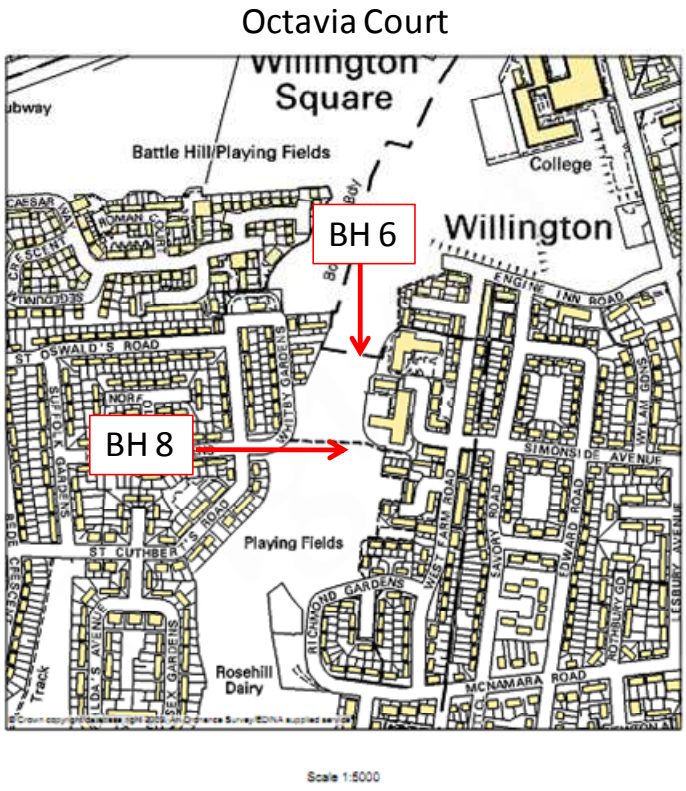


Fig 5.3 Sampling locations for Octavia Court Landfill Site

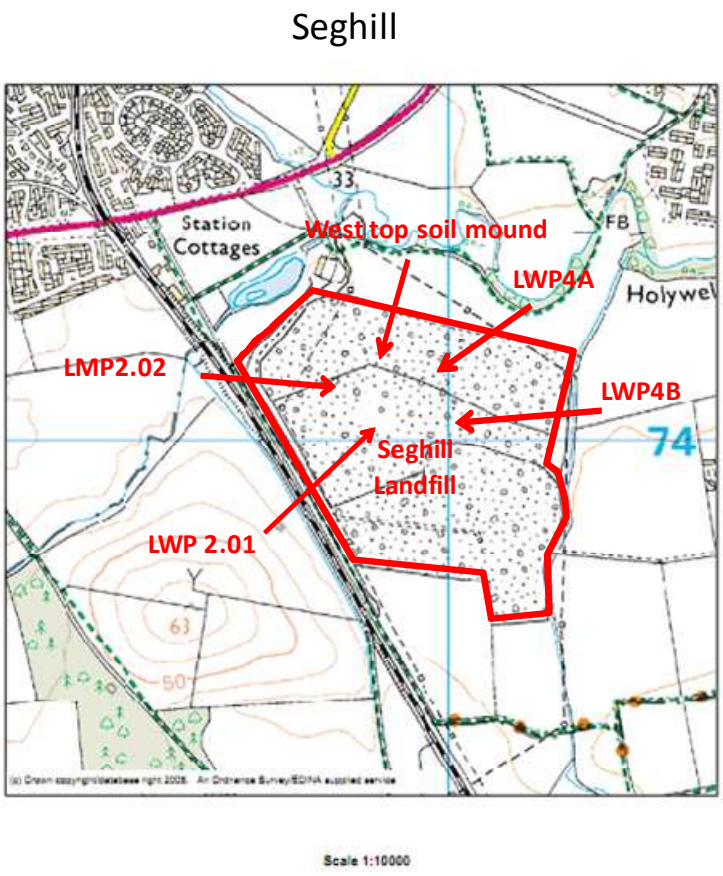


Fig 5.4 Location map for Seghill Landfill sampling points.

5.3 Goals

The goal of this phase of the research was to test the ability of BHP analysis to identify specific bacterial activity and therefore to test hypothesis 1;

The BHP fingerprint of a soil indicates the current bacterial population.

Goal 6

Identify the presence of methanotrophs and ammonia oxidising bacteria in the BHP profile.

Landfill cover soils have been shown to have a high methane oxidising capacity due to the presence of methane oxidising bacteria (e.g. Whalen et al., 1990, Mandernack et al 2000, Crossman et al., 2004, Lee et al., 2009). Previous work has identified that cover soils contained both type I and type II methanotrophs, with type II dominating.
The landfill sites study investigates the influence of methane production by landfills on the bacterial populations in the overlying cover soils by looking for the presence of aerobic methane oxidising bacteria and their characteristic BHP biomarkers aminotetrol (**1h**; type I and II, e.g. Neunlist and Rohmer, 1985a; Cvejic et al., 2000) and aminopentol (**1c**; Type I, e.g. Neunlist and Rohmer, 1985a; b; Cvejic et al., 2000; Talbot et al., 2001).
Yu et al., (2009) investigated the activity of ammonia oxidising bacteria in landfill cover soils and found that *Nitrosomonas europaea* type species dominated in cover soils without a HDPE liner and that *Nitrospira* type ammonia oxidising bacteria dominated in sites with a HDPE liner. The differences are believed to be related to lower N_2O flux from site lined with HPDE.
Elevated concentrations of ammonia oxidising bacteria would be expected to be present in the landfill cover soils (Yu et al., 2009) and will be identified by the presence of the BHP markers for *Nitrosomonas europaea* ammonia oxidising bacteria, i.e. hopane lactone (**1t**) and adenosylhopane (**1e**). The sites at Chicken Road, West Allotment and Octavia Court are not expected to be capped with a HDPE liner, due to the dates that the sites were capped and therefore the soils above these would

be expected to contain the highest levels of these ammonia oxidising bacteria markers. The site at Seghill is covered with a HDPE liner and would be expected to have *Nitrospira* linked ammonia oxidising bacteria (Yu et al., 2009). No *Nitrospira* have been tested for BHPs at this time, so the BHP profile, if any, is as yet unknown.

5.4 Sampling Methodology

Samples were collected in June 2006 from the Chicken Road, West Allotment and Octavia Court sites. Samples from Seghill were collected in July 2007. All samples were collected using the methodology described in section 2.1. The weather was hot and dry and the ground conditions were very dry with no rain for the previous month. This resulted in soils that were hard and dry with a uniform grey colour. This made collection difficult and in some cases only 10 cm depth was achieved.
The soil at Chicken Road and Octavia Court contained large amount of brick rubble and other building detritus whereas these materials were absent in the soil at Seghill and West Allotment. The samples were identified by the closest water or gas monitoring point.

5.5 Results

5.5.1 Chicken Road

The results from Chicken Road (Fig. 5.5) show a low concentration of BHPs when compared to the Palace Leas (Chapter 3) and Hack Hall Farm (Chapter 4) samples with a maximum concentration in BH1 of 224 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ (0-5 cm, Fig 5.5) decreasing down to 92 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 5-10 cm layer (no 10-15 cm layer was obtained for BH1). The concentration in BH5/1 (Fig 5.5) is 113 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 0-5 cm layer and decreasing to 40 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 10-15 cm layer. The number of BHPs identified was also lower than in the Palace Leas and Hack Hall Farm samples with 12 in the 0-5 cm layer of BH1, 13 in the 5-10 cm layer and 11 in the 10-15 cm layer. In BH5/1 15 BHPs were identified in the 0-5 cm layer and 11 in the 5-10 cm layer. There is a general decrease in both BHP concentration and number of structures with depth (Fig. 5.5).

The TOC in both cores was relatively high, 8.2% in BH5/1 0-5 cm, 7.8% in 5-10 cm and 8.0% in 10-15 cm whereas in BH1 the TOC was 6.9% in 0-5 cm and 4.6% in 5-10 cm layer. These results are comparable with the other sites investigated.

Both sets of results are dominated by 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**), similar to other soils investigated. Both aminotriol (**1f**) and BHT cyclitol ether (**1d**) show very significant decreases below the 0-5 cm layer. The concentrations of all 5 major BHPs in the 0-5 cm layer of BH1 are much higher than in the 0-5 cm layer of BH5/1 with an approximate 505 increase in the concentrations.

All other BHPs are minor constituents of the total BHP concentration and contain similar BHPs to those seen in other soils (Chapters 2 and 3). Most noticeable is the very low level of BHhexol cyclitol ether (**1k**) in these samples when compared to the other soils investigated. Guanidine substituted BHT (**1s**) is also present whereas it is absent in the Hack Hall Farm samples (Chapter 4) and rarely observed in Palace Leas (Chapter 3).

Both 2-Me BHT (**2a**) and 2-Me BHT cyclitol ether (**2d**) were present as minor components and decreased with depth.

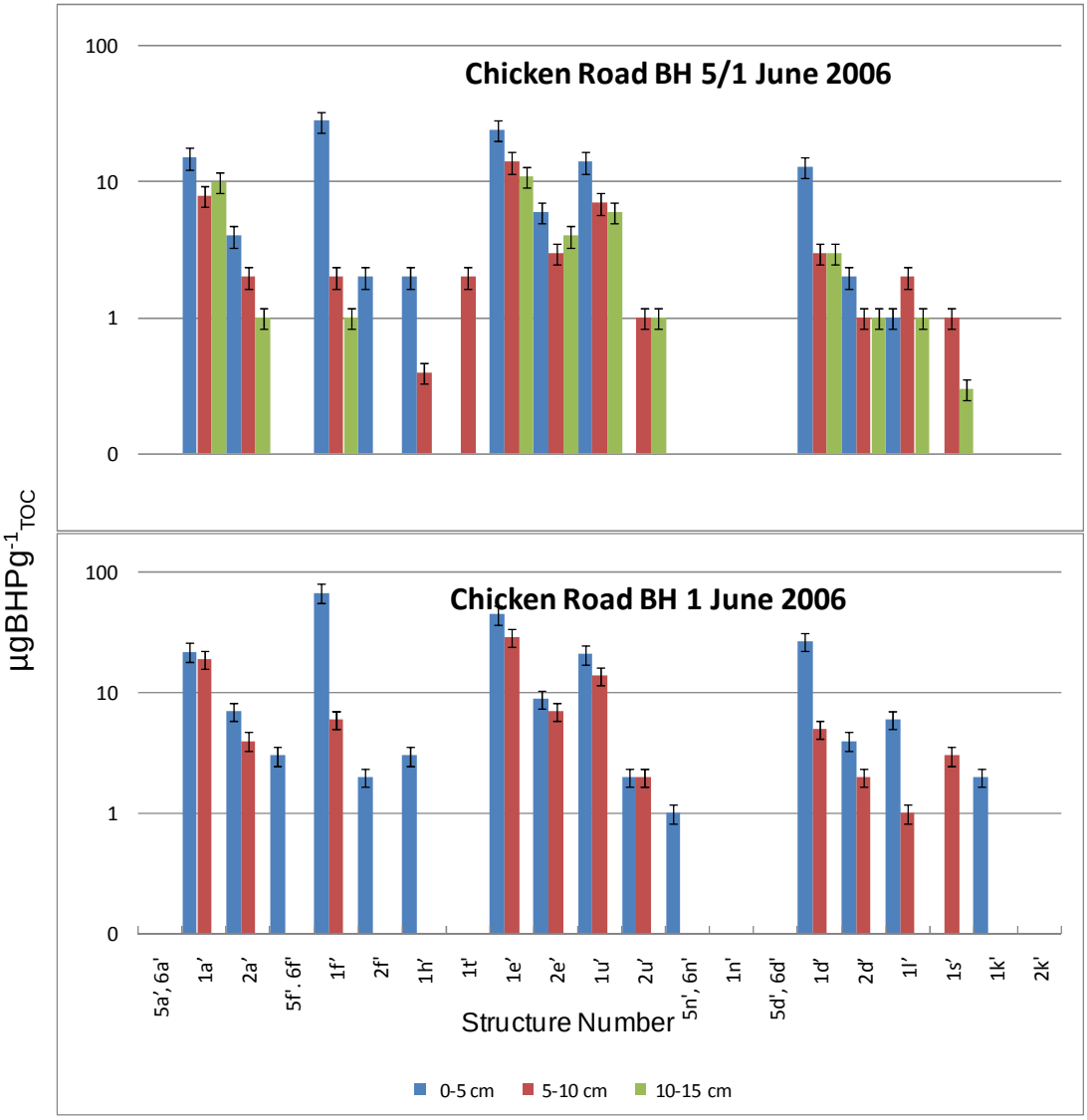


Fig. 5.5 Log graph of semi-quantitative BHP concentration for ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) Chicken Road BH5/1, June 2006.

5.5.2 West Allotment

The results for the West Allotment samples are similar to those observed in the Chicken Road samples. The total BHP concentration is very low with 71 $\mu g_{BHP} g^{-1}{}_{TOC}$ in BH3/4, 0-5 cm (Fig. 5.6) and 66 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in BH3/4, 5-10 cm. BH4/1 contained at total concentration of 64 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in 0-5 cm layer and 33 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in 5-10 cm layer (Fig. 5.6). In both cases no 10-15 cm layer was obtained. The total number of BHPs identified was also very low, 9 in BH3/4 0-5 cm layer and 6 in the 5-10 cm layer. BH4/1 contained 10 different BHPs in 0-5 cm and 11 in the 5-10 cm layer.

BH 3/4 was dominated by the same 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**). All decreased with depth except aminotriol (**1f**) and BHT cyclitol ether (**1d**) which increased significantly in the 5-10 cm layer. Adenosylhopane type-1 (**1u**) was absent in the 5-10 cm layer. Both depth layers had very high concentrations of BHT (**1a**) when compared to the other BHPs.

Again BHhexol cyclitol ether (**1k**) and 2-Me BHhexol cyclitol ether (**2k**) were absent from these samples but guanidine substituted BHT (**1s**) was present in the 0-5 cm layer.

The TOC was similar to that observed in the Chicken Road samples (6.3% in 0-5 cm and 7.6% in 5-10 cm). The TOC in BH4/1 was again comparable with the other samples, 8.9 in 0-5 cm and 9.0 in 5-10 cm.

BH4/1 had an almost identical BHP distribution although the concentrations of aminotriol (**1f**) and BHT cyclitol ether (**1d**) were much lower. These BHPs also increased again with depth. Adenosylhopane type-1 (**1u**) was present in the 5-10 cm layer unlike BH3/4.

2-Me BHT (**2a**) decreased with depth but 2-Me BHT cyclitol ether (**2d**) was only present in the 5-10 cm layer.

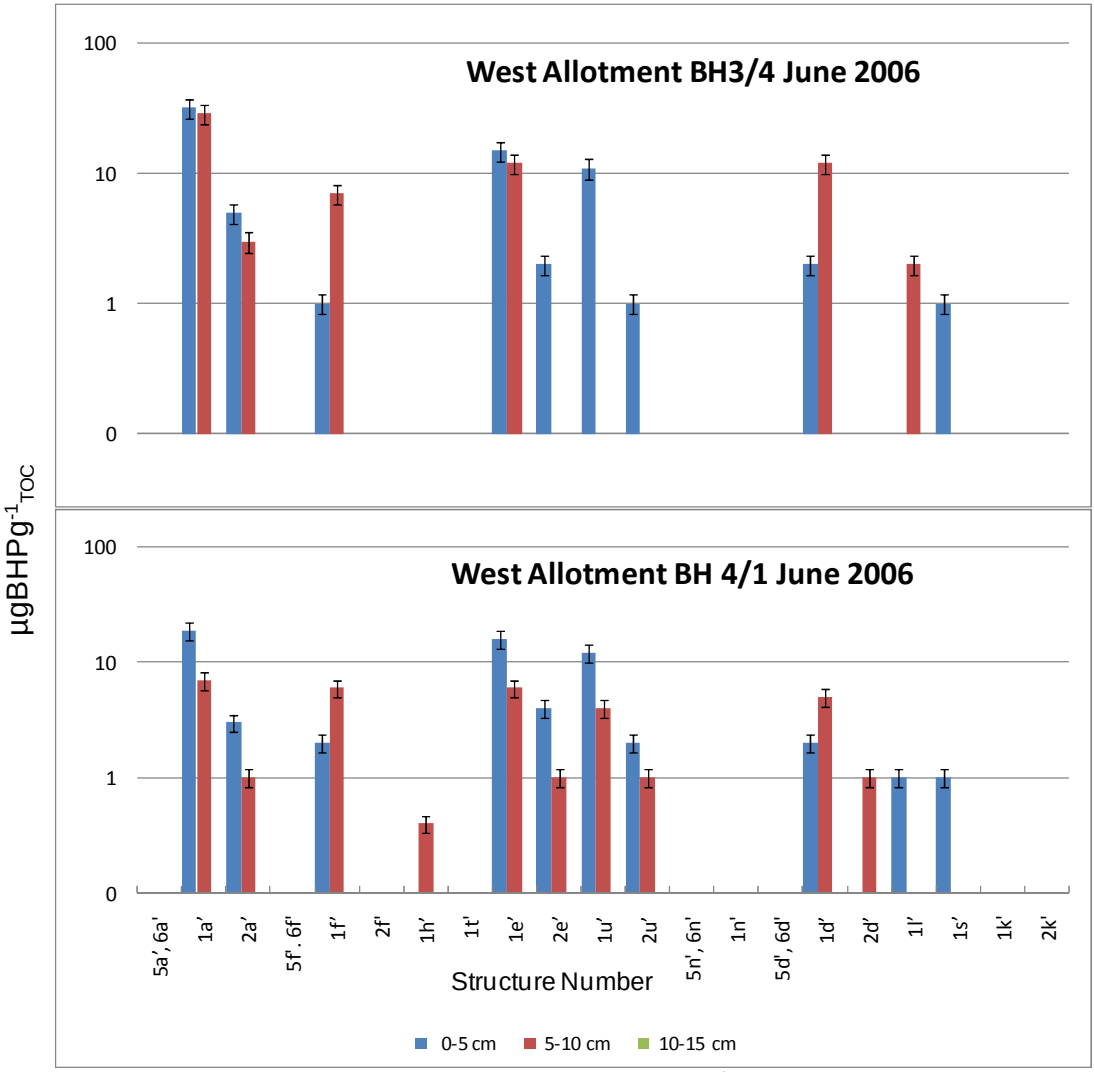


Fig. 5.6 Log graph of semi-quantitative BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) for West Allotment BH3/4, June 2006.

5.5.3 Octavia Court

The results from Octavia Court (Fig. 5.7), as with the other landfill sites, showed a very low BHP concentration; BH8 0-5 cm 77 $\mu g_{BHP}\ g^{-1}{}_{TOC}$, falling to 22 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 5-10 cm layer. The number of BHPs identified was low with 8 in the 0-5 cm layer and only 4 in the 5-10 cm layer. In BH6 an opposite trend was observed with the BHP concentration increasing from 75 $\mu g_{BHP}\ g^{-1}{}_{TOC}$, 0-5 cm, to 124 $\mu g_{BHP}\ g^{-1}{}_{TOC}$, 5-10 cm layer and number of BHPs identified increasing from 11 (0-5 cm) to 13 (5-10 cm). In both cases a 10-15 cm layer was not obtained. The TOC values were comparable to the other landfill samples, BH8; 7.6% (0-5 cm) and 5.0% (5-10 cm), BH6; 9.6% (0-5 cm) and 7.0% (5-10 cm).

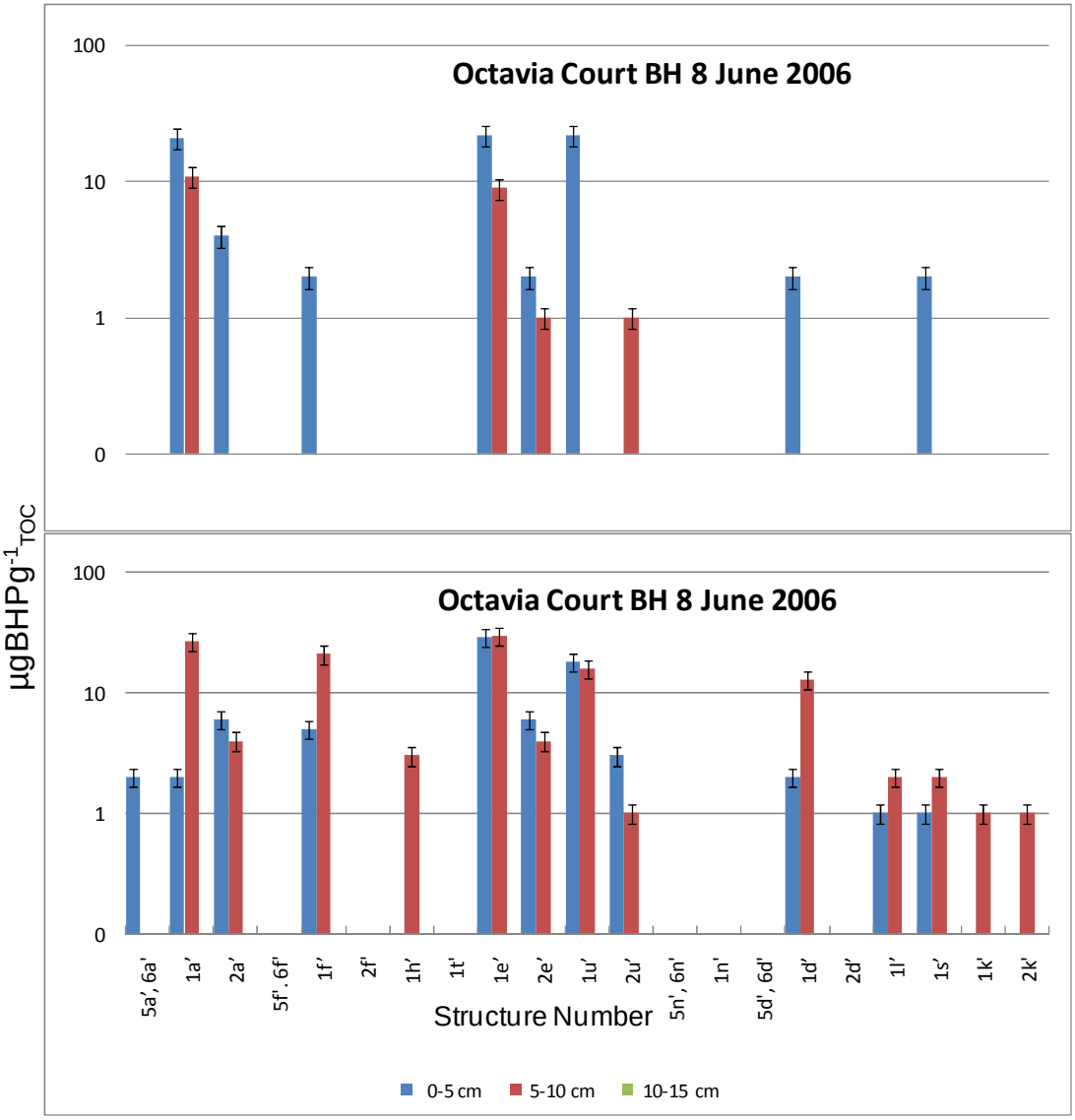


Fig 5.7 Log graph of semi-quantitative BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) for Octavia Court BH8, June 2006.

The BH8 results were dominated by 3 BHPs; BHT (**1a**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**) with the concentrations falling rapidly below the 0-5 cm layer and adenosylhopane type-1 (**1u**) being absent in the 5-10 cm layer.

BH6 was dominated by 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**) and contained a much more diverse group of BHPs than BH8. The concentrations of BHT (**1a**), aminotriol (**1f**) and BHT cyclitol ether (**1d**) all increased significantly in the 5-10 cm layer whereas the concentration of adenosylhopane (**1e**), 2-Me adenosylhopane (**2e**) and adenosylhopane type-1 (**1u**) remained consistent. The other BHPs showed different trends with depth with aminotetrol (**1h**), BHpentol cyclitol ether (**1l**), guanidine

substituted BHT (**1s**), BHhexol cyclitol ether (**1k**) and 2-Me BHhexol cyclitol ether (**2k**) all increasing with depth, whereas the other minor BHPs decreased with depth.

5.5.4 Seghill

The results from the Seghill are again similar to the other landfill sites with a low BHP concentration and a low number of BHP structures identified (Fig. 5.8). The TOC from the Seghill sites is much lower than that seen in the other landfill sites with a maximum of 4.7% in the 10-15 cm layer of LW4A and a minimum of 1.3% in LWP4B. There is a general decrease in TOC with depth in the samples (Appendix).

Core LW4A has a total BHP concentration that decreased with depth from 103 μg_{BHP} $g^{-1}{}_{TOC}$ in the 0-5 cm layer to 87 μg_{BHP} $g^{-1}{}_{TOC}$ in the 5-10 cm layer and 42 μg_{BHP} $g^{-1}{}_{TOC}$ in the 10-15 cm layer (Fig. 5.8). The number of BHP structures identified is 9 (0-5 cm layer), 10 (5-10 cm layer) and 6 (10-15 cm layer). Two structures, BHT (**1a**) and adenosylhopane (**1e**) dominate the BHP profile and all the structures decrease in concentration with depth.

The concentration of BHPs in the west topsoil mound samples is approximately double that seen in LW4A with 255 μg_{BHP} $g^{-1}{}_{TOC}$ in the 0-5 cm layer, 211 μg_{BHP} $g^{-1}{}_{TOC}$, 5-10 cm layer and 89 $\mu gBHPg^{-1}{}_{TOC}$, 10-15 cm layer (Fig. 5.8). The number of BHPs is similar to that in LW4A with 11 in 0-5 cm layer, 10, 5-10 cm layer, and 7 in 10-15 cm layer. The BHP profile is dominated by 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**). All these structures show a decrease with depth which is mirrored in the distribution of the minor BHPs except for 2 methyl adenosylhopane type-1 (**2u**) which increases with depth but is absent in the 10-15 cm layer.

The BHP concentrations in LWP2.01 (Fig. 5.8) are similar to those observed in west topsoil mound samples with 230 μg_{BHP} $g^{-1}{}_{TOC}$ in 0-5 cm layer and 197 μg_{BHP} $g^{-1}{}_{TOC}$ in 5-10 cm layer, no 10-15 cm layer was obtained. Only 7 BHP structures were identified in 0-5 cm layer and 8 in the 5-10 cm layer. The BHP profile was dominated by 2 BHPs; BHT (**1a**) and adenosylhopane (**1e**) and these 2 BHP concentrations decreased with depth. The minor BHPs remained relatively consistent with depth showing either small decreases or small increases.

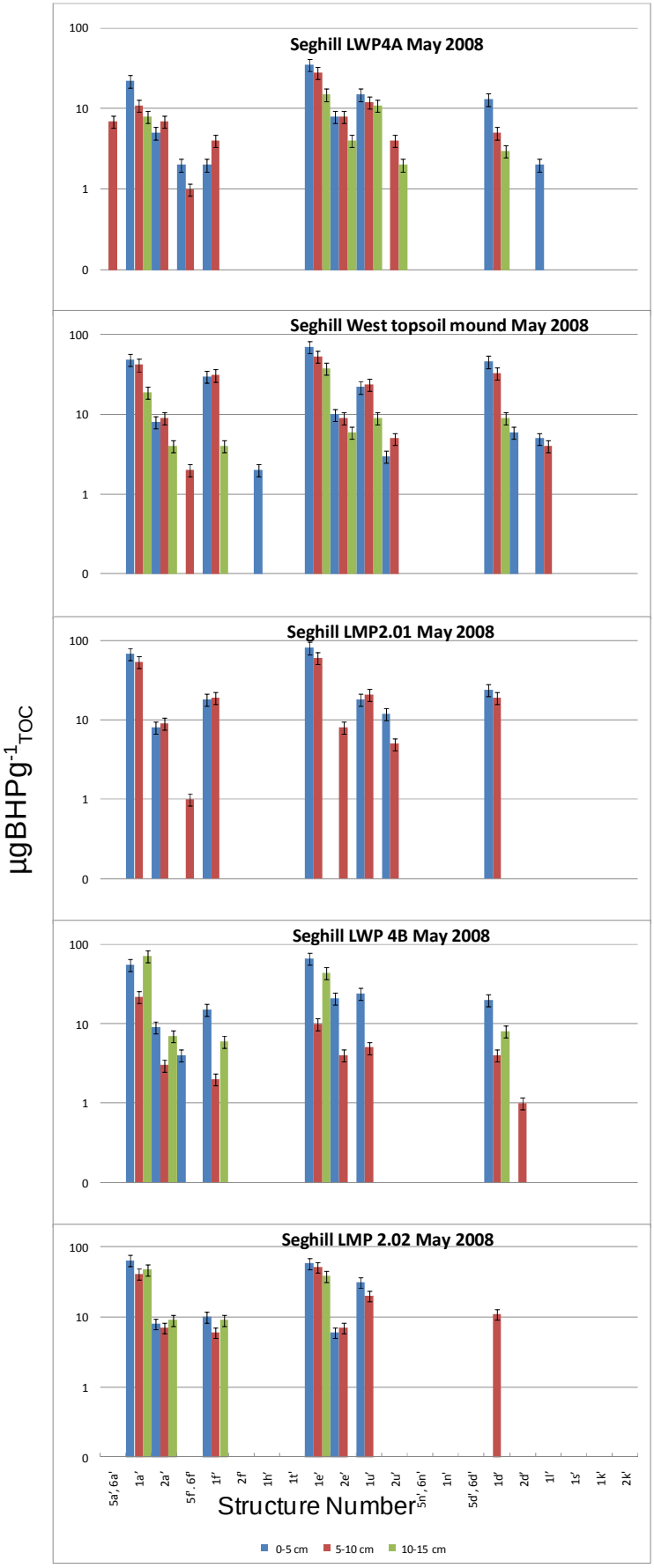


Fig. 5.8 BHP distribution Seghill, June 2007. ($\mu g_{BHP}\, g^{-1}{}_{TOC}$)

The BHP concentration of the 0-5 cm layer in core LWP4B was 214 $\mu g_{BHP}\ g^{-1}{}_{TOC}$, this dropped to 51 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 5-10 cm layer before rising to 136 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 10-15 cm layer (Fig. 5.8). The number of BHP structures identified was low at 8, 0-5 cm and 5-10 cm, and only 5 in the 10-15 cm layer. The BHP profile was dominated by 2 BHPs; BHT (**1a**) and adenosylhopane (**1e**). The changes in total concentration of BHPs are mirrored in the changes in the individual concentrations with falls in the 5-10 cm layer and a subsequent rise in the 10-15 cm layer except of 2 methyl adenosylhopane (**2e**) and adenosylhopane type-1 (**1u**) which decrease with depth and are absent in the 10-15 cm layer.

The total concentration of BHPs in LMP2.02 is similar to that seen in west topsoil mound core with 177 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in 0-5 cm layer, 144 $\mu g_{BHP}\ g^{-1}{}_{TOC}$, 5-10 cm, and 102 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in 10-15 cm (Fig. 5.8). However the number of BHP structures identified is much lower at 6 in 0-5 cm, 7 in 5-10 cm and only 4 in the 10-15 cm layer. The BHP profile is dominated by 3 BHPs; BHT (**1a**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**). There is a general trend of decrease in concentration with depth except for 2 methyl BHT (**1a**), which has a slight rise in concentration with depth.

5.6 Discussion

A total of 21 BHPs were identified in the landfill samples with the maximum in any one sample being 13 BHP structures (Chicken Road BH5/1, 5-10 cm and Octavia Court BH6, 5-10 cm) and the minimum being 4 (Seghill LMP2.02, 10-15 cm). These numbers are approximately half those observed in the Palace Leas (Chapter 3) and Hack Hall Farm (Chapter 4) indicating a much lower bacteria diversity than that seen in Palace Leas (Chapter 3) and Hack Hall Farm (Chapter 4).

The total BHP concentration was low with a maximum of 255 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ (Seghill, west topsoil mound, 0-5 cm) and a minimum of 22 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ (Octavia Court BH8, 5-10 cm). These concentrations are approximately 50% of the total BHP concentration in Hack Hall Farm (Chapter 4) and 25% of the total BHP concentration in the Palace leas Plots (Chapter 3).

These results are similar to those observed in the University Garden samples (Cooke et al., 2008b) where the total BHP concentration was 50 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ and the number of structures identified was 9. These low concentrations are accompanied by TOC

levels that are similar to those seen in Palace Leas and Hack Hall Farm and therefore the organic content of the soil must not be high in BHP producing bacteria but is instead likely to be dominated by fungi and non-BHP producing bacteria (Cooke et al., 2008b). The low bacterial population and high fungal population have been identified in unfertilised grassland whereas fertilised soils contained a higher microbial population (Bargett et al., 1999). This is in broad agreement with observations in the unfertilised landfill cover soils when compared to the fertilised Palace Leas plots (Chapter 3), although the untreated Palace Leas plot 6 does not show a reduction in BHPs when compared to the other treated Palace Leas Plots.

Alternatively the environmental conditions are such that BHPs are not being produced in abundance due to environmental stress such as that seen in plots 2, low stress, and plot 7, high stress, on the Palace Leas plots. The weather conditions at the time of sampling were hot and dry and the arid conditions may have resulted in a reduction in the bacterial population. Drought has been shown to significantly decrease soil microbial population (Kassem et al., 2008) but the same report identified that elevated CO_2 levels increased the soil microbial population and ameliorated the effect of drought. The high levels of CO_2 produced by the landfill may have a similar effect on the cover soil microbial population. It must also be considered that the environmental conditions could only be described as a hot summer and not a drought and that a temporary drying of the soil may have a detrimental effect on the bacterial population either by cell death or by elucidating a bacterial response to osmotic stress which has been shown to involve the production of more BHPs to condense the cell membrane and prevent desiccation (Poralla et al., 2000). These points indicate that the dry weather is probably not having a detrimental effect on the bacterial population in the cover soil and that the microbial population is most likely dominated by fungi.

In almost all the landfill samples the BHP profile is dominated by 2 major BHPs; BHT (**1a**) and adenosylhopane (**1e**) which are the only to BHPs identified in every sample and account for on average 27.0% (BHT; **1a**) and 29.6% (adenosylhopane; **1e**) (Figs. 5.5 to 5.8). The other BHPs identified as major components of the BHP profile only accounted for a relatively small % of the total BHP contribution; aminotriol (**1f**; 8.1% on average), adenosylhopane type-1 (**1u;** 12.4% on average) and BHT cyclitol ether (**1d**; 8.1% on average) although in some samples their BHP concentrations were higher than BHT (**1a**) and adenosylhopane (**1e**; Figs. 5.5 to 5.8).

These contributions to the total BHP concentration are different to the contributions of these 5 BHPs seen in the Palace Leas and Hack Hall Farm samples (Table 5.1).

Table 5.1. Average % contribution of BHPs to total BHP concentration for Palace Leas, Hack Hall Farm and Landfill sites

BHP	Palace Leas (%)	Hack Hall Farm (%)	Landfills (%)
BHT (**1a**)	5.4	10.5	27.0
Aminotriol (**1f**)	29.7	25.1	8.1
Adenosylhopane (**1e**)	14.0	15.9	29.6
Adenosylhopane type - 1(**1u**)	9.3	8.6	12.4
BHT cyclitol ether (**1d**)	22.2	22.3	8.1

It is clear from these results that there is a relative increase in the contribution of BHT (**1a**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**) to the BHP profile in landfill soils and a decrease in the relative contribution of aminotriol (**1f**) and BHT cyclitol ether (**1d**) when compared to the Palace Leas and Hack Hall Farm samples, although it must be remembered that the overall BHP concentration is low so that in many cases the absolute concentration of BHT (**1a**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**) may have decreased or remained at levels similar to those seen in Palace Leas and Hack Hall Farm.

5.6.1 BHPs as Indicators of bacterial Communities

Using the relationship between BHP and source bacteria (Table 2.2) the bacterial population of the Chicken Road landfill samples is dominated by BHPs from various sources and by PNSB and N_2 and NH_3 fixing bacteria (Table 5.2). The BHPs from various sources are dominated by BHT (**1a**) and the high concentration of adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**) in these samples accounts for the high level of the PNSB and the N_2 fixing and NH_3 oxidising bacteria. The level of cyanobacteria is lower than that observed for the Palace Leas samples (Chapter 3) and comparable with that in the Hack Hall Farm Common fir and Scots pine samples where lack of light penetration was believed to restrict the activity of cyanobacteria (Chapter 4).

Table 5.2 Distribution of BHP producing bacteria in Chicken Road samples, identified by presence of BHPs

Bacteria	BHPs (abbreviated name)	Chicken Road BH5/1 0-5 cm	Chicken Road BH5/1 5-10 cm	Chicken Road BH5/1 10-15 cm	Chicken Road BH1 0-5 cm	Chicken Road BH1 5-10 cm
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)				
Various sources	655, 714, 1000, 1002ce, 1060ce	[a]58 [b](51.2)	15 (32.3)	16 (39.2)	123 (54.9)	32 (34.6)
Purple non sulphur bacteria	627, 712, 746, 761	39 (34.2)	22 (45.5)	18 (44.0)	69 (30.7)	42 (46.1)
[c] without adenosylhopane and hopanelactone		15 (12.8)	8 (15.0)	6 (15.3)	23 (10.4)	14 (14.8)
N_2 and NH_3 fixing bacteria	627,746, 760	30 (26.8)	19 (37.3)	15 (37.6)	57 (24.4)	35 (38.3)
[c] without adenosylhopane and hopanelactone		6 (5.3)	5 (6.9)	4 (8.9)	9 (4.1)	7 (7.1)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 2Me 1016, 2Me 1132	8 (4.8)	3 (5.1)	2 (3.6)	16 (5.3)	6 (4.4)
Methanotrophs	772	2 (2.1)	0.3 (0.7)	0 (0.0)	3 (1.3)	0 (0.0)
Acetic Acid Bacteria	653	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Methylotrophs	1086	0 (0.0)	0.7 (1.6)	0.4 (1.1)	0 (0.0)	3 (3.0)
Unknown	775, 1118	0.4 (0.4)	1 (5.7)	0.8 (1.9)	4 (1.9)	2 (2.5)

. [a][BHP] ($\mu gBHPg^{-1}_{TOC}$) , [b](% ave BHP contribution)

[c] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid**.**

Methanotroph levels are low and are only indicated by aminotetrol (**1h**) as aminopentol (**1c**) was absent from all samples. This indicates a dominance of Type II methanotrophs as suggested by Crossman et al., (2004), although the concentrations of aminotetrol (**1h**) were elevated when compared to Palace Leas (Chapter 3) and Hack Hall Farm (Chapter 4) and aminotetrol (**1h**) has been identified in almost every sample analysed during this research. Acetic acid bacteria are absent from all samples and only unsaturated BHT (**5a or 6a**) was identified as an acetic acid BHP marker, with no 3-Me BHPs identified in any of the landfill sites. The level of methylotrophs was relatively high compared to Palace Leas and Hack Hall Farm due to relatively

high levels of guanidine substituted BHT (**1s**) when compared to the Palace Leas and Hack Hall Farm sites.

The bacterial population of the West Allotment samples (Table 5.3) is very similar to that observed in the Chicken Road site, being dominated by BHT (**1a**) and adenosylhopane (**1e**) to give a high contribution from PNSB and N_2 and NH_3 fixing bacteria. Acetic acid bacteria are again absent and the levels of methanotrophs are very low, compared to Palace Leas or Hack Hall Farm samples, only being identified in one sample.

Table 5.3 Distribution of BHP producing bacteria in West Allotment samples, identified by presence of BHPs.

Bacteria	BHPs (abbreviated name)	West Allotment BH 3/4 0-5 cm	West Allotment BH 3/4 5-10 cm	West Allotment BH 4/1 0-5 cm	West allotment BH 4/1 5-10 cm
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)			
Various sources	655, 714, 1000, 1002ce, 1060ce	[a]35 [b](49.7)	51 (77.2)	25 (38.5)	18 (54.4)
Purple non sulphur bacteria	627, 712, 746, 761	26 (36.7)	12 (18.5)	29 (44.8)	10 (30.4)
[c] without adenosylhopane and hopanelactone		11 (16.0)	0 (0.0)	13 (19.6)	4 (11.4)
N_2 and NH_3 fixing bacteria	627,746, 760	17 (23.7)	12 (18.5)	20 (31.8)	8 (23.0)
[c] without adenosylhopane and hopanelactone		2 (3.1)	0 (0.0)	4 (6.5)	1 (4.0)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 2Me 1016, 2Me 1132	5 (7.0)	3 (4.3)	3 (4.9)	2 (5.5)
Methanotrophs	772	0 (0.0)	0 (0.0)	0 (0.0)	0.4 (1.4)
Acetic Acid Bacteria	653	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Methylotrophs	1086	1 (1.8)	0 (0.0)	1 (2.0)	0 (0.0)
Unknown	775, 1118	1 (1.7)	0 (0.0)	2 (3.3)	0.5 (1.6)

[a][BHP] ($\mu gBHPg^{-1}{}_{TOC}$) , [b](% ave BHP contribution)

[c] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid**.**

This bacterial population distribution is repeated in the Octavia Court samples with a dominance of PNSB, N_2 and NH_3 fixing bacteria and various bacterial sources due to

the presence of adenosylhopane (**1e**) and BHT (**1a**). The contribution to the bacterial population is very similar to the other sites although acetic acid bacteria are identified by the presence of unsaturated BHT (**5a** or **6a**) in one sample (Table 5.4).

Table 5.4 Distribution of BHP producing bacteria in Octavia Court samples, identified by presence of BHPs.

Bacteria	BHPs (abbreviated name)	Octavia court BH8 0-5 cm	Octavia court BH8 5-10 cm	Octavia Court BH6 0-5 cm	Octavia court BH6 5-10 cm
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)			
Various sources	655, 714, 1000, 1002ce, 1060ce	[a]25 [b](32.2)	11 (47.3)	10 (12.8)	62 (49.6)
Purple non sulphur bacteria	627, 712, 746, 761	44 (57.8)	9 (40.8)	47 (63.3)	46 (37.2)
[c] without adenosylhopane and hopanelactone		22 (28.5)	0 (0.0)	18 (24.0)	16 (12.8)
N_2 and NH_3 fixing bacteria	627,746, 760	25 (32.0)	10 (46.7)	35 (46.7)	34 (27.6)
[c] without adenosylhopane and hopanelactone		2 (2.7)	1 (5.9)	6 (7.4)	4 (3.1)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 2Me 1016, 2Me 1132	4 (5.3)	0 (0.0)	6 (8.0)	5 (4.2)
Methanotrophs	772	0 (0.0)	0 (0.0)	0 (0.0)	3 (2.3)
Acetic Acid Bacteria	653	0 (0.0)	0 (0.0)	2 (2.5)	0 (0.0)
Methylotrophs	1086	2 (2.0)	0 (0.0)	1 (1.6)	2 (1.9)
Unknown	775, 1118	0 (0.0)	1 (5.9)	3 (4.4)	2 (1.7)

[a][BHP] ($\mu gBHPg^{-1}{}_{TOC}$) , [b](% ave BHP contribution)

[c] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid.

The bacterial population identified using BHPs at Seghill is again very similar to that seen for the other landfills (Tables 5.5a to 5.5c) with a very high contribution from adenosylhopane (**1e**) and BHT (**1a**) giving a dominance to PNSB and N_2 and NH_3 fixing bacteria. The contribution from other bacteria to the population is even less

varied than on the other landfills with acetic acid bacteria and methanotrophs only being identified in one sample and methylotrophs being entirely absent.

Table 5.5a Distribution of BHP producing bacteria in Seghill samples, identified by presence of BHPs.

Bacteria	BHPs (abbreviated name)	Seghill LW4A 0-5 cm	Seghill LW4A 5-10 cm	Seghill LW4A 10-15 cm	Seghill west of top soil mound 0-5 cm	Seghill west of top soil mound 5-10 cm	Seghill west of top soil mound 10-15 cm
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)					
Various sources	655, 714, 1000, 1002ce, 1060ce	[a]39 [b](37.6)	20 (22.4)	11 (25.9)	130 (51.6)	110 (52.0)	31 (35.2)
Purple non sulphur bacteria	627, 712, 746, 761	52 (50.3)	42 (47.3)	26 (61.1)	93 (36.9)	79 (37.3)	47 (52.8)
[c] without adenosylhopane and hopanelactone		17 (16.1)	14 (15.6)	11 (25.2)	22 (8.9)	26 (12.3)	9 (9.9)
N_2 and NH_3 fixing bacteria	627,746, 760	43 (41.6)	36 (41.1)	19 (44.8)	81 (32.2)	62 (29.2)	44 (49.9)
[c] without adenosylhopane and hopanelactone		8 (7.3)	8 (9.3)	4 (8.9)	11 (4.1)	9 (4.2)	6 (7.1)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 2Me 1016, 2Me 1132	5 (4.8)	7 (8.0)	0 (0.0)	14 (3.0)	9 (4.2)	4 (4.9)
Methanotrophs	772	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.7)	0 (0.0)	0 (0.0)
Acetic Acid Bacteria	653	0 (0.0)	7 (8.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Methylotrophs	1086	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	775, 1118	0 (0.0)	4 (4.6)	2 (4.1)	3 (1.1)	5 (2.3)	0 (0.0)

[a][BHP] ($\mu gBHPg^{-1}_{TOC}$) , [b](% ave BHP contribution)

[c] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid.

Table 5.5b Distribution of BHP producing bacteria in Seghill samples, identified by presence of BHPs.

Bacteria	BHPs (abbreviated name)	Seghill LWP 2.01 0-5 cm	Seghill LWP 2.01 5-10 cm	Seghill LWP 4B 0-5 cm	Seghill LWP 4B 5-10 cm	Seghill LWP 4B 10-15 cm
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}_{TOC}$) (% ave BHP contribution)				
Various sources	655, 714, 1000, 1002ce, 1060ce	[a]111 [b](48.1)	91 (46.3)	90 (41.9)	28 (54.5)	85 (62.6)
Purple non sulphur bacteria	627, 712, 746, 761	100 (43.4)	83 (41.9)	94 (44.0)	15 (29.0)	44 (32.1)
[c] without adenosylhopane and hopanelactone		18 (8.0)	22 (11.1)	28 (13.0)	5 (10.3)	0 (0.0)
N_2 and NH_3 fixing bacteria	627,746, 760	81 (35.3)	69 (35.1)	88 (41.0)	14 (27.6)	44 (32.1)
[c] without adenosylhopane and hopanelactone		0 (0.0)	9 (4.3)	21 (10.0)	5 (8.8)	0 (0.0)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 2Me 1016, 2Me 1132	8 (3.4)	10 (4.8)	9 (4.1)	4 (5.7)	7 (5.3)
Methanotrophs	772	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Acetic Acid Bacteria	653	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Methylotrophs	1086	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	775, 1118	12 (5.2)	5 (2.7)	0 (0.0)	0 (0.0)	0 (0.0)

[a][BHP] ($\mu gBHPg^{-1}_{TOC}$) , [b](% ave BHP contribution)

[c] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid.

Table 5.5c Distribution of BHP producing bacteria in Seghill samples, identified by presence of BHPs.

Bacteria	BHPs (abbreviated name)	Seghill LMP 2.02 0-5 cm	Seghill LMP 2.02 5-10 cm	Seghill LMP 2.02 10-15 cm
		Ave [BHP] ($\mu g_{BHP}\ g^{-1}{}_{TOC}$) (% ave BHP contribution)		
Various sources	655, 714, 1000, 1002ce, 1060ce	[a]75 [b](42.2)	59 (41.1)	55 (54.0)
[c] without adenosylhopane and hopanelactone	627, 712, 746, 761	89 (50.1)	70 (49.0)	38 (37.0)
N_2 and NH_3 fixing bacteria		31 (17.5)	20 (13.8)	0 (0.0)
[c] without adenosylhopane and hopanelactone	627,746, 760	64 (36.0)	58 (40.3)	38 (37.0)
[c] without adenosylhopane		6 (3.3)	7 (5.1)	0 (0.0)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 2Me 1016, 2Me 1132	8 (4.3)	7 (4.8)	9 (8.9)
Methanotrophs	772	0 (0.0)	0 (0.0)	0 (0.0)
Acetic Acid Bacteria	653	0 (0.0)	0 (0.0)	0 (0.0)
Methylotrophs	1086	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	775, 1118	0 (0.0)	0 (0.0)	0 (0.0)

[a][BHP] ($\mu gBHPg^{-1}{}_{TOC}$) , [b](% ave BHP contribution)

[c] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid.

5.6.2 Statistical Analysis

Using the same PCA statistical analysis techniques employed on the Palace Leas and Hack Hall Farm sites and removing the contribution from "various sources" and "unknown bacteria" the loading plot (Fig. 5.9) shows a clear influence of adenosylhopane (**1e**) with PNSB and N_2 and NH_3 fixing bacteria being positively correlated with respect to the first component whereas the second component is correlated positively with respect to acetic acid bacteria, methylotrophs and cyanobacteria. Methanotrophs are negatively correlated with respect to the first component but the relationship with cyanobacteria is not as seen in other locations

(Palace Leas and Hack Hall) where the 2 groups have opposite effects on the PCA loading.

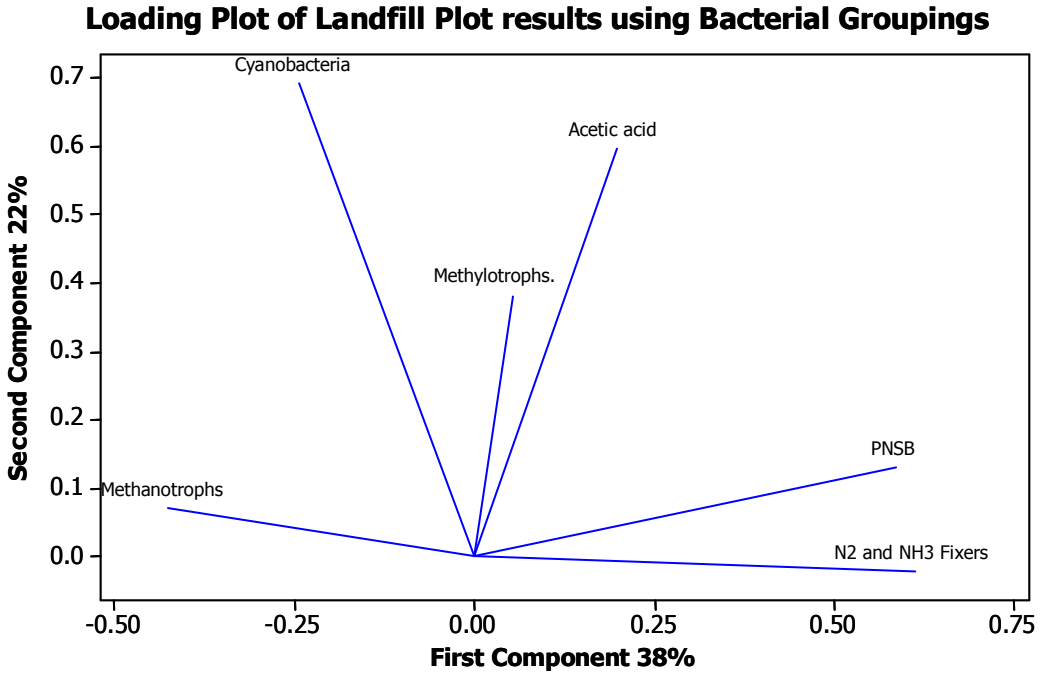


Fig. 5.9 PCA Loadings Plot for landfill sites

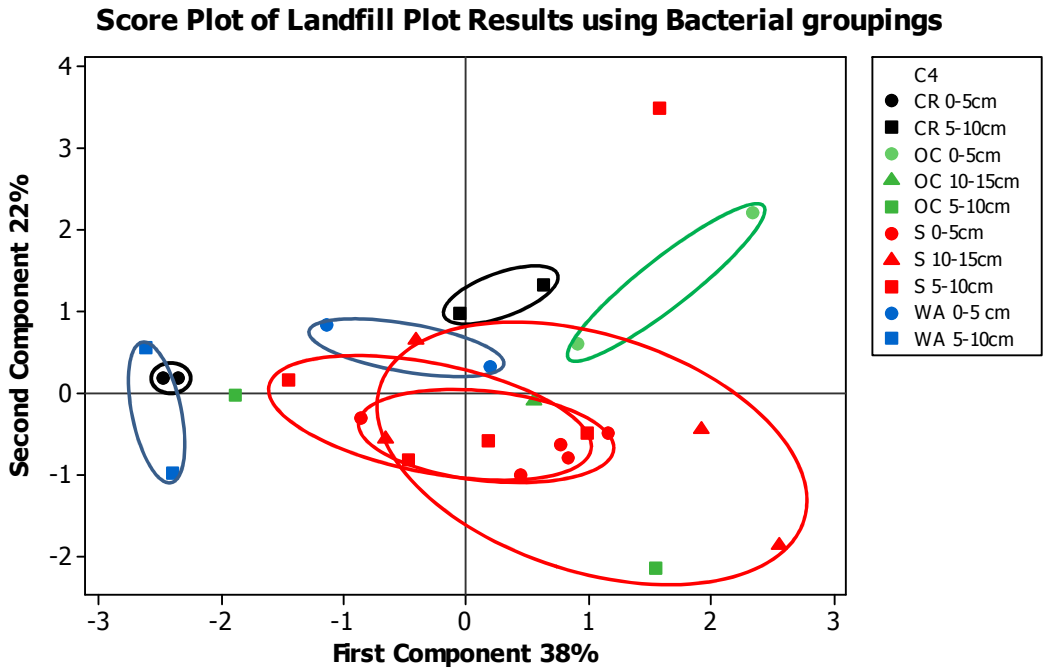


Fig 5.10 PCA Score Plot for landfill sites
CR = Chicken Road; OC = Octavia Court; S = Seghill; WA = West allotment

The resulting scores plot (Fig. 5.10) shows that there is significant overlap between the sites with most results being sited in the centre of the plot indicating no significant influence by any of the bacterial groups. The West Allotment site is influenced by the presence of methanotrophs, especially in the 5-10 cm layer, as in the 0-5 cm layer of the Chicken Road site. The Seghill samples are widely distributed but appear to be the most influenced by the presence of adenosylhopane (**1e**) when compared to the other locations.

5.7 Summary

Goal 6

> *Identify the presence of methanotrophs and ammonia oxidising bacteria in the BHP profile.*

There is no evidence from the BHP results from any of the sampled landfill cover soils that there is an increase in the population of methanotrophs and when compared to other sites (Palace Leas and Hack Hall Farm, Chapters 3 and 4) the level of methanotrophs is reduced. Given that all the sites except Chicken Road are known to have high elevated CH_4 levels this result is somewhat unexpected. The enhanced gas collection and control methods found in a modern well operated landfill such Seghill may account for the very low levels of methanotrophs at this site. The low levels in methanotrophs markers at the much more poorly controlled Octavia Court and West allotment sites may be due to the use of till in the cover soil. This is highly impermeable when compacted and is used for landfill lining material due to this impermeability (Murray 1993). This impermeabilty may be restricting the release of CH_4 through the cover soil. This was not the case with the landfill cover soils investigated by Crossman et al., (2004). They tested 2 different cover soils, a sand based and clay based cap. In both cases there were elevated methanotroph populations that could only exist in the elevated CH_4 concentration found in the cover soils although they do note that there is a reduction in methanotroph activity associated with compacted clay sections of the cover soil.

The dominance of fungi in the soils, as observed in the low levels of BHPs and the relatively high TOC values, may prevent the methanotrophs from achieving a viable

population. Analysis of the heavy metal contamination levels from these soils (Perradeau, 2010) has shown that there is no significant contamination in the cover soils that could hinder bacterial growth.

The BHP profiles are dominated by BHT (**1a**) and adenosylhopane (**1e**), whilst BHT (**1a**) can be attributed to various bacterial sources adenosylhopane (**1e**) is linked to PNSB, N_2 fixing and NH_3 oxidising bacteria. All other BHPs are at significantly reduced concentrations or absent when compared to other sites (Palace Leas and Hack Hall Farm). This indicates that the cover soil BHP producing bacterial population is dominated by PNSB, N_2 fixing and NH_3 oxidising bacteria in accordance with the observations of Yu et al., 2009 (NH_3 oxidising bacteria only).

5.8 Conclusions

The BHP profiles of landfill cover soils are distinct from other sites investigated due to the very low BHP concentrations, (maximum of 255 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 0-5 cm layer of Seghill LW4A and minimum of 22 $\mu g_{BHP}\ g^{-1}{}_{TOC}$ in the 5-10 cm of Octavia Court BH8), and the low number of different BHP structures identified, maximum of (13 BHP structures (Chicken Road BH5/1, 5-10 cm and Octavia Court BH6, 5-10 cm and the minimum of 4 Seghill LMP2.02, 10-15 cm).

The TOC values are similar to other sites at 6-8%, except Seghill which is lower at 2-4%, so the low BHP concentration indicates that the soil microbial population is probably dominated by fungi (Bargett et al., 1999) or non BHP producing bacteria. The effect of the dry weather could either lead to a decrease in the bacterial population or an increase in the number of BHPs being produced. The number of observed BHPs and this concentration implies that there is no significant increase in BHPs, especially the more functionalised BHPs that would increase membrane condensation (Poralla et al., 2000; Joyeux et al., 2004) although it must be noted that the work by Joyeux was not on drought affected bacteria but on temperature increases that would increase membrane fluidity.

The low number of BHP structures indicates a low diversity in the BHP producing bacterial population and the high concentration of adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**) indicates that this population is dominated by PNSB, N_2 fixing and NH_3 oxidising bacteria.

The till cover soil appears to be restricting the release of methane from the landfill and may be the reason that there is an unexpectedly low methanotroph population in these cover soils.

6 Global River Estuary Systems

6.1. Introduction

The transport and subsequent deposition of terrestrially derived organic matter (TOM) into the ocean, is a key but poorly constrained aspect of the modern global carbon cycle influencing Earth System Processes over a wide spatial and temporal spectrum (e.g. Aumont et al., 2001). The transport of TOM to the ocean is complex depending on e.g. transport mode (fluvial vs aeolian), rate of export across climate zones, temporal variability, land-ocean interface transformation processes, terrestrial geology, vegetation cover and hydrology with soil organic carbon (SOC) accounting for 66% of the total terrestrial carbon budget (Batjes, 1996). Sediments collected from river estuaries may therefore contain a terrestrial BHP signal in addition to the marine one. In regions associated with large river systems the input of TOM is likely to be much more complex than commonly considered and very difficult to trace using established geochemical proxies.

Recent advances using soil-specific archaeal branched and isoprenoid tetraether lipids (BIT index, e.g. Hopmans et al., 2004) support the relevance of TOM for the marine carbon budget (Weijers et al., 2006 and references therein) and show a way forward in investigating the transport and subsequent burial of SOC using specific biomarkers that can be clearly related to specific source materials or biogeochemical processes.

n-alkanes have been used as a biomarker to measure the movement of TOM from land to sea. *n*-alkanes are derived from the surface waxes of terrestrial higher plant leaves and can be leached from leaves by rain or eroded with soils and subsequently transported by rivers to the coastal marine environment (Eglinton and Hamilton, 1963). Transportation of TOM as dust by prevailing winds to marine environments can also be measured (e.g. Gagosian et al., 1981; Poynter et al., 1989).

Sterols have been used to indicate sources of higher plants was C_{29} sterols were thought to be dominant in higher plants when compared to C_{27} and C_{28} (Huang and Meinschiem, 1976). However it has been subsequently shown that C_{29} sterols are ubiquitous in marine algae (Volkman, 1986) and therefore sterols cannot be used effectively as a biomarker for the transport of TOM to marine settings.

These 3 classes of biomarkers have enabled insights into the transport of some forms of TOM to the marine environment and have also show the problems with biomarkers

both due to alternative sources, as in sterols, or in only identifying a specific part of the TOM. It is therefore important to develop new proxies for different sources of TOM that target the movement and fate of TOM and BHPs may be able to fulfil the role.

The analysis of BHP distribution in the Congo estuary (West Africa), Rhone estuary (Mediterranean), and 7 rivers draining into the Arctic Ocean; (Ob, Yenisey, Lena, Indigirka, Kolyma, Yukon and Mackenzie Rivers) was undertaken to investigate the differences between these sediments, soils and to investigate the transport of soil organic matter (SOM) from terrestrial to marine environments. The results from the Congo and Rhone were reported to the International Meeting of Organic Geochemists (IMOG) 2007 and an abridged set of the Congo results subsequently published (Cooke et al., 2008a). The results from the Arctic rivers were published in 2009 (Cooke et al., 2009). This chapter builds on those papers to provide a more comprehensive assessment of the data.

Analysis of the intact BHP profile using HPLC-MS^n of sediment has been conducted by several researchers (e.g. Talbot and Farrimond, 2007; Pearson et al., 2009a) and the typical sediment BHP profile is significantly different from a typical soil BHP profile (Cooke et al., 2008b, Xu et al., 2008, Redshaw et al., 2008) with significantly fewer BHP structures identified in sediment and a dominance of BHT (**1a**), aminotriol (**1f**) and BHT cyclitol ether (**1d**) in the sediment BHP profile.

There is a measurable decrease in BHPs with depth in sediments (Talbot et al., 2003) that can be attributed to diagenesis. The diagenesis of BHPs and the subsequent formation of geohopanoids has been widely studied (e.g. Rohmer et al., 1980; Quirk et al., 1984; Bissaret et al., 1997; Innes et al., 1997; Rodier et al., 1999; Tritz et al., 1999; Watson and Farrimond, 2000). The incorporation of hopaniods into kerogen, a form that cannot be extracted by the methods used in this study, can occur rapidly (Farrimond et al., 2003). A diagenetic mechanism proposed by Costantino et al., (2001) and Schaeffer et al., (2008, 2010) that can be identified using the modified Bligh and Dyer extraction employed here is the cyclisation of BHT (**1a**) to form anhydroBHT (**1j**) *via* acid hydrolysis.

An alternative fate of BHPs may exist where BHPs are preserved unaltered at depth, either free in the sediment, as sulphur bound BHPs or in intact microbial cells. The results from the Palace Leas soils (Chapter 3) indicate that BHPs are rapidly broken down in aerobic condition but in anaerobic conditions preservation of intact BHPs

may occur, e.g. van Dongen et al., (2006) identified intact BHT (**1a**) in 65 Ma sediments indicating that intact BHPs may be preserved. Recent investigations have identified that BHPs can be produced by anaerobic bacteria (e.g. Sinninghe Damste et al., 2004; Fischer et al., 2005; Blumenberg et al. 2006) so any identification of intact BHPs in ancient sediments must be treated with caution as it may have been produced in situ. If a BHP known to only be produced by aerobic bacteria can be identified at depth then this will prove that BHPs can be preserved in ancient sediments enabling them to be used as biomarkers for palaeo studies.

6.2 Site Background Information

6.2.1 Rhone Shelf

The Rhone River is the major freshwater input into the Mediterranean with an annual discharge of 7-10 10^6 tonnes (Kim et al., 2006) and discharges in to the Gulf of Lions. The Gulf of Lions is located in the northwestern Mediterranean basin with a crescent shaped continental shelf that is marked by numerous submarine canyons. The sedimentation rate is relatively high, approx 20 cm y^{-1}, and sediment is derived from 2 main sources, the river and Aeolian dust from the Sahara (Kim et al., 2006). The sampling locations are shown in Fig. 6.1.

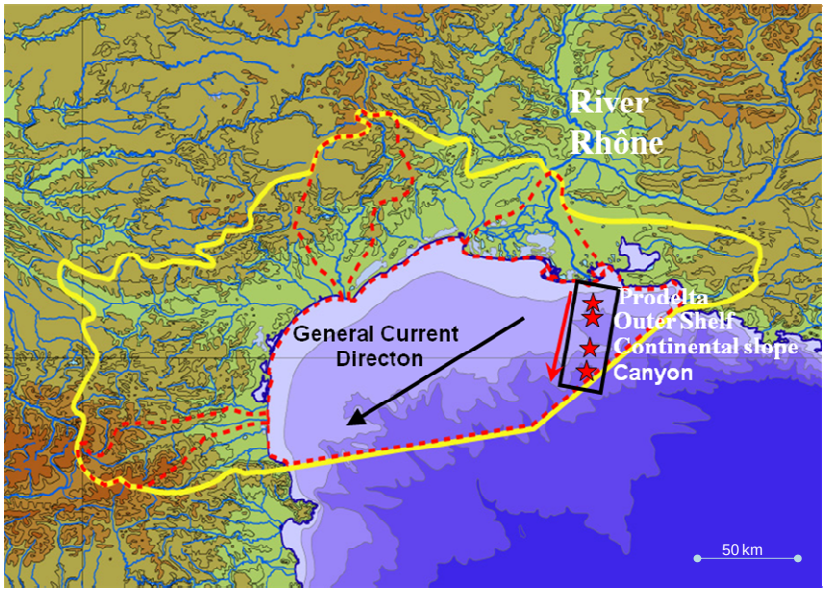


Fig. 6.1 Location map for Rhone shelf and deep canyon samples

6.2.2 Congo Marine Fan and Shelf

The Congo River catchment is the second largest catchment area in the world, accounting for more than one third of the runoff from the African continent per year (N'Kounkou and Probst, 1987) and approximately 4% of the total terrigenous input into the world's oceans (Martins and Probst, 1991). Therefore the Congo has a major impact on the content of the sediment and the sedimentation rate off West Africa. The submarine canyon associated with the river starts within the river mouth and therefore leads to direct deposition of terrestrial organic matter at times of low sea level (Holtvoeth et al., 2001). This geography enables the Congo estuary sediments to be used in climate studies and it was selected to be the site of Ocean Drilling Program (ODP) leg 175 (Schulz and Scientific Party (1998). The location of ODP site 1075 and 4 additional surface samples, from the Bremen core repository which were also analysed are shown in Fig. 6.2.

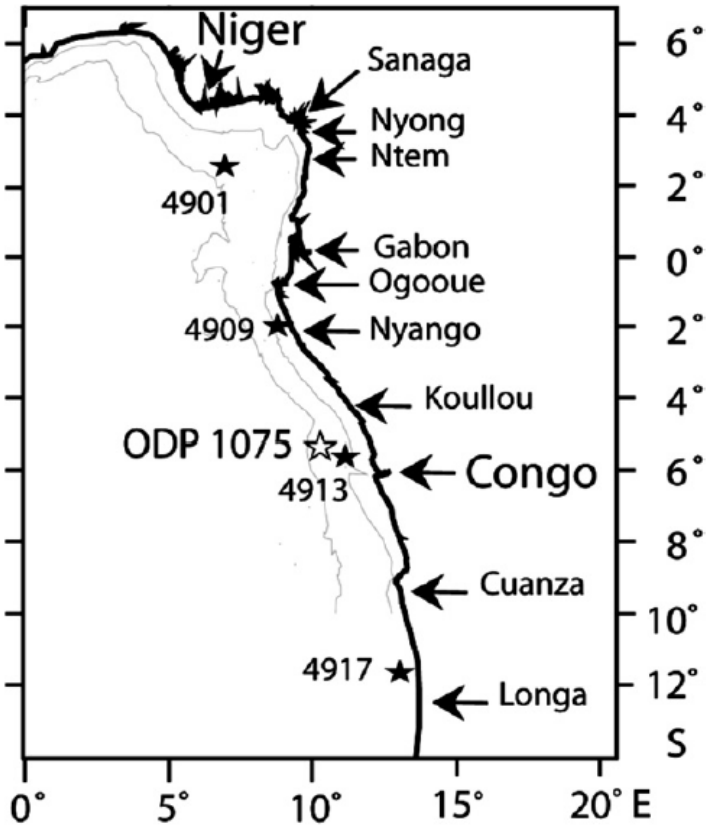


Fig. 6.2 Map of location of sites including core (ODP leg 175, site 1075) indicated by open star and core top samples from stations 4901, 4909, 4913 and 4917, indicated by closed star.

6.2.3 Arctic River sediments

The Arctic Ocean, accounts for only 1% of the world's ocean volume (Menard and Smith, 1966), yet it receives 11% of the freshwater and dissolved organic material (Aagaard and Carmack, 1989). It is estimated that up to 33% of global soil organic matter (SOM), predominantly present as taiga and tundra soils, is stored in the Arctic terrestrial regions, matching the total amount held in the atmosphere as CO_2 (Waelbroeck et al., 1997; Oechel et al., 2000). Climate models (e.g. Zwiers, 2002) forecast an amplification of global warming in the continental Arctic region. Projected and observed Arctic warming may result in destabilisation of permafrost (Stendel and Christensen, 2002; Jørgenson et al., 2006) and an increase in river discharge (e.g. Savelieva et al., 2000; Peterson et al., 2002), which could lead to accelerated fluvial release of the stored TOM and changes in the input flux and composition of TOM to the Arctic Ocean (e.g. Dittmar and Kattner, 2003; Stein and Macdonald, 2004; Guo et al., 2004, 2007; Zou et al. 2006; van Dongen et al., 2008). This makes it reasonable to believe that the Arctic region is one of the first areas where climate warming effects on large-scale biogeochemical cycles will be observed, with potential implications for the entire global climate system (Macdonald, 1996; Dickson et al., 2000; Serreze et al., 2000; ACIA, 2004; IPCC, 2007). It is therefore important to study the composition of river transported TOM from these regions to better understand the effect of climate changes on the stability of OM in terrestrial systems, its transport to and subsequent fate in marine sediments.

Several studies have been conducted at a molecular compositional level on the OM of rivers that discharge into the Arctic Ocean. These include studies on the Mackenzie – Beaufort Sea system (e.g. Yunker et al., 1993, 1995, 2002; Goñi et al., 2000, 2005; Belicka et al., 2004; Drenzek et al., 2007; Guo et al., 2007) and the Great Russian Arctic Rivers (GRARs; Peulve et al., 1996; Fahl and Stein, 1997; Zegouagh et al., 1998; Fernandez and Sicre, 2000; Fahl et al., 2003; Stein and Macdonald, 2004; Guo et al., 2004; Elmquist et al. 2008; van Dongen et al., 2008).

The major objective of the study was to provide a detailed and coherent benchmark of the BHP composition of the TOM exported coastally by the major Arctic Rivers; Ob, Yenisey, Lena, Indigirka, Kolyma, Yukon and Mackenzie (Fig. 6.3). Investigation of these sediments offers the possibility of identifying Arctic-scale trends in both the

terrestrial and marine processes mediated by hopanoid-producing bacteria, which are recorded in the BHP signature of the deposited OM.

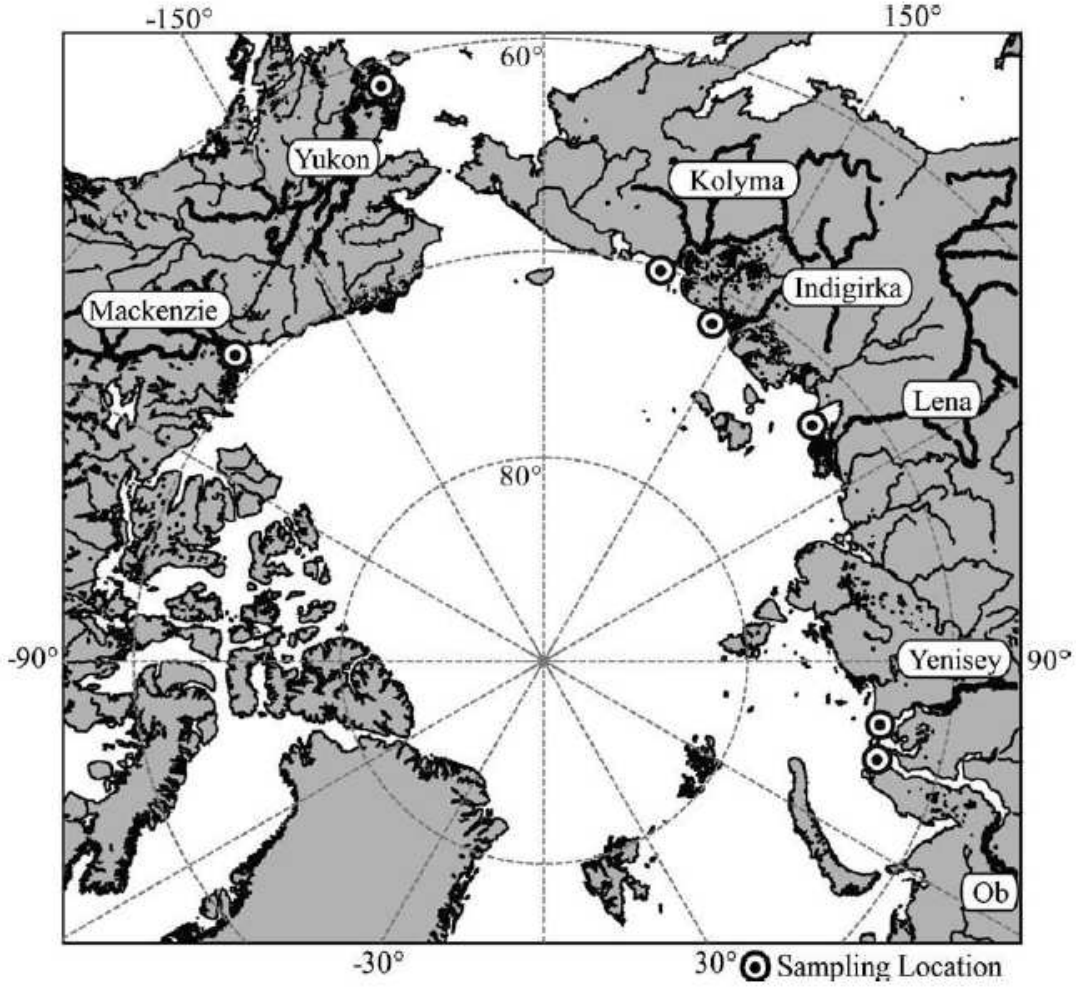


Fig. 6.3. Location of sampling site for the 7 Arctic rivers.

6.3 Goals

The investigation into the river estuary sediments tested hypothesis 2

Complex intact BHPs are preserved in ancient marine sediments

By achieving 3 goals

Goal 7

Identify complex intact BHPs from aerobic bacteria in ancient marine sediments.

Analysis of BHPs in sediments has shown that they decrease rapidly with depth (Watson et al., 2002; Talbot et al., 2003) as a result of rapid diagenesis (Rohmer et al., 1980; Quirk et al., 1984; Innes et al., 1997). Research into BHP producing bacteria has identified that they are predominantly produced in aerobic conditions (e.g. Rohmer et al., 1984; Zundel and Rohmer, 1985; Neunlist et al., 1985; Ourisson et al., 1987; Summons et al., 1999). However BHPs have been identified in obligate anaerobes (e.g. Ourison et al., 1984; Sinninghe Damste et al., 2004; Fischer et al., 2005; Blumenberg et al., 2006) confirming production in anaerobic conditions. Therefore not all BHP production can be linked to aerobic conditions and therefore the presence of BHPs in deep sediments cannot be directly linked to preservation after burial and may be produced in situ (Saito and Suzuki, 2007).

However, specific BHPs are used as biomarkers for aerobic processes, e.g. aminopentol (**1c**) as a marker for aerobic methane oxidation which occurs in the water column and surface sediments (e.g. Neunlist and Rohmer, 1985a; b; Cvejic et al., 2000; Talbot et al., 2001) and aminotetrol (**1h**; e.g. Neunlist and Rohmer, 1985a; Cvejic et al., 2000). The presence of aminopentol (**1c**) and aminotetrol (**1h**) at depth in the sediment must be as a result of preservation after burial.

Goal 8

*Identify the distribution of potential soil organic carbon (SOC) markers adenosylhopane (**1e**) and its related homologues (the soil markers) in sediments to test their preservation potential and to track movement of SOC via rivers to marine systems.*

Analysis of the BHP distribution of a wide range of soils from around the world (Cooke et al., 2008b; Xu et al., 2008; Redshaw et al., 2008 and this thesis) has shown that adenosylhopane (**1e**), 2-Me adenosylhopane (**2e**), adenosylhopane type-1 (**1u**) and 2-Me adenosylhopane type-1 (**2u**) (the soil marker BHPs from herein) comprise

on average 28% of all soil BHPs (Cooke et al., 2008a; 2008b). In a survey of over 40 different recent marine and non-marine settings only 3 lacustrine sediments known to have large TOM supply from their catchments were found to contain these markers (Talbot and Farrimond, 2007).

The presence of these soil markers in the estuary sediments will indicate the presence of soil derived material in the sediment and a decrease in concentration of these soil markers with distance from estuary will confirm that the source of these markers is the terrestrial environment.

Goal 9

> *Compare the BHP profile with other biomarkers such as sterols and n-alkanes, and compare with the carbon cycling across the 7 Arctic rivers.*

Surface sediment analysis using py-GC/MS of the 7 arctic rivers shows a shift in the pyrolysate of furfurals, products from polysaccharides, towards the east, indicating an increase in the presence of more labile TOM and thus a lower degree of degradation of the TOM (Guo et al., 2004). In addition, a recent study showed an increase in the relative abundance of terrestrial compounds with functional groups, such as sterols and *n*-alkanoic acids eastwards, further supporting the decreasing degree of decomposition of the river-exported OM along this west-east sequence (van Dongen et al., 2008). The larger abundance of permafrost in the east, in combination with the shorter average length of the summer thaw is probably the cause for the observed differences. Changes in BHP profile relating to the transport of terrestrial organic matter will therefore potentially reflect these changes and show a decrease in degradation moving to the east.

6.3. Sampling Methodology

Samples of core sediments from the Congo Fan (ODP leg 175, site 1075, 2 km water depth) (Fig. 6.1.) were obtained from the ODP core repository in Bremen. The four core top samples (4901, 4909, 4913, 4917) from a N-S transect along the tropical African continental margin were provided by Dr Matthias Zabel (University of Bremen) (Fig. 6.1). Details of sampling are reported by Schulz and Scientific Party

(1998). The stratigraphy of core 1075 has been obtained from by Holtvoeth et al. (2001). The samples were freeze dried prior to arrival at Newcastle.

The Rhone samples were provided by Dr Jung-Hyun Kim (NIOZ) and Dr Roselyne Buscail (Université de Perpignan) and comprised a transect from the pro-delta to the canyon (Fig. 6.2) and were freeze dried prior arrival at Newcastle.

The pan-Arctic coastal samples were collected from five Siberian rivers and in two North American rivers (Fig. 6.3), and are described in detail by van Dongen et al. (2008) and Elmquist et al. (2008). A brief summary of the sites is given in Table 6.1.

All sediment samples were taken from a central location in the coastal river plume. The Indigirka sediments were obtained from 8-11 m water depth, whereas the samples from the other Russian Arctic rivers were obtained at even shallower depths using a small boat deployed from the mother ship

Sediments from the Yukon and Mackenzie rivers were collected and sub-sampled from the riverbed about 5-10 m away from the riverbank using a stainless steel hand shovel during June and July 2004, respectively, as described by Guo et al. (2007). The Yukon River sediment was collected near the town of Pilot Station (Alaska, USA) and the Mackenzie River sediment from near the Arctic Red River, Canada (Table 6.1). All samples were stored at -20^{o}C prior to freeze drying in preparation for extraction.

All freeze dried samples were extracted using the methodology described in chapter 2.

Table 6.1 Sampling Locations and total organic carbon (TOC) content of the Arctic river estuary samples.

	Ob	Yenisey	Lena	Indigirka	Kolyma	Yukon	Mackenzie
Sample	1	2	N-8	IK43- 46[a]	IK-71	Pilot Station	Red River
Major contribution geological and physiographic regions	West Siberian Lowlands (plain)	West Siberian Lowlands (plain) /Central Siberian Plateau	Central Siberian Plateau	East Siberian Highlands	East Siberian Highlands	North American Cordillera	North American Cordillera/ Interior Plain/ Canadian Shield
Latitude; Longitude	72°65'N; 73°44'E	72°61'N; 79°86'E	71°96'N 129°54' E	72°06'N; 150°46'E– 71°02'N; 152°60'E	70°00'N; 163°70'E	61°50'N; 162°53'W	67°26'N; 130°45'W
TOC (mg/g)[c]	9.2 ± 0.2	19.4 ± 0.3	4.8 ± 0.2	14.6 ± 0.2	17.3 ± 1.1	12.4 ± 0.5	20.2 ± 0.3

6.5. Results

6.5.1 Rhone River Results

Only 8 different BHPs were identified in the Rhone fan and deep marine canyon surface transect (Fig. 6.4) with the number of BHPs decreasing with distance from river estuary. BHT (**1a**) dominates the BHP profile and reaches a maximum in the shelf samples. 2-Me BHT (**2a**) and aminotriol (**1f**) are present in all samples. Adenosylhopane (**1e**) in only present in the pro-delta and shelf samples but 2 other BHPs, adenosylhopane type-1 (**1u**) and 2-Me adenosylhopane type-1 (**2u**), are present in the outer shelf sample (Fig. 6.7), all three were not detected in the canyon sample. BHT cyclitol ether (**1d**) is only present in the pro-delta and shelf samples and BHpentol cyclitol ether (**1l**) is only present in the pro-delta sample

The concentrations are low in all samples when compared to soil samples (e.g. Palace Leas samples, Chapter 3) and the ODP 1075 samples (Fig. 6.6.) but are comparable to the concentration in the Congo surface samples (Fig. 6.5).

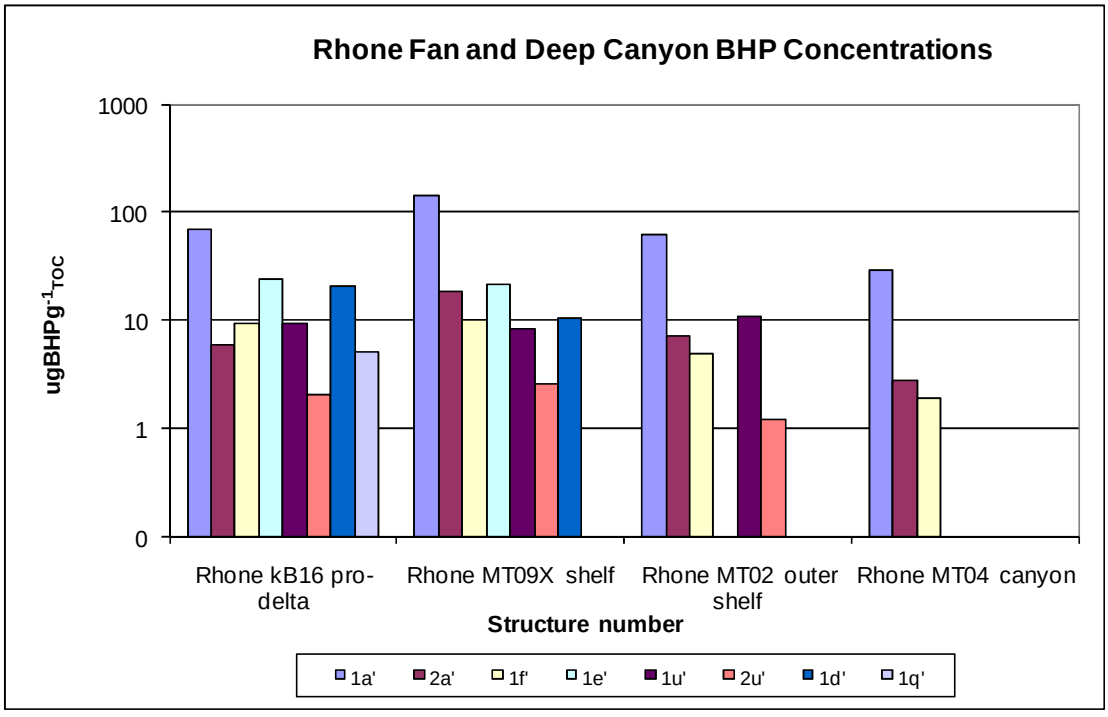


Fig. 6.4. Log graph of semi-quantitative BHP concentration ($\mu gBHPg^{-1}_{TOC}$) in Rhone estuary samples

6.5.2 Congo Fan and West African Coast Results

Analysis of the surface samples from 4 sites (cores 4901, 4909, 4913 and 4917; Fig. 6.5) identified only 4 different BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**). The highest total concentration was observed in core 4913, the site closest to the mouth of the Congo and the site of ODP core 1075. Core 4909, the next closest to the Congo had the 2nd highest BHP concentration. Adenosylhopane (**1e**) is present in these 2 samples with the highest values being found in core 4913. The 2 most distant samples from site 1075, 4901 and 4917, show no evidence of any adenosylhopane (**1e**) and only contain BHT (**1a**) and a small concentration of aminotriol (**1f**) in core 4917 only (Fig. 6.4).

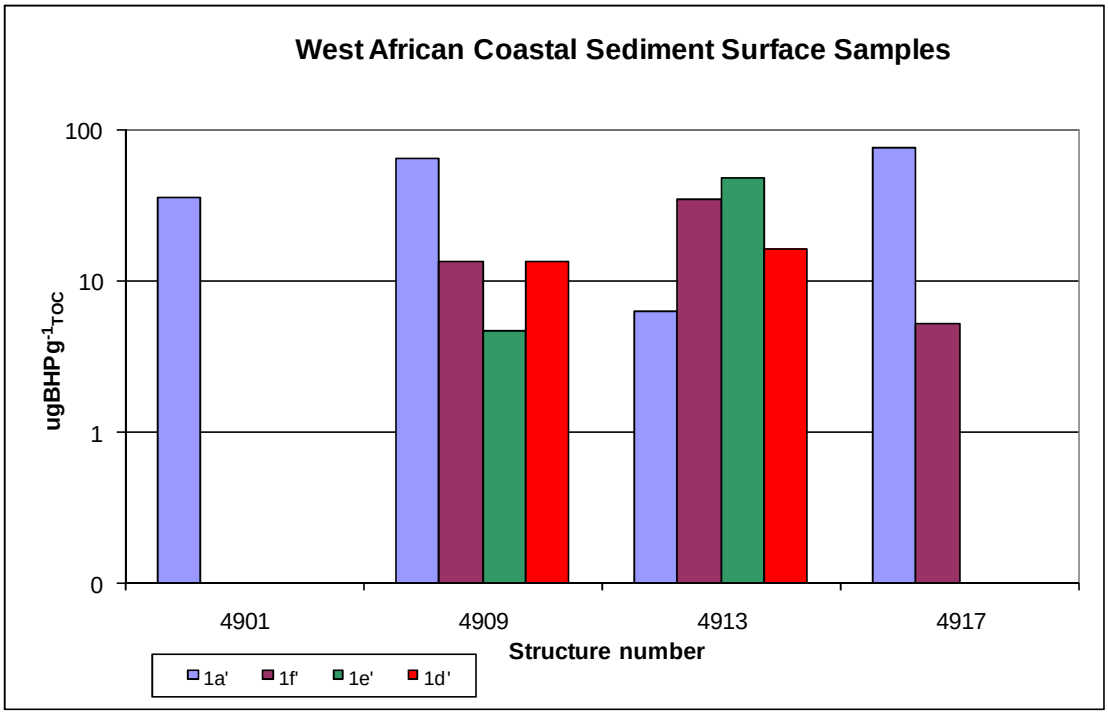


Fig. 6.5 Log graph of semi-quantitativeBHP concentration (µgBHPg$^{-1}_{TOC}$) in surface samples of cores 4901, 4909, 4913 and 4917.

Analysis of 22 Late Quaternary sediments from the Congo deep sea fan (ODP 1075) identified the presence of 15 different BHPs with the common sediment associated BHPs; BHT (**1a**), aminotriol (**1f**) and BHT cyclitol ether (**1d**) dominating all samples (Fig. 6.6). There is a general rise in BHP concentration down the core peaking at

55.22 m followed by a decrease down to 69.05 m and a further rise to the base of the core.

The adenosylhopane (**1e**) was present in samples analyzed down to 89 metres below sea floor (mbsf) corresponding to an age of 847.9 ka before present (bp), with 2-methyl adenosylhopane (**2e**) being found intermittently down to 49 m, There are distinct peaks of these structures at about 21, 34 and 60 mbsf (Fig 6.5). Several other BHPs common to soils; aminotetrol (**1h**), 2-Me BHT cyclitol ether (**2d**) and BHT glucosamine (**1g**) **(**Cooke et al., 2008b, Xu et al., 2008, Redshaw et al., 2008, this thesis) were also identified in the core (Fig. 6.6). These three BHPs have been found in over 150 soil samples analysed from numerous global locations (Chapters 3, 4, 5 and 7).

Aminopentol (**1c**) is found in all samples except 9.65 m and 21.15 m. and the changes in concentration mirror the changes in total BHP concentration (Fig 6.7). AnhydroBHT (**1j**) is present in all samples and increases in concentration with depth (Fig. 6.6). BHpentol cyclitol ether (**1l**) is found in all samples except the bottom 2 core samples

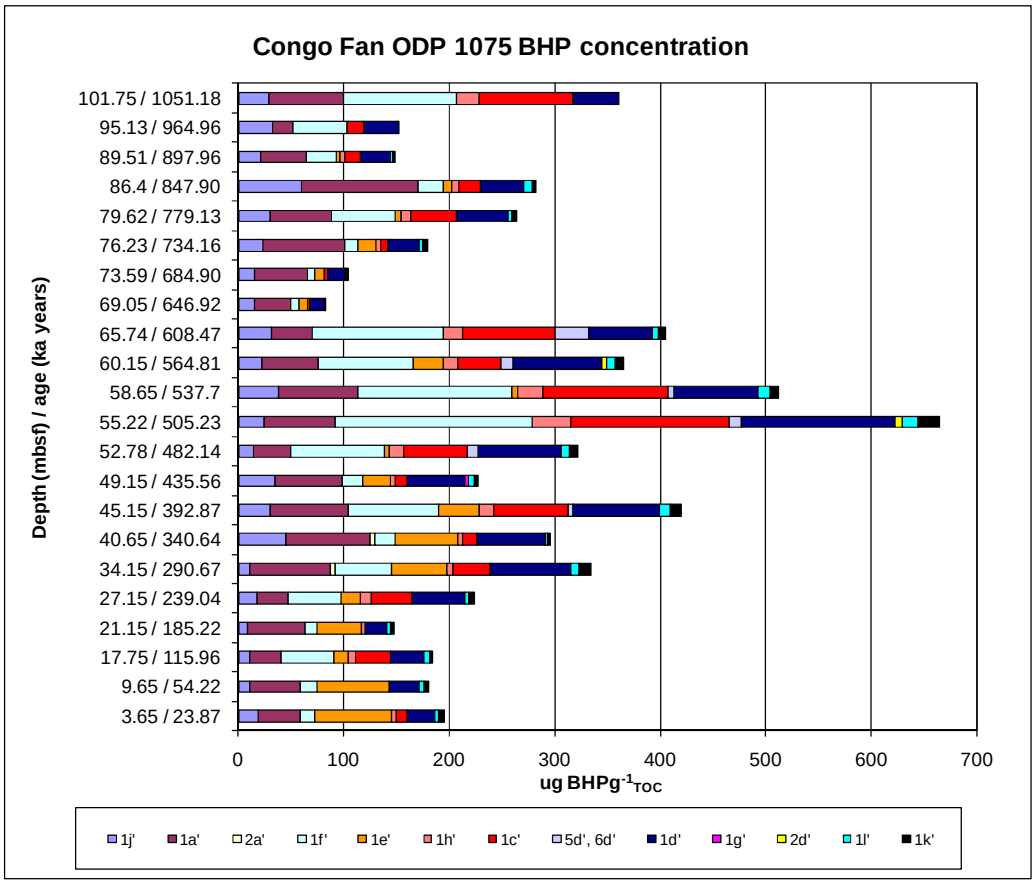


Fig. 6.6 Semi-quantitative BHP concentration ($\mu gBHPg^{-1}_{TOC}$) down core for ODP 1075.

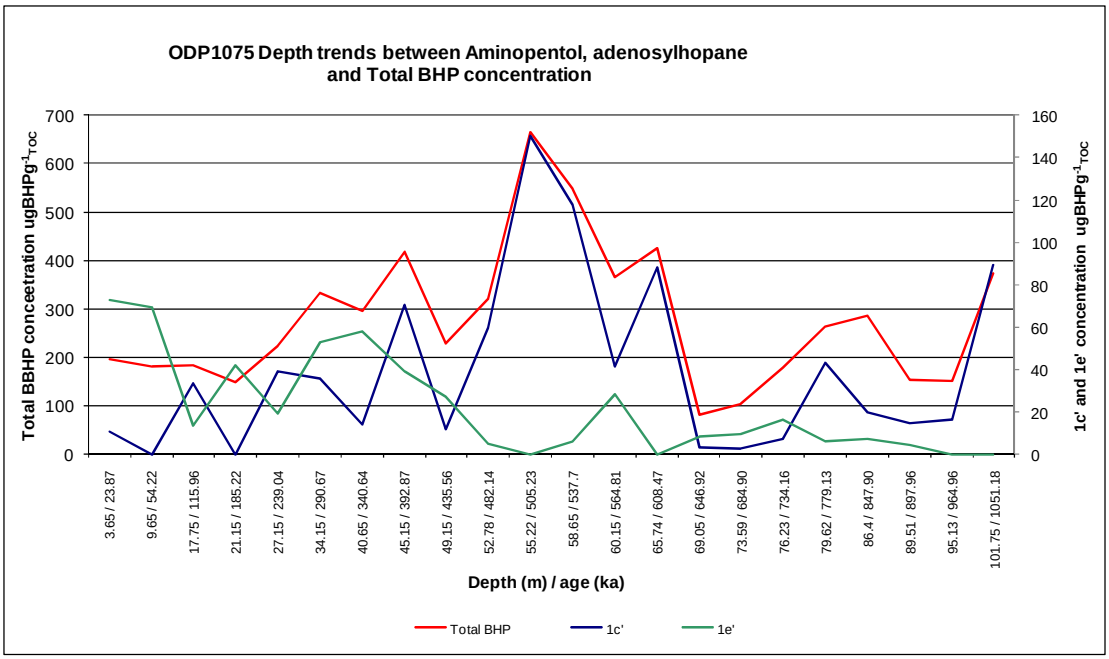


Fig. 6.7 Depth trends between aminotriol (**1f**), adenosylhopane (**1e**) and total semi-quantitative BHP concentrations ($\mu g BHP g^{-1}_{TOC}$) in ODP1075

6.5.3 Arctic Rivers Samples

LC-MS analysis revealed that up to 15 different BHP could be identified and quantified in the Arctic river and coastal sediments (Fig. 6.8). The total concentrations vary from 89 $\mu g_{BHP}\ g^{-1}_{TOC}$ (Mackenzie River sediment) to 613 $\mu g_{BHP}\ g^{-1}_{TOC}$ (Kolyma River estuary sediment; Table 2). The levels are low compared to soils (e.g. Cooke et al., 2008b and unpublished data), with the values from the Arctic sediments being approximately 25% to 50% of those in soils, similar to those found in the Congo Delta (Cooke et al., 2008a). A general increase in the total concentration of BHPs moving from west to east along the continental-scale climosequence of the Siberian Arctic can be observed, with the lowest concentration for the Ob River estuary sediment (236 $\mu g_{BHP}\ g^{-1}_{TOC}$) and the highest for the Kolyma River estuary sediment (613 $\mu g_{BHP}\ g^{-1}_{TOC}$). The two North American river sediments show significant differences in the total amounts. The sediment of the Yukon River has a total concentration of 552 $\mu g_{BHP}\ g^{-1}_{TOC}$, similar to the Kolyma River estuary sediment, and the Mackenzie River a total concentration of 89 $\mu g_{BHP}\ g^{-1}_{TOC}$, considerably lower than all the GRAR estuary sediments.

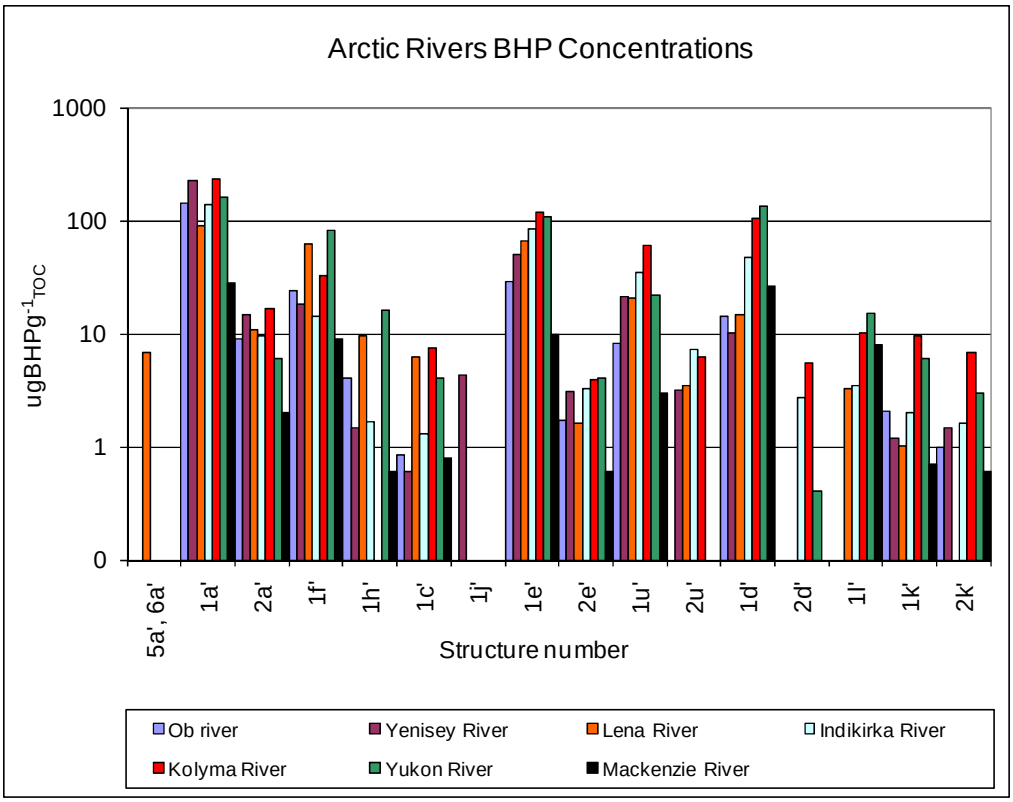


Fig. 6.8 Semi-quantitative BHP concentration ($\mu gBHPg^{-1}_{TOC}$) in Arctic River Sediments

Although some differences among them can be observed, all sediments generally have similar BHP assemblages. The distributions are all dominated by 5 different structures with the most abundant being bacteriohopanetetrol, with a contribution between 28% (Yukon) and 64% of total BHPs (Yenisey). The other abundant BHPs are aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**). Concentrations vary but, combined, these 5 BHPs comprise between 85% and 93% of the total The relative combined amount of adenosylhopane (**1e**), 2-Me adenosylhopane (**2e**), adenosylhopane type-1 (**1u**) and 2-Me adenosylhopane type-1 (**2u**) varies from 15 % (Mackenzie River sediment) to 37% (Indigirka River estuary sediment) of the total amount of BHPs.

6.6. Discussion

6.6.1 Rhone River

Analysis of the Rhone river sediments clearly demonstrates a reduction in soil marker BHPs (adenosylhopane (**1e**), 2-Me adenosylhopane (**2e**), adenosylhopane type-1 (**1u**)

and 2-Me adenosylhopane type-1 (**2u**)) with distance from the river mouth (Fig. 6.9). This distribution would be expected if the river mouth was the point source of the soil marker BHPs, with the reduction being due to dilution in the Mediterranean and degradation in the water column and surface sediments.

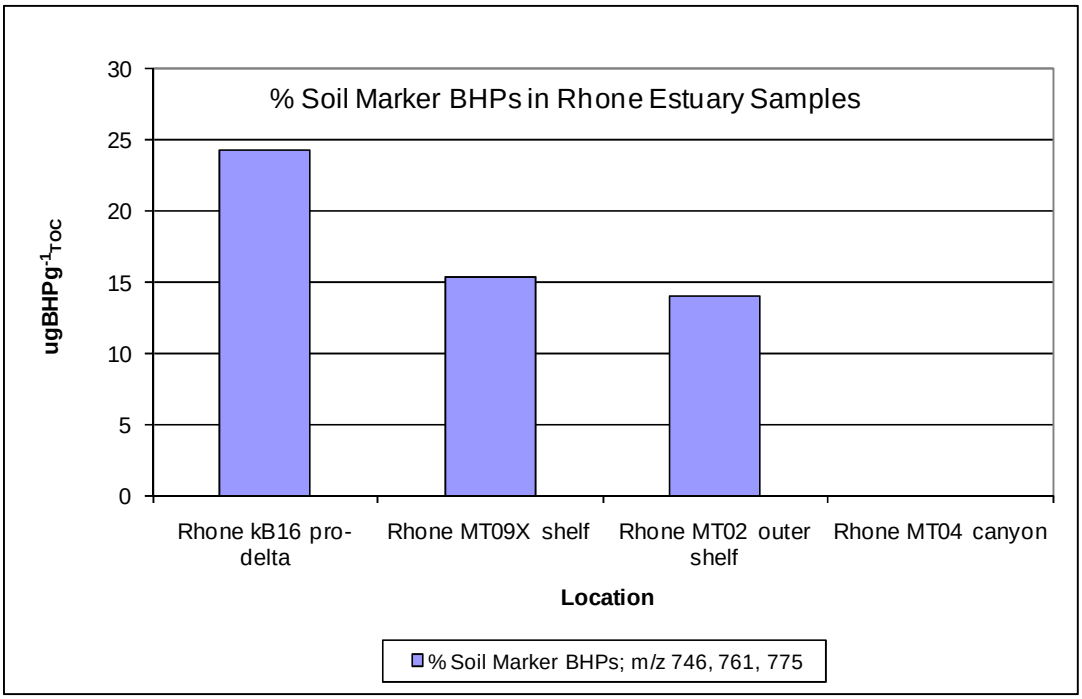


Fig. 6.9 Semi-quantitative soil Marker BHP concentration (% total BHPs) in Rhone estuary samples

6.6.2 Congo River

The concentration of adenosylhopane (**1e**) in the Congo fan surface samples (Figs. 6.5 and 6.6) clearly shows a decrease in adenosylhopane (**1e**) concentration with distance from the river mouth (Fig. 6.1). This is in accordance with the source of these BHPs being fluvially transported soil derived material (Cooke et al., 2008a). The decrease in the number of BHP structures identified with distance from river mouth and the rise in dominance of BHT (**1a**) corresponds with the BHP signature found in other non-marine sediments (e.g. Talbot and Farrimond, 2007) where sedimentary BHP profiles are dominated by BHT (**1a**), aminotriol (**1f**) and BHT cyclitol ether (**1d**) and therefore a sediment derived BHP signature whereas sample 4913 implies a mixed signature with some terrestrial derived BHPs and some sediment derived BHPs.

The remarkable preservation of BHPs in the ODP 1075 core results in the most diverse range of BHP structures found in samples of this age (Cooke at al., 2008a). This is the first time that adenosylhopane (**1e**), aminotetrol (**1h**), aminopentol (**1c**), BHpentol cyclitol ether (**1l**) and BHhexol cyclitol ether (**1k**) have been observed in a continuous sedimentary sequence to an age of ~0.85 Ma years (~89 mbsf). A limited number of less source-specific BHPs have previously been observed in sediments from the Benguela Upwelling to an age of ~4.5 Ma (Watson 2002; Talbot et al., unpublished data) and bacteriohopanetetrol has been identified in sediments up to 50 Ma years old (van Dongen et al., 2006). Although the recalcitrance of the triterpenoid ring system is well established the survival of the side chain functional groups is less clear. If diagenetic transformation of the side chain occurs quickly then the presence of intact BHPs at depth could indicate the presence of living deep sub-surface bacteria (Saito and Suzuki, 2007). However, the preservation at depth of markers for aerobic methane oxidising bacteria aminotetrol (**1h**; e.g. Neunlist and Rohmer, 1985b; Cvejic et al., 2000) and aminopentol (**1c**; e.g. Neunlist and Rohmer, 1985a; b; Cvejic et al., 2000; Talbot et al., 2001), indicative of water column or surface sediment processes, suggests that the rate of diagenetic transformation of the side chain in these samples is slow, enabling BHPs to be used to study ancient systems. The mirroring of changes in aminopentol (**1c**) concentrations with the total BHP concentrations (Fig. 6.6) indicates that the 2 concentrations are linked, whereas there is no direct relationship with adenosylhopane (**1e**) which decreases with depth (Fig. 6.6 and 6.9). A high input of organic material into the estuary could result in a high sedimentation rate which will promote the burial of organic material and the release of methane. This methane will subsequently be oxidised in the water column by methanotrophs giving a rise in aminopentol (**1c**) concentration. Therefore the BHP profile in ODP 1075 indicates an input of terrestrial BHPs (e.g. adenosylhopane; **1e**), water column BHPs, aminopentol (**1c**), and sediment derived BHPs, e.g. the reduced number of BHP structures when compared to soil and the dominance of BHT (**1a**).

Analysis of a wide range of soils (Cooke et al., 2008b, Xu et al., 2008, Redshaw et al., 2008 and this thesis) indicates that on average the soil marker BHPs (adenosylhopane; **1e**) account for approximately 28% of the total BHP concentration. In the Congo sediment samples there is decrease in contribution of the soil marker BHPs to the total sediment BHP content with depth, from 38% in the surface to 0% in the deepest samples. This is mirrored by a corresponding rise in the contribution of anhydroBHT

(**1j**; Fig. 6.8). AnhydroBHT (**1j**) has been found in numerous sediments at depth (e.g. Watson 2002, Bednarcyzk et al 2005) and may be a product of the diagenesis of the non-methylated soil markers by the reductive removal of adenine from adenosylhopane (**1e**). However anhydroBHT (**1j**) could be derived from other BHPs such as BHT (**1a**), the most common BHP in sediments, via a cyclisation / dehydration reaction (e.g. Costantino et al., 2001; Schaeffer et al., 2008). These Congo sediment samples show an increase in anhydroBHT (**1j**) reflects the reduction in adenosylhopane (**1e**) (Fig. 6.9), whereas there is no clear change in concentration in BHT (**1a**) with depth (Fig. 6.9).

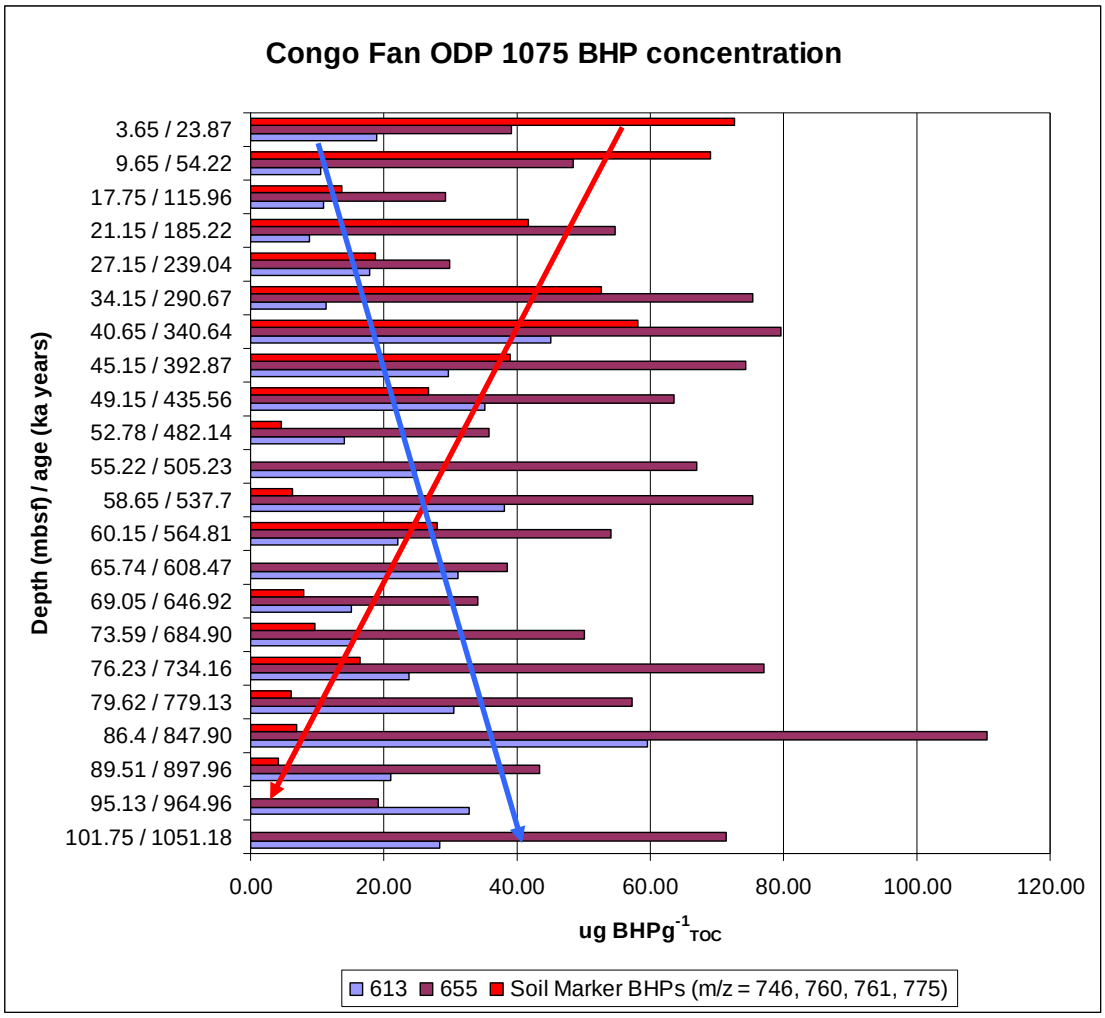


Fig. 6.10 Relationship between AnhydroBHT (**1j**), BHT (**1a**) and semi-quantitative soil marker BHPs concentration ($\mu gBHPg^{-1}_{TOC}$) down ODP 1075

The variation in soil marker concentration moving down the core (Fig. 6.11) shows a general decrease with depth and age due to diagenesis (Cooke et al., 2008a). There are

4 distinct peaks that appear to indicate increases in flux of SOC into the sediment indicating climatic conditions that would promote erosion and soil transport.

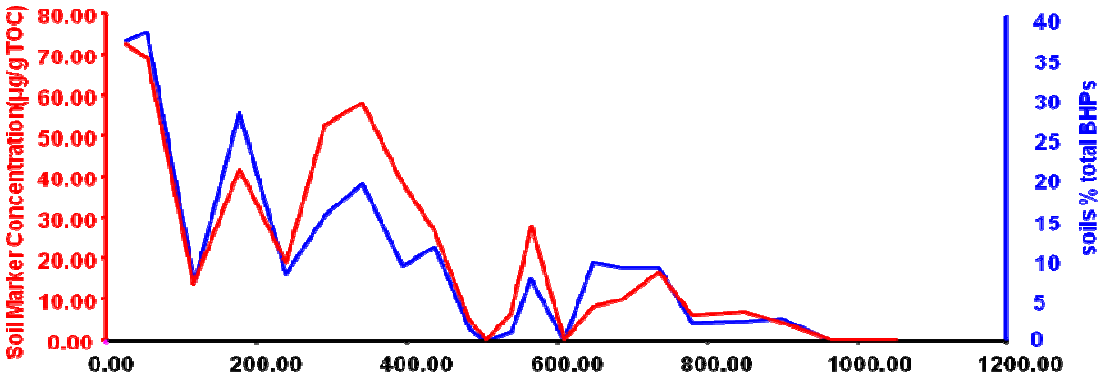


Fig. 6.11 Semi-quantitative soil BHP marker concentration in late Quaternary sediments from the Congo fan (ODP 1074)

6.6.3 Arctic River Samples

Up to 15 different BHPs were found in the Arctic river and coastal sediments, with different potential origins (Table 2.2). The most abundant BHPs; BHT (**1a**), aminotriol (**1f**) and BHT cyclitol ether (**1d**), are commonly found in most environments and have been linked to many different organisms (e.g. Talbot and Farrimond, 2007).

Adenosylhopane (**1e**) and the related soil markers have been identified in purple non-sulfur bacteria (e.g. Talbot et al., 2007), two proteobacteria *Nitrosomonas europaea* (ammonia oxidising bacterium; Seemann et al., 1999) and *Bradyrhizobium japonicum* (nitrogen fixing bacterium; Bravo et al., 2001). 2-Methyl adenosylhopane (**2e**) has been identified in *Bradyrhizobium japonicum* (Talbot et al., 2007). More importantly, these three structures, and a fourth with no known bacterial source, have been linked together as markers for SOM (Talbot and Farrimond, 2007; Cooke et al., 2008a).

A known marker for Type I methanotrophs is aminopentol (**1c**), but this component is rarely found in soil (Redshaw et al, 2008, Cooke this report). In contrast, aminotetrol (**1h**), another less specific marker for methanotrophs, is commonly observed in soil samples (Cooke et al., 2008b). However, it is also biosynthesised by some *Desulfovibrio* spp. (Blumenberg et al., 2006; Blumenberg 2009) Hence, it is the presence of aminopentol (**1c**) that is most directly indicative of methanotroph activity.

With the exception of aminotetrol (**1h**) in the Kolyma River estuary sediment, both methanotroph markers are present in all sediments (Fig. 6.8). Although they only account for an average of 2% of the BHPs this is comparable with soils studied to date where these methanotroph markers account for approximately 3% of the BHPs (Redshaw et al., 2008 and this thesis). Methanotrophs generally inhabit water columns, surface sediments, soils and peats (e.g. Hanson and Hanson, 1996). The source of these BHPs in the samples may therefore be from the water column or from methanotrophs in the surface sediment and not necessarily from the soils. Strong methane out gassing from thawing sub-sea permafrost has recently been demonstrated for the Siberian Arctic coastal zone (Semiletov, 1999; Shakhova et al., 2005; Semiletov et al., 2007; Shakhova and Semiletov, 2007). However, an origin for the methanotroph-derived BHPs further up the river system cannot be discounted at this stage as the methane rich-wetland and permafrost drainage basins of these rivers would also be a methanotroph-rich environment (Knoblauch et al., 2008). Further investigation of the river sediments and surrounding terrestrial environment will give a clear indication as to the origin of the methanotroph-derived BHPs. Overall BHP analysis indicates a contribution to the Arctic coastal and river sediments from a variety of different bacteria including purple non-sulfur bacteria, cyanobacteria and methanotrophs.

The general distribution patterns of BHPs in the Arctic samples are comparable to that found in the Congo Estuary (Cooke et al., 2008a), but show on average a lower number of different components than observed in soils (Cooke et al., 2008b). Soils contain a wider range of typically up to 22 BHPs (Cooke et al., 2008b; Redshaw et al., 2008; Xu et al., 2009) with tetra-, penta- and hexafunctionalised components being routinely present. Here, the number of structures identified is limited to between 11 (Ob estuary sediment) and 15 (Indigirka and Mackenzie estuary sediments). The BHP signature of all river estuary sediments is dominated by 5 components; bacteriohopanetetrol, aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**). This is consistent with the BHP signatures of soils (Cooke et al., 2008b and unpublished data). As discussed above the soil marker BHPs have been identified as being present in soils and absent from open marine and most lacustrine sediments, except where there is a high soil input (Talbot and Farrimond, 2007; Cooke et al., 2008a). These soil marker BHPs are all present in the estuary sediments and account for between 15% (Mackenzie) and 37% (Indigirka) of the total

BHPs (Fig. 6.8). This is consistent with the levels found in a wide range of soils studied to date, where the average level of these soil-specific components is 28% (Cooke et al., 2008a, b, Xu et al., 2008, Redshaw et al., 2008 and this report). Presently, no specific BHP concentrations from the tundra and taiga soils or peats of the studied areas are available, so any relationships between the sediment BHP profile and this potential source material BHP profile can only be described as tentative at this stage. The other BHPs in the Arctic estuary sediments, which account for up to 85% of the total, include the only three known to occur commonly in marine settings, BHT (**1a**), aminotriol (**1f**) and BHT cyclitol ether (**1d**; Talbot et al., unpublished data), but these are also present in soils, and others more commonly associated with lacustrine or terrestrial environments (Talbot and Farrimond, 2007). Presently, these BHPs cannot be readily used to identify the environmental origin of the bacterial source directly.

Several earlier studies already suggested a predominance of terrOM in Arctic sediments (e.g. Yunker et al., 1995, 2002; Peulve et al., 1996; Fahl et al., 2003; Guo et al., 2004; Stein and Macdonald, 2004; Semilitov et al., 2005; Drenzek et al., 2007; van Dongen et al., 2008). Analysis of the lipid biomarker composition (*n*-alkanes, *n*-alkanoic acids, *n*-alkanols, triterpenoids, glycerol dialkyl glycerol tetraether lipids and steroids), geochemical bulk parameters (total organic carbon/total nitrogen [TOC/TN] ratios and $\delta^{13}C_{TOC}$) and pyrolysis gas chromatography-mass spectrometry (Py-GC/MS) analysis of estuary sediments along the same continental-scale Russian climosequence as used herein, already revealed a predominance of terrestrially derived OM in these sediments (Guo et al., 2004; Semiletov et al., 2005; van Dongen et al., 2008). Furthermore, the overwhelming presence of terrestrial biomarkers, in combination with the complete absence or only minor presence of marine biomarkers, has also been shown in several other Russian, as well as Canadian/American, Arctic sediment studies (e.g., Yunker et al., 1995, 2002; Peulve et al., 1996; Fahl et al., 2003; Stein and Macdonald, 2004). The BHP compositions in the present study further support the significance of terrOM in coastal Arctic sediments. The distribution indicates that, although the OM is predominantly terrestrially derived, it is possible that some transformation and diagenesis has occurred, reducing the number of BHP structures present.

Whilst diagenesis must be considered as one of the explanations for the observed BHP profile other observations indicate that there may be other explanations. The

results for the Indigirka have the highest soil marker BHP level (37%) and lowest level of tetra-functionalised BHPs (60%) of all the samples (Fig. 6.12), implying the least diagenesis/transformation, yet the Indigirka samples were taken from the greatest water depth (8-11 m). Further investigation of the river sediments is required to compare the source material with the estuary sediments as a wide variation is known to occur (Yunker et al., 1993, 1995). A further possibility is that this BHP distribution is typical of the Arctic river drainage basins, where permafrost conditions may limit the range of bacteria active in the soil and therefore reduce the number of BHP structures present.

A relative increase in the percentage levels of soil marker BHPs from west to east along the transect can be observed (Fig. 6.11). There is also a corresponding drop in the percentage levels of tetra-functionalised BHPs, specifically BHT (**1a**), with those of the penta- and hexa-functionalised BHPs remaining relatively consistent at a combined total of less than 5% along the same transect (Fig. 6.12). The general increase in the abundance could be caused by an increase in microbial activity, enhanced contribution of SOM to the sediment or enhanced preservation of BHPs as a result of the environmental conditions in the eastern Siberian sampling sites. Although an increase in microbial activity cannot be excluded, the latter options are probably more likely. Earlier studies already showed a lower degree of decomposition of the OM towards the east (Guo et al. 2004; van Dongen et al. 2008). The relative increase in the percentage levels of soil marker BHPs eastwards can be caused by a relative increase in the input of soil-derived material to the sediment. However, van Dongen et al. (2008) have shown, using both bulk geochemical and a broad suite of biomarker analyses, that the OM across the entire continent-scale climosequences of the coastal Siberian Arctic are overwhelmingly dominated by terrestrial sources. This makes it very unlikely that a higher input of soil-derived material is the main cause of the observed increase, although such a contribution cannot totally be excluded. Therefore, the general increase in the relative contribution of soil BHPs may also reflect a decrease in extent of diagenesis from west to east.

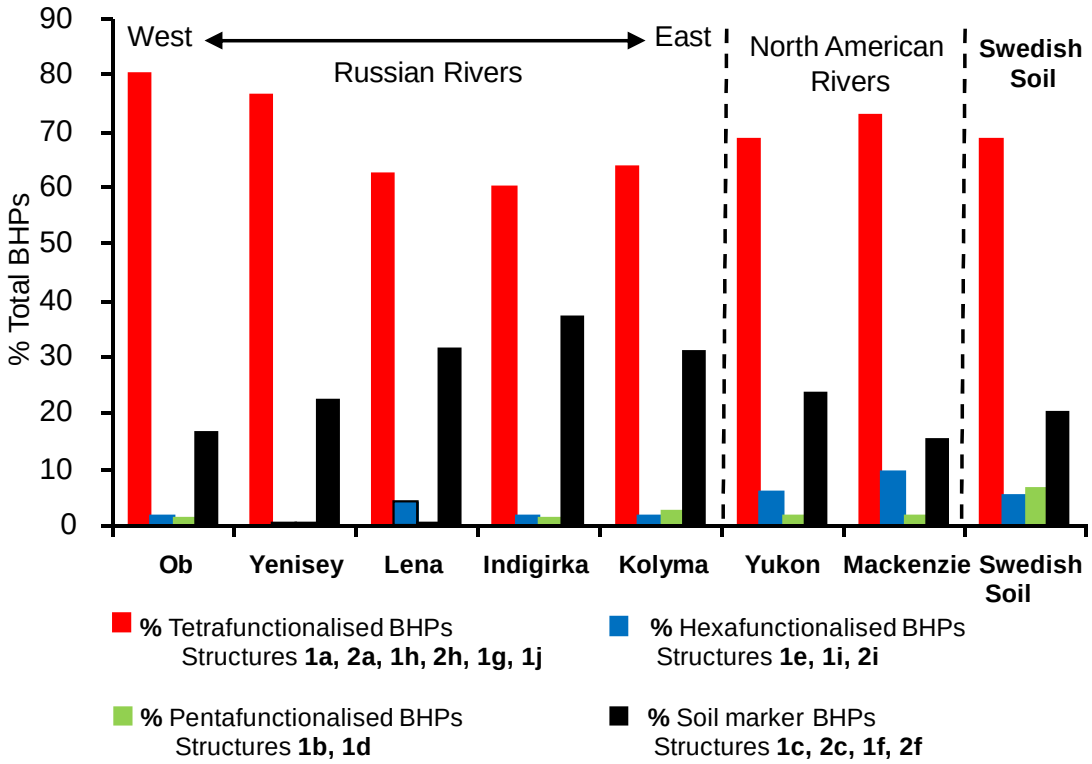


Fig. 6.12 Variation in % soil marker, Tetra-, Penta- and hexafunctionalised BHPs across the climate sequence. From Cooke et al., 2009.

Like the GRAR estuary sediments, the BHP signatures of the Yukon and Mackenzie River sediments indicate a predominant soil origin that has undergone some degree of functional group reduction and transformation via diagenesis. The Mackenzie River sediment contains 14 different structures and a relatively low percentage of soil-specific BHP marker compounds (15%), whereas the Yukon River sediment contains 13 different structures and contains 23% soil-specific BHP marker compounds (Figs. 6.11).

There is a significant difference in BHP concentrations between the sediments of the Yukon River, west of the Mackenzie Mountains, and the Mackenzie River to the east of the mountain range. For the Mackenzie, the low BHP level (89 $\mu g_{BHP}\ g^{-1}{}_{TOC}$), coupled with the low percentage of BHP soil markers, may indicate that there is low terrestrial inputs to the sediment at this location or that the overall BHP-producing bacterial signal in this river is weak. The relatively high number of BHP structures indicates that this reduction is unlikely to be due to degradation of the BHP signature. The Yukon has a much higher BHP level (552 $\mu g_{BHP}\ g^{-1}{}_{TOC}$) and soil marker BHP percentage, suggesting that, compared with the Mackenzie River, there is a much greater input of terrOM, including soil-derived material to the sea. The number of

BHP structures in the Yukon River sediment is comparable to that in the Mackenzie River and the eastern GRAR estuary sediments (Fig. 2). This implies a relatively high degree of preservation of old soil organic carbon, which could result from the large amount of the permafrost present (Guo and Macdonald, 2006; Guo et al., 2007) and the relatively shorter summer thaw when compared with the Ob River, where there are fewer preserved structures.

6.7. Summary

Goal 7

> *Identify complex intact BHPs from aerobic bacteria in ancient marine sediments.*

The identification of aminopentol (**1c**), a marker for aerobic methane oxidation at all depths in the ODP1075 core (Fig. 6.5) indicates that complex BHP structures are preserved intact during burial to an age of at least 1 million years in marine sediments. There is also an underlying diagenetic trend of reduction in concentration of BHPs, including the soil markers, over time together with a corresponding increase in the possible adenosylhopane (**1e**) diagenetic product anhydroBHT (**1j**; Schaeffer et al., 2008).

The variability of the BHPs with depth may be linked to periods of high soil input into the sediment indicating periods of increased soil instability. These initial results suggest that the new BHP markers have the potential to serve as a powerful tracer for SOM to measure both the current and ancient movement of soils by rivers

Goal 8

> *Identify the distribution of potential soil organic carbon (SOC) markers Adenosylhopane (**1e**) and its related homologues (the soil markers) in sediments to test their preservation and potential to track movement of SOC via rivers to marine systems.*

Identification of proposed soil specific BHP marker compounds adenosylhopane (**1e**), 2 methyl adenosylhopane (**2e**), adenosylhopane type-1 (**1u**) and 2 methyl adenosylhopane type-1 (**2u**) in all the estuary sample and the obvious decrease in concentration with distance from river mouth in the Congo and Rhone samples supports these recalcitrant molecules to be used as proxies for the movement and burial of SOC by rivers. The decrease in concentration with distance indicates progressive dilution by marine derived organic matter or diagenesis in the water column.

Goal 9

> *Compare the BHP profile with other biomarkers and compare with the carbon cycling across the 7 Arctic rivers.*

The increase in the BHP concentration and specifically the soil marker BHPs moving to the East in the Arctic Rivers is in agreement with other investigations into polysaccharides (Guo et al., 2004), sterols and *n*-alkanoic acids (van Dongen et al., 2008) which show an increase in labile TOM moving to the east where there is a larger abundance of permafrost and a shorter average length of the summer thaw. This will increase the preservation of these terrestrial structures in rivers and therefore give a greater TOM signal when compared to the western samples where the relatively warmer climate enables a longer period of microbial activity and a greater time to enable degradation of TOM.

6.8 Conclusions

Analysis of ODP 1075 shows a remarkable preservation of intact BHP structures to an age of 1Ma which contradicts previous studies suggesting rapid degradation due to diagenesis and the subsequent formation of geohopanoids (e.g. Rohmer et al., 1980; Quirk et al., 1984; Bissaret et al., 1997; Innes et al., 1997; Rodier et al., 1999; Tritz et al., 1999; Watson and Farrimond, 2000), or the rapid incorporation into kerogen (Farrimond et al., 2003). A total of 15 different structures were identified and adenosylhopane (**1e**), aminotetrol (**1h**), aminopentol (**1c**), BHpentol cyclitol ether (**1l**) and BHhexol cyclitol ether (**1k**) were all identified continually down the core (Fig.

6.5), indicating that there is significant preservation of intact BHPs. There is no clear trend in total BHP concentration with depth, possibly indicating that the peaks in BHP concentration are related to peaks organic matter input into the sediment. The research into soils (Chapters 3 and 4) indicates that there is rapid turnover in BHPs in aerobic conditions and therefore the anaerobic conditions that would be expected in these sediments must enable preservation of the intact BHPs.

The decrease in adenosylhopane (**1e**) with distance from river mouth or both the Rhone and the Congo (Figs. 6.4 and 6.7) indicates that adenosylhopane (**1e**) is a terrestrially derived BHP. These results appear to confirm that the observed soil marker BHPs, and possibly the unobserved but related 2 methyl adenosylhopane (**2e**), are all markers for terrestrial organic matter.

The investigation of BHPs in the Arctic region has demonstrated that it is possible to use BHPs as markers to examine the export of TOM across the Arctic land-sea interface. The general BHP distribution patterns were comparable to those recently observed in Congo estuary sediments. The Arctic estuary surface sediments contain soil-specific BHP marker compounds at levels comparable to those in soils themselves, indicating a significant soil input to the sediment. This is in agreement with earlier studies suggesting a high flux of fluvially transported TOM to the Arctic shelf systems. In addition, the BHP signatures indicate that the TOM transported via the estuaries had undergone a degree of transformation of the functional groups by way of diagenesis. This resulted in a reduction in the number of BHPs relative to the number found in a typical soil.

Comparable to earlier studies, clear trends in molecular composition can be observed along the continental-scale climosequence of the Siberian Arctic. Both a general increase in the total abundance of BHPs and a relative increase in the percentage of soil marker BHPs can be observed from west to east along the transect. This implies most likely a greater preservation of TOM due to the higher abundance of permafrost, in combination with a shorter average length of the summer thaw period in the east.

The predicted amplified warming in the Arctic continental region, particularly in east Siberia, could have consequences, including an increase in summertime soil active-layer thickness and a reduction in the volume of the permafrost. This may have consequences for the release and degree of decomposition of the TOM, including the BHP concentrations and patterns in these regions and consequently could affect the composition of the TOM delivered to the Arctic Shelf.

Combined with Siberian Arctic and North American Arctic river samples, a general pan-Arctic distribution pattern of BHPs can be identified, with the Indigirka River basin being the epicentre with the highest amounts soil marker BHPs, a trend similar to the distribution and abundance of permafrost in the Arctic region.

Overall these results clearly demonstrate the potential for BHPs to be used as a biomarker when investigating the transport of TOM via rivers to marine environments and agree with the results obtained using the BIT index for the Congo Fan and West Africa (Hopmans et al., 2004) and the Rhone (Kim et al., 2010). These global results will enable soil marker BHPs to be correlated with the BIT index and will provide an additional piece to the jigsaw that is TOM enabling a fuller understanding of the sources and fate of TOM as the soil marker BHPs and BIT index have different source organisms and relate to different terrestrial activities.

7. Investigation in to Global Soils

7.1 Introduction

It has been demonstrated in Chapters 3, 4 and 5 that soils from the NE of England all have BHP profiles that are dominated by 4 or 5 BHPs and have up to 20 other minor BHPs that complete the profile. The differences between the BHP profiles have enabled the use of PCA to differentiate between the soils (Figs. 3.38 and 4.15). These soils are from geographically similar locations with a temperate climate and with a similar geological history being formed from post glacial till at the end of the Devensian glaciations, approximately 10,000bp (Woodcock and Strachan, 2000). Therefore any conclusions drawn from these results are at best region specific. To be able to apply the data obtained to the global scale a much wider range of soils with a variety of geological and geographical origins must be analysed for their BHP profile. This will enable the assumptions made regarding the source bacteria of the BHPs to be further tested in a variety of environments where different bacterial processes could be dominating. The BHP results obtained will be compared to the current understanding of the bacterial activity in these soils to further confirm the use of the BHPs as markers for specific bacterial activity.

Soils were obtained from a N-S transect of Sweden, a forest fire affected soil in Seville, Southern Spain, Vietnamese paddy fields, Lake District peat deposits, SW England allotments treated with human sewage waste and Canadian soils from the province of Alberta. All samples were collected by other researchers before being extracted and analysed in accordance with the method described in chapter 2. The results from the SW allotments were subsequently reported by Redshaw et al., 2008, and the Canadian soils were reported by Xu et al., 2008 and will not be discussed in detail in this thesis although their BHP profiles will be used during the statistical analysis of the data.

7.1.1 Goals

The broad aim of this phase of the research is to test hypothesis 1

The BHP fingerprint of a soil indicates the current bacterial population.

By achieving the following goal

Goal 10

Identify the distinct BHP profiles of soils from different global locations.

It has been previously demonstrated that although soil BHP profiles are very similar being dominated by 4 or 5 major BHPs, there are sufficient differences between soils to enable differentiation of these soils; Palace Leas (Chapter 3), Hack Hall Farm (Chapter 4) and landfill sites (Chapter 5). It would be expected that by analysing a much more disparate group of soils these differences will increase and a clear separation between the soils will be obtained simply by using the BHP profile.

7.2 Swedish Soils

7.2.1 Introduction

This study represents the geographically widest range of samples analysed in this thesis with samples taken in a north – south transect of Sweden (Table 7.1 and Fig. 7.1) covering a range of approximately 600 km from southern Sweden to within the Arctic Circle. Research has shown that there are major differences between the soil microbial population in colder northern environments where fungal processes dominate to the warmer southern environments where the contribution by bacteria increases, although still less than the fungal activity (Schroter et al., 2003). This is analogous to the differences seen in upland and lowland pasture where the more extreme upland climate promotes fungal dominated biomass (Grayston et al., 2001). This change in composition of biomass is closely related to soil fertility with low fertility environments being dominated by fungi and high fertility environments by bacteria (Grayston et al., 2001; Pennanen et al., 1999). For the tundra environments in the Arctic two seasonal shifts can be observed that promote bacterial activity in the soil at the expense of fungal activity, a post snow melt shift and a summer shift (Bjork et al., 2008).

The permafrost in the more northern regions also acts to preserve the soil organic matter which upon thawing can be transported in a preserved state to marine environments (van Dongen et al., 2008; Cooke et al., 2009) or can be degraded to release methane (Schuur et al., 2009). The release of methane from thawing permafrost will promote the growth of methanotrophs, and the identification of a strong BHP methanotroph signature in the form of aminotetrol (**1h**) and aminopentol (**1c**) would indicate this process.

The Swedish soil samples were collected by Dr Bart van Dongen (University of Manchester) between 29/5/2005 and 4/6/2005. At each location 2 samples were taken of varying depths down to 25 cm, above the permafrost layer, and a variety of soils were encountered from rich humic soils in the top layer to sands and gravels in the bottom layer (Table 7.1). All samples were delivered to Newcastle as air dried samples and were subsequently extracted and analysed in accordance with the methods described in chapter 2.

Table 7.1. Sampling locations and soil types for the Swedish soils

Sample	Location	GPS (N)	GPS (E)	depth (cm)	comments
1a	Sättra	59° 37.603'	18° 26.299'	0-15	light brown, contains plenty of pine needles
1b				15-25	dark black, almost no pine needles
2a	ca 20km North of Gävle	60° 54.366'	17° 01.252'	0-15	black, humus rich, pine needles
2b				15-20	brown , clear colour distinction from upper layer
3a	ca 20km North of Sundsval	62° 32.341'	17° 33.817'	0-5	black, humus rich, roots and grass
3b				5-15	gray-bright brown, mineral
4a	ca 50 km South of Umea	63° 27.520'	19° 15.552'	0-5	black, humus
4b				5-10	gravel and stones
5a	20 km North of Pitea	64° 53.150'	21° 01.667'	0-5	humus, dark
5b				5-20	gray and yellow-brown, sand
6a	Kamlunge	66° 0.124'	22° 50.756'	0-15	roots, humus, black-brown
6b				15-25	mineral, brown
7a	Polar circle, Lulea - Jokkmok road	66° 33.247'	20° 06.902'	0-4	humus, roots
7b				4-15	sand, brown and partly orange

Fig. 7.1 Sample locations for Swedish soils. Map from www.umsl.edu.

7.2.2 Results

Site 1 contained 19 different BHP structures (Fig. 7.2) with a concentration of 532 $\mu g_{BHP}\ g^{-1}$dry soil in the 0-15 cm layer and 705 $\mu g_{BHP}\ g^{-1}$dry soil in the 15-25 cm layer. The profiles are dominated by 4 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**), accounting for 75.7% of the total BHP concentration. All BHPs have an increase in concentration with depth except for aminotetrol (**1h**) and unsaturated BHT pentose (**5n** or **6n**), both of which decrease with depth.

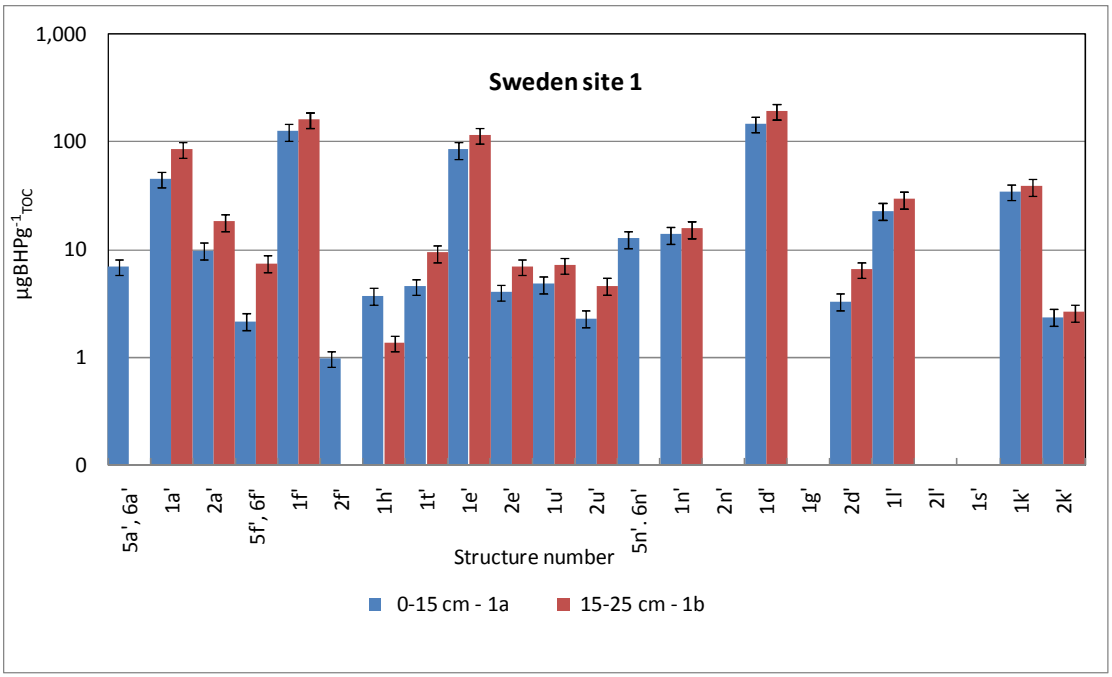


Fig 7.2Log graph distribution of semi-quantitative BHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 1

Site 2 (Fig. 7.3) also contains 19 different BHP structures but has a lower BHP concentration than site 1 with the 0-15 cm layer having a BHP concentration of 204 μg_{BHP} g^{-1}dry soil and the 15-20 cm layer 45 μg_{BHP} g^{-1}dry soil. The BHP profile is dominated by aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) and all BHP concentrations decrease with depth although there is no decrease in the observed number of structures.

Site 3 (Fig. 7.4) again contains 19 BHP structures and is dominated by BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**). The BHP concentrations are 20 μg_{BHP} g^{-1}dry soil, 0-5 cm layer, and 27 μg_{BHP} g^{-1}dry soil in 5-15 cm layer. Again there is a decrease in BHP concentration with depth for all BHPs and no decrease in number of BHP structures with depth. Unlike sites 1 and 2 BHT glucosamine is present as a minor component of the BHP profile.

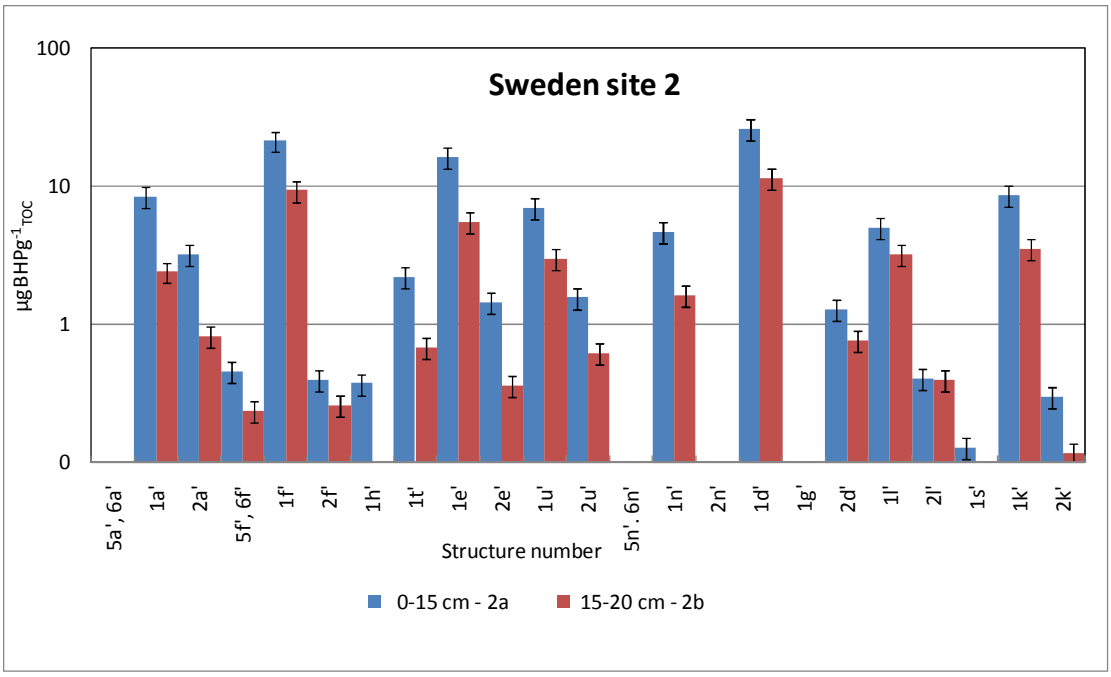


Fig 7.3 Log graph distribution of semi-quantitative BHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 2

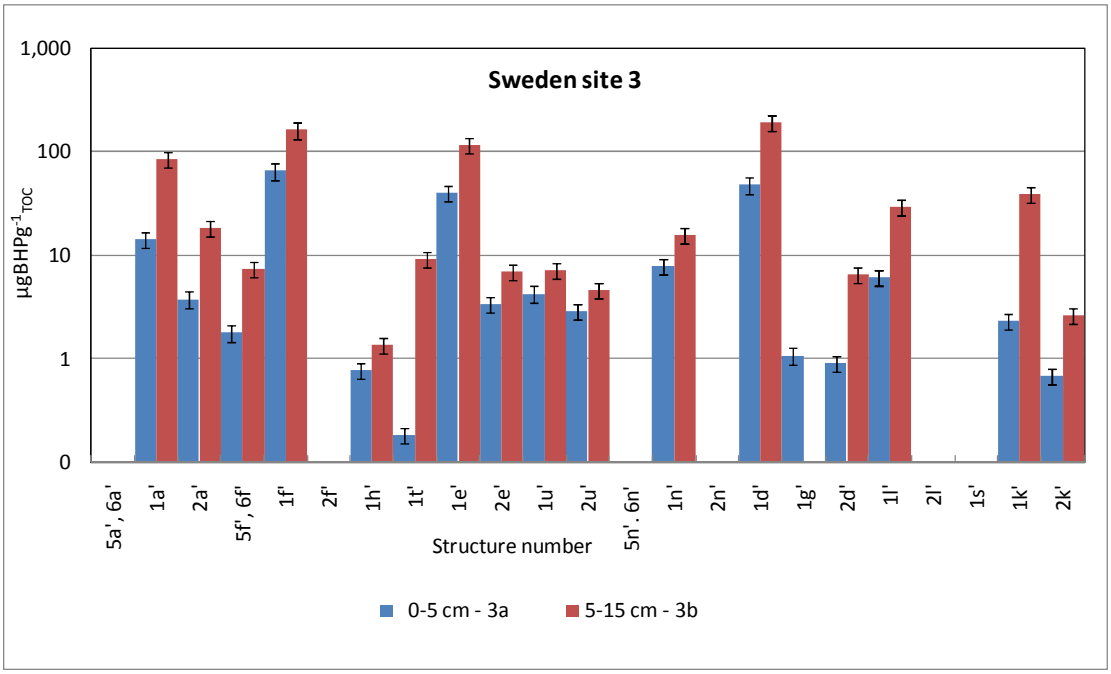


Fig 7.4 Log graph distribution of semi-quantitative BHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 3

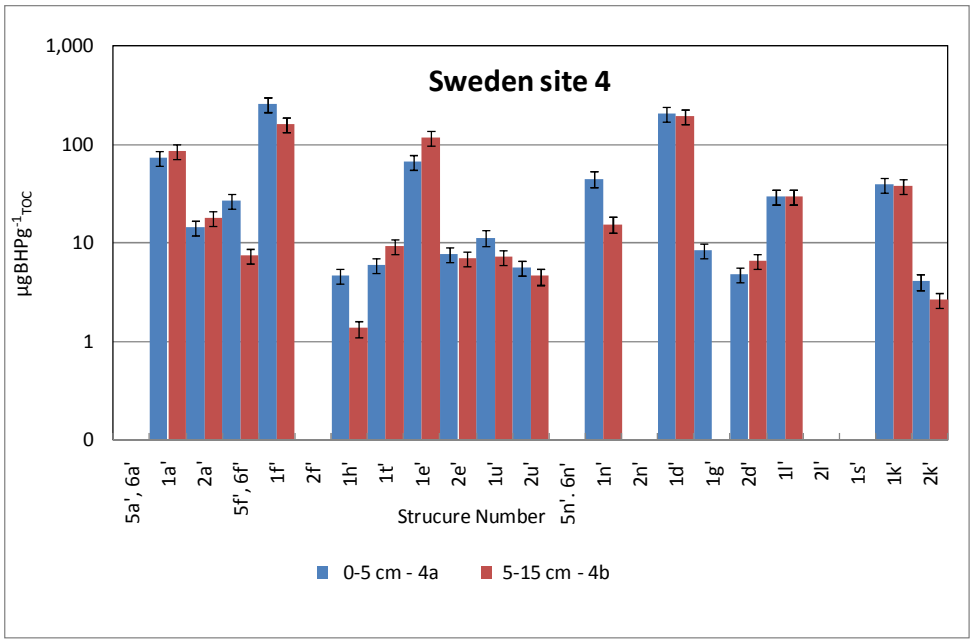


Fig 7.5 log graph distribution of semi-quantitative BHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 4

Sites 4, 5 and 6 all have a dramatic decrease in the concentration between the top 0-5 cm layer (0-15 cm in site 6) and the underlying layer where the concentration of BHPs is between 1.6% (site 4) and 6.9% (site 6) of the overlying layer. Despite these significant decreases with depth the actual number of BHP structures rises in site 5 from 12 in 0-5 cm, to 17 in 5-20 cm and in site 6 from 10 to 16 structures. All the sites are dominated by aminotriol (**1f**) and BHT cyclitol ether (**1d**).

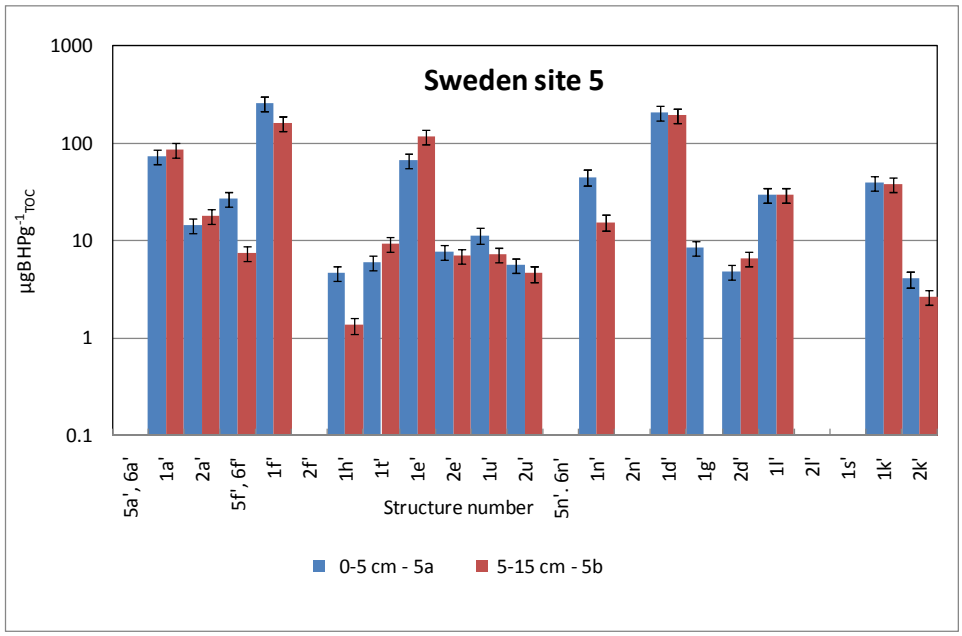


Fig 7.6 Log graph distribution of semi-quantitaitveBHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 5

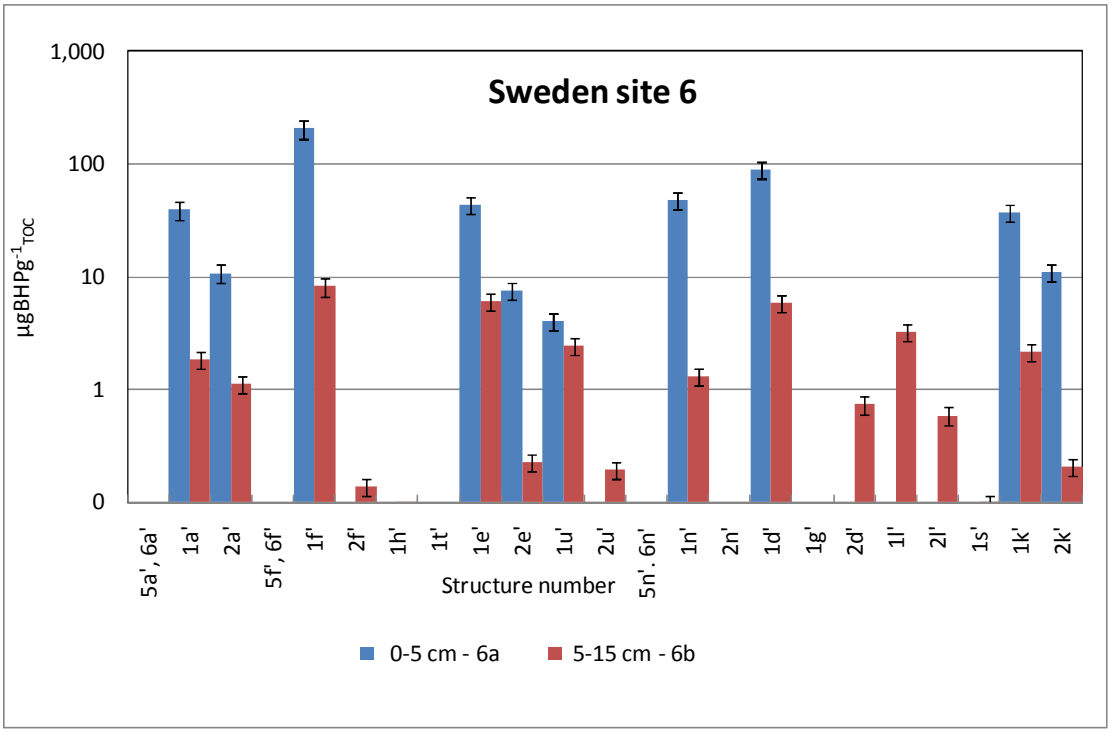


Fig 7.7 Distribution of BHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 6

Site 7 (Fig. 7.8) is the most northern site just inside the Arctic Circle. The BHP profile contains 12 BHPs in both depth layers and there is an increase in the BHP concentration for all BHPs in the lower 4-15 cm layer. The BHP profile is dominated by aminotriol (**1f**) which accounts for 53% of the total BHP concentration in both the 0-4 cm and 4-15 cm layers. BHT (**1a**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) are the next most significant BHPs and account for 36% in top layer and 34% in bottom layer of total BHP concentration.

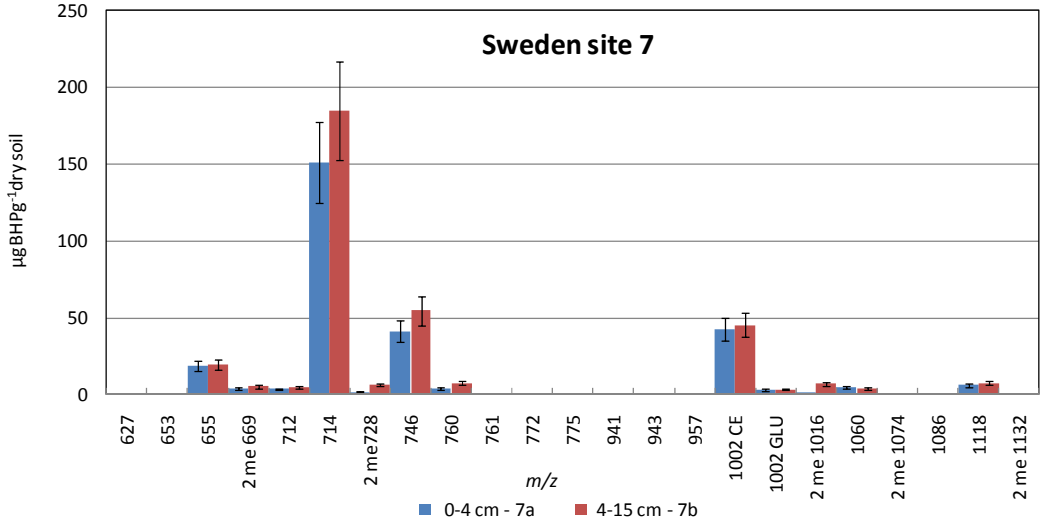


Fig 7.8 Distribution of BHP concentration (μg_{BHP} g^{-1}dry soil) for Sweden soil 7

7.2.3 Discussion

The depth of each top sample was based the depth to the next visible soil type (table 7.1) and this variation appears to provide an insight in to the observed BHP concentrations. Site 1 showed an increase in BHP concentration with depth and the description of the soils is of an upper light brown layer overlying a dark lack layer. Dark soil colour is an indicator of an organic carbon rich soil (White, 2006) and a possible accumulation of organic carbon that is leached from the upper layer (although without TOC values this cannot be verified). This observation appears to be confirmed by the presence of cyanobacteria marker BHPs in the lower layer (Table 7.2a), although at a slightly reduced % contribution to the BHP total concentration than in the top layer (7.5% in top layer compared to 5.6% in lower layer). Cyanobacteria are associated with near surface environments (White, 2006) and the occurrence of these BHPs at below 15 cm indicates leaching and accumulation. The methanotroph marker aminotetrol (**1h**) is present in greater quantities in the top layer than in the lower layer indicating that the majority of CH_4 oxidation is occurring in this layer, although the concentration is low when compared to other locations such as Palace Leas (Chapter 3) and Hack Hall Farm (Chapter 4). This implies that there is little CH_4 generation in this soil which is unexpected as the black sub soil would be expected to be anoxic and organic carbon rich (White, 2006). Methanotroph activity can be inhibited by high levels of NH_3 which compete for the CH_4-binding site in

methane mono-oxygenase (Seghers et al., 2003; Hutsch 1998) so a high level of ammonia oxidising bacteria would indicate an inhibition of methane oxidation.

The level of BHPS indicating PNSB, N_2 and NH_3 fixing bacteria in this site is comparable to that seen in other sites, e.g. Palace Leas and Hack Hall. It has been previously reported that the soils in Southern Sweden arboreal forests contain levels of ammonia oxidising bacteria between 1 and 6 orders of magnitude higher than in other forest soils (Hermansson et al., 2004) so it would have been expected that these soils would have contained higher levels of these bacteria, although the level of forestation at the sites it not known at this time.

Sites 2, 3, 4, 5 and 6 all have similar profiles with relatively high BHP concentrations in the top layer and very low BHP concentrations in the bottom layer. In all cases the top layer is described as a humic layer (Table 7.1) with the underlying layer being brown or grey mineral soils at sites 2, 3 and 6. Site 4 is underlain by a layer of sand and gravel and site 5 a grey sand. Humic soils have a high TOC and would therefore be expected to have high BHP content whereas all the base soils are usually associated with lower TOC levels. In all these cases it appears that the bacterial activity is limited to the top layer and the underlying soil is a receptor for leeched organic carbon. This is again confirmed by the consistent levels of cyanobacteria indicating BHPs in both layers and the similarity in the BHP profiles between the top and bottom layers. Once again the level of methanotroph activity is very low indicating that there is little methane oxidation occurring in the soil and the levels of ammonia oxidising bacteria sourced BHPs are comparable with or lower than other sites, so there appears to be no significant increased inhibition of methanotrophs.

Table 7.2a Distribution of BHP producing bacteria in Swedish soil samples.

Bacteria	BHPs	Swe 1a	Swe 1b	Swe 2a	Swe 2b	Swe 3a	Swe 3b	Swe 4a	Swe 4b
		[BHP] (μg_{BHP} g^{-1}dry soil) (%BHP contribution)							
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	342 (64.2)	471 (66.8)	61 (55.7)	26 (59.5)	135 (66.0)	17 (61.6)	575 (70.8)	9 (65.9)
Purple non sulphur bacteria	627, 712, 746, 761	96 (18.1)	140 (20.0)	26 (23.8)	9 (21.2)	47 (22.9)	6 (21.4)	112 (13.7)	2 (16.6)
[a]without adenosylhopane or hopanelactone		7 (1.3)	15 (2.1)	7 (6.8)	3 (7.2)	6 (3.0)	1 (3.2)	38 (4.7)	0.4 (2.9)
N_2 and NH_3 fixing bacteria	627,746, 760	93 (17.5)	133 (18.8)	20 (18.3)	7 (14.8)	44 (21.6)	5 (20.0)	81 (10.6)	2 (14.3)
[a]without adenosylhopane or hopanelactone		4 (0.8)	7 (1.0)	1 (1.3)	0.4 (0.8)	3 (1.6)	0.5 (1.9)	8 (1.0)	0.1 (0.5)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	43 (8.0)	43 (6.1)	10 (9.4)	4 (9.0)	13 (6.5)	2 (5.8)	68 (8.4)	1 (9.0)
Methanotrophs	772	4 (0.7)	1 (0.2)	0.4 (0.3)	0.1 (0.2)	0.8 (0.4)	0.2 (0.7)	5 (0.6)	ND (0.0)
Methylotrophs	1086	ND (0.0)	ND (0.0)	0.1 (0.1)	ND (0.0)	ND (0.0)	0.2 (0.7)	ND (0.0)	ND (0.0)
Unknown	775, 1118,	37 (6.9)	43 (6.1)	10 (9.4)	4 (9.3)	5 (2.6)	2 (8.1)	45 (5.5)	1 (8.0)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

Site 7 has a thin humic layer on top of an orange-brown sand but this site has an unexpected increase in the BHP concentration with depth. This site is within the Arctic Circle and a shallow frozen layer immediately below the sandy layer may prevent leaching of BHPs through the sand and therefore promote accumulation. This is indicated, again, by the cyanobacteria BHP markers which increase as a % of total BHP concentration with depth. This location has the highest concentration of BHPs from various sources (78% for 7a and 73.5% for 7b; Table 7.2b). This could indicate a lower range of bacterial diversity compared to the other sites, but the absolute number of observed structures is similar to the other sites (12 structures in both layers; Fig 7.8). No clear trend in the BHP concentration or number of structures can be observed

in the results and therefore it is difficult to draw any conclusions as to the variation in bacterial and fungal activity in these soils.

Table 7.2b Distribution of BHP producing bacteria in Swedish soil samples.

Bacteria	BHPs	Swe 5a	Swe 5b	Swe 6a	Swe 6b	Swe 7a	Swe 7b
		[BHP] (μg_{BHP} g^{-1}dry soil) (%BHP contribution)					
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	206 (67.9)	9 (51.1)	334 (67.4)	19 (55.6)	221 (78.0)	257 (73.5)
Purple non sulphur bacteria	627, 712, 746, 761	28 (9.4)	4 (22.8)	47 (9.6)	8 (24.7)	45 (15.9)	59 (17.0)
[a]without adenosylhopane or hopanelactone		ND (0.0)	2 (9.1)	4 (0.8)	2 (7.1)	4 (1.3)	5 (1.3)
N_2 and NH_3 fixing bacteria	627,746, 760	31 (10.1)	3 (15.7)	51 (10.3)	6 (18.3)	45 (16.0)	62 (17.8)
[a]without adenosylhopane or hopanelactone		2 (0.8)	0.3 (1.9)	7 (1.5)	0.2 (0.7)	4 (1.4)	7 (2.1)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	41 (13.5)	3 (15.7)	69 (14.0)	4 (11.8)	7 (2.5)	18 (5.3)
Methanotrophs	772	ND (0.0)	ND (0.0)	ND (0.0)	0.1 (0.2)	ND (0.0)	ND (0.0)
Methylotrophs	1086	ND (0.0)	0.04 (0.2)	ND (0.0)	0.1 (0.3)	ND (0.0)	ND (0.0)
Unknown	775, 1118,	27 (8.8)	1 (8.3)	37 (7.5)	2 (6.8)	6 (2.2)	7 (2.1)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

7.3 Spanish Soils

7.3.1 Introduction

The Spanish soils were collected by Dr Elisa Lopez-Capel (Newcastle University) from Andalusia in 2004, 2005 and 2006 as part of an investigation into the effect of a forest fire, in 2004, on soil carbon (De La Rosa et al., 2008). Forest fires can have severe effects on soil composition by removal of organic matter from the soil, destruction of soil structure, specifically porosity which affects the soil's ability to absorb and retain water, loss of nutrients, increased erosion and leaching and significant changes to the soil microbial composition (Certini, 2005). During a forest

fire the soil can reach a sufficiently high temperature to sterilise the top surface with the immediate subsurface microbial biomass falling by 50% with only a slow recovery observed (Prieto-Fernandez et al., 1998). The bacterial recovery is further hindered by the production of toxic organic compounds such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) which hinder bacterial growth (Kim et al., 2003).

The 4 sampling sites were all in the Sierra Aznocolar, Andalusia (Fig.7.9) and all were subjected to a forest fire in 2004 except the control site, Barranca de los Laurels (Fig.7.10). All samples were the 0-15 cm layer of the soil. As can be clearly seen in all locations there was significant re-growth of vegetation in all the fire affected locations during the sampling period (Figs. 7.11 – 7.13).

The BHP profile was analysed from samples taken 1 month after the fires, in 2004, and at the same time in the subsequent 2 years to investigate the increase in soil bacterial population as part of the recovery of the soil. The recovery of the sites, post forest fire, can be clearly seen by the increase in the vegetation at each site (Figs.7.10 – 7.13). As previously discussed BHPs are markers for a wide range of bacteria (Table 2.2), therefore, as the soil bacterial population increases there will be an accompanying rise in the BHP concentration. It must be noted that BHP concentration is not directly related to bacterial population and that stressed bacteria produce more BHPs than unstressed bacteria. This is shown in Palace Leas plots 2 and 7 (Chapter 3) where plot 2 has the higher bacterial population than plot 7, but plot 7 has the highest BHP concentration and variety of BHP structures.

Fig. 7.9 Location map of Sierra Aznacolar (Lopez-Capel, 2007)

2004 2005 2006

Fig. 7.10 Barranca de los Laureles sampling sites (photographs courtesy of E. Lopez-Capel)

2004 2005 2006

Fig. 7.11 Arroyo de las Canadas sampling sites (photographs courtesy of E. Lopez-Capel)

2004 2005 2006

Fig. 7.12 Casa de las Canadas sampling sites (photographs courtesy of E. Lopez-Capel)

2004 2005 2006

Fig. 7.13 Carril de la Osa sampling sites (photographs courtesy of E. Lopez-Capel)

7.3.2 Results

A total of 22 different BHPs were identified in the Spanish samples with the maximum in any sample being 18 (Barranca de los Laureles, 2005, and Arroyo de las Canadas, 2005). The control sample, Barranca de los Laurels (Fig. 7.14) is dominated by 3 BHPs; aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**). There is a decrease in BHP concentration in 2005 from 22 μg_{BHP} g^{-1}dry soil to 10 μg_{BHP} g^{-1}dry soil, followed by an increase in 2006 to 44 μg_{BHP} g^{-1}dry soil. This increase in 2006 is due to significant rises in the concentrations of BHT (**1a**), aminotriol (**1f**) and adenosylhopane (**1e**). Conversely there is an increase in the number of BHP structures identified in 2005 to 18 when compared to 2004, 14 structures, and 2006, 16 structures. The other BHPs observed are similar to those seen in other soils with a dominance of 4 or 5 dominant BHPs and all the other BHPs being minor contributors.

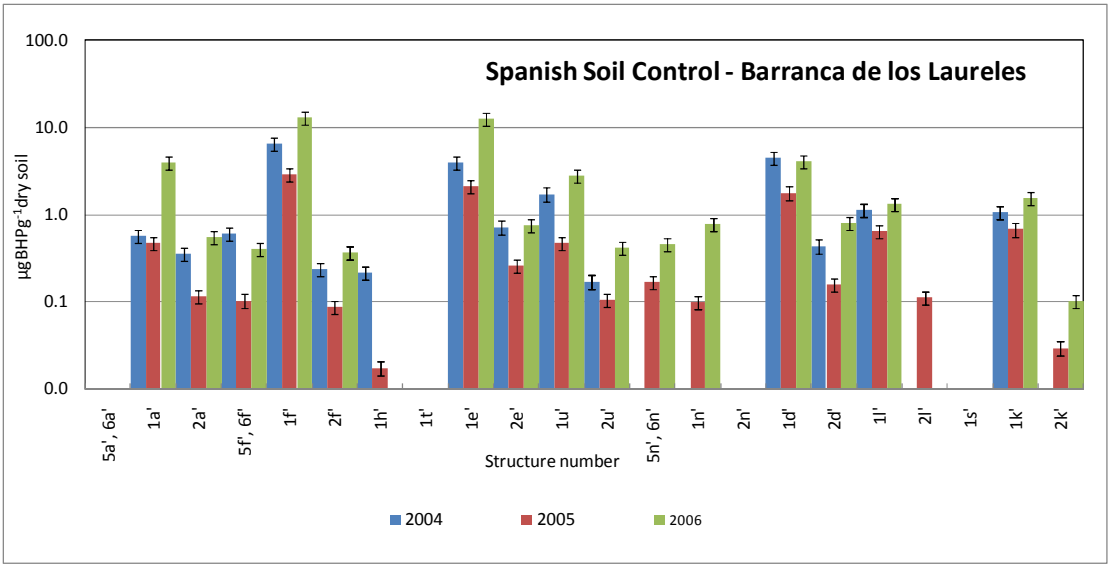


Fig 7.14 Log graph distribution of semi-quantitative BHP concentration (μg_{BHP} g^{-1}dry soil) for Barranca de los Laureles

The 3 fire affected soils; Arroyo de las Canadas, Casa de las Canadas and Carril de la Osa (Figs. 7.15, 7.16 and 7.17) all have very low BHP concentrations in 2004 (maximum of 2 μg_{BHP} g^{-1}dry soil in Carril de la Osa) and very low numbers of BHP structures (maximum of 10 in Carril de la Osa) and there is no overall dominance of any BHPs in these samples. In all cases the BHP concentration and number of structures rises dramatically in 2005 to a maximum of 12 μg_{BHP} g^{-1}dry soil in Carril

de la Osa, a 6 fold increase, and 18 structures in Arroyo de las Canadas. In 2006 the number of structures remains relatively consistent and the BHP concentration continues to rise except for Carril de la Osa (Fig 7.17) where if falls from to 7 µgBHPg^{-1}dry soil. During this time the BHP profile changes such that aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) all become dominant BHPs.

The increase in the number of structures is predominantly due to the rise in cyanobacterially related BHPs in 2005 and 2006 (unsaturated BHT pentose (**5n** or **6n**), BHT pentose (**1n**), 2-Me BHT cyclitol ether (**2d**) and 2-Me BHpentol cyclitol ether (**2l**)). In addition there is an increase in the presence of adenosylhopane type-1 (**1u**) which is absent in all 3 fire effected soils in 2004 but increases in all cases to be the 4th most abundant BHP in the soils. Adenosylhopane type-1 (**1u**) is known to be produced by PNSB (Neunlist et al., 1985; Talbot et al., 2007a) and its absence could be indicative of the loss of PNSB from the soil during the fire and its subsequent recovery. This is also indicated by the increase in concentration of the other PNSB markers; hopanelactone (**1t**), unsaturated aminotriol (**5f** or **6f**) and adenosylhopane (**1e**; Table 2.2). There are also smaller increases in the presence and concentration of aminotetrol (**1h**) and 2-Me adenosylhopane type-1 (**2u**) in all the fire affected soils.

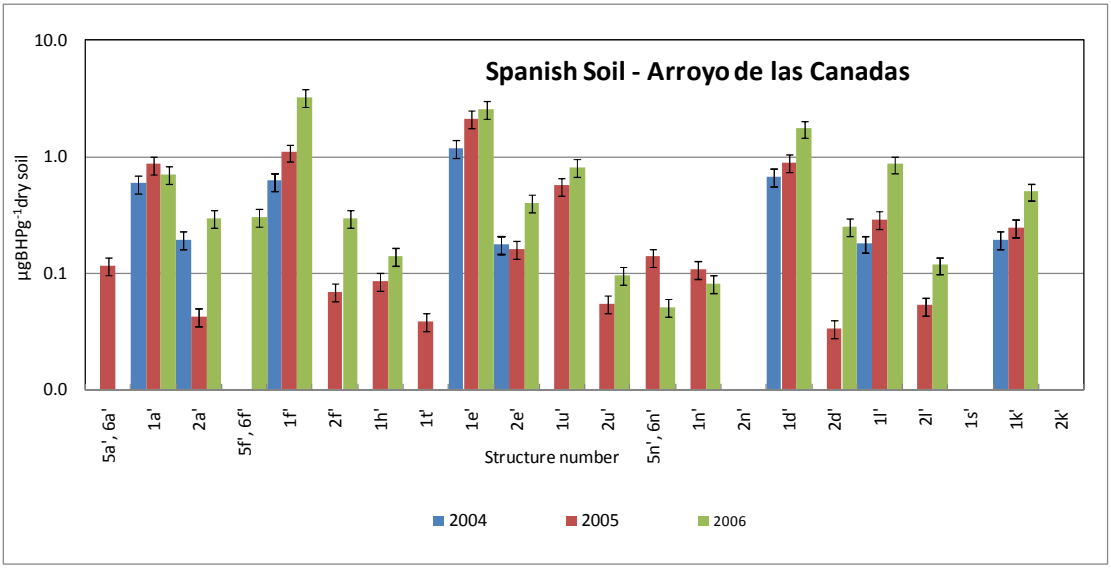


Fig 7.15 Log graph distribution of semi-quantitative BHP concentration (µg_{BHP} g^{-1}Dry soil) for Arroyo de las Canadas

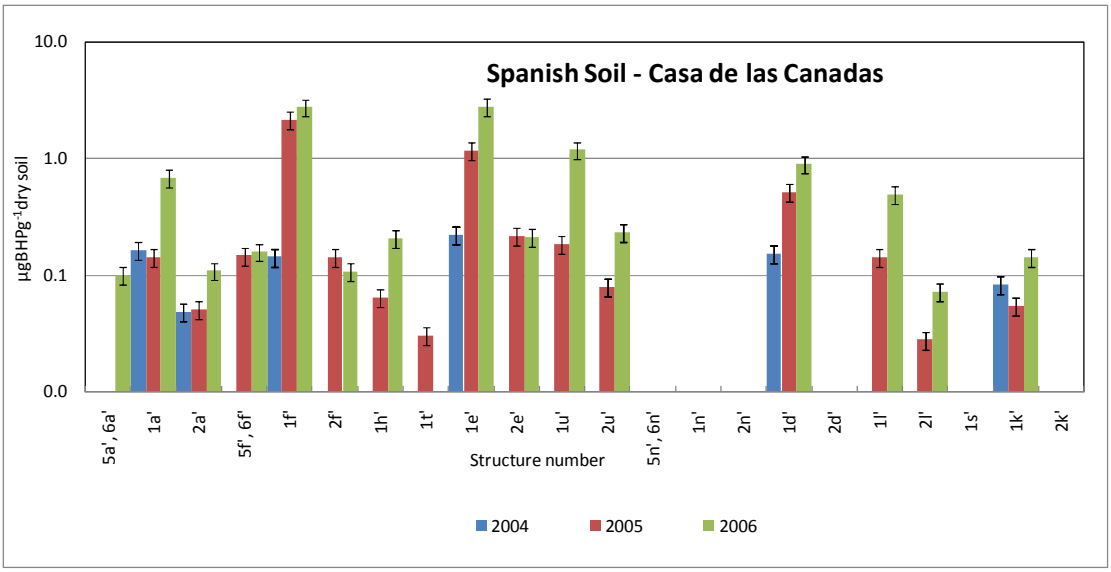


Fig 7.16 Log graph distribution of semi-quantitiave BHP concentration (μg_{BHP} g^{-1}Dry soil) for Casa de las Canadas

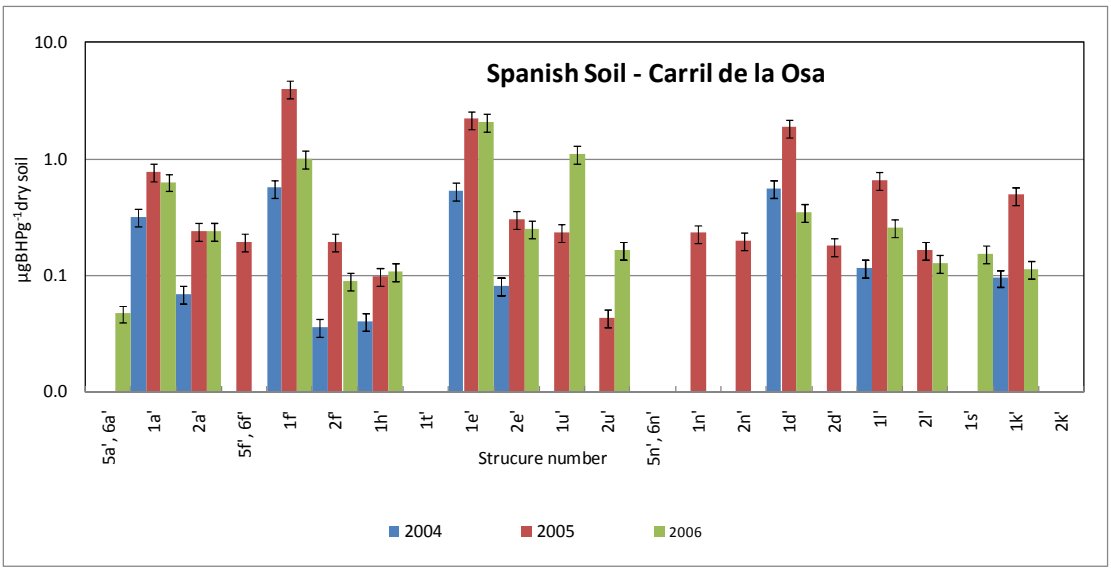


Fig 7.17 Distribution of BHP concentration (μg_{BHP} g^{-1}Dry soil) for Carril de la Osa

7.3.3 Discussion

There is a clear and dramatic rise in the BHP concentration and number of observed BHP structures in all the fire affected soils as the soils recover and vegetation increases in 2005 and 2006. The BHP concentration increases between 2004 and 2006 by 75% in Arroyo de las Canadas (Fig. 7.15), 800% in Casa de las Canadas (Fig. 7.16) and 250% in Carril de la Osa (Fig. 7.17) whereas the control increases by 100% (Fig 7.14). The accompanying increase in the number of structures is 212%, Arroyo

de las Canadas, 275%, Casa de las Canadas, and 150% in Carril de la Osa whereas the number of structures identified in the control, Barranca de los Laureles is only 14%. This clearly indicates that the BHP producing bacterial population is increasing and is in agreement with Prieto-Fernandez et al., (1998) who identified a slow but significant rise in the microbial biomass in the years following a forest fire.

The differences in the bacterial population (Tables 7.3a and 7.3a) indicate that this change is driven by an overall rise in all BHP concentrations, with an increase in the concentration of BHPs from various sources of 350% (Arroyo de las Canadas; Table 7.4)) and 1000% (Casa de las Canadas; Table 7.3b). The increase in the number of identified BHP structures is driven by a rise in cyanobacteria related BHP in Arroyo de las Canadas and Carril de la Osa and also by a rise in methanotrophs in Arroyo de las Canadas and Casa de las Canadas. In all 3 sites there is also a rise in the PNSB population due to the large rises in the concentration of adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**).

It is clear from the increase in methanotroph and cyanobacteria that the increase in BHPs is due to an overall increasing population and not due to stressed conditions in the soil promoting the production of more BHP by the small bacterial population.

Table 7.3a Distribution of BHP producing bacteria in Spanish soil samples, identified by BHPs.

Bacteria	BHPs	Barranca de los Laureles			Arroyo de las Canadas		
		2004	2005	2006	2004	2005	2006
		[BHP] ($\mu g_{BHP}\ g^{-1}$Dry soil) (%BHP contribution)					
Various sources	655, 714, 1000, 1002ce, 1060ce	13 (57.6)	6 (56.1)	22 (50.6)	2 (54.2)	3 (45.0)	7 (52.6)
Purple non sulphur bacteria	627, 712, 746, 761	6 (28.1)	3 (26.2)	16 (36.3)	1 (30.9)	3 (39.3)	4 (29.4)
[a]without adenosylhopane or hopanelactone		2 (10.4)	0.6 (5.6)	3 (7.4)	ND (0.0)	0.6 (8.7)	1 (8.9)
N_2 and NH_3 fixing bacteria	627,746, 760	5 (20.9)	2 (23.2)	13 (30.6)	1 (35.6)	2 (33.4)	3 (23.7)
[a]without adenosylhopane or hopanelactone		0.7 (3.2)	0.3 (2.5)	0.8 (1.7)	0.2 (4.6)	0.2 (2.8	0.4 (3.2)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	1 (4.6)	0.8 (7.4)	3 (6.9)	0.2 (5.1)	0.4 (6.4)	1 (8.8)
Methanotrophs	772	0.2 (10)	0.02 (0.2)	ND (0.0)	ND (0.0)	0.1 (1.2)	0.1 (1.1)
Acetic Acid Bacteria	653,	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	0.1 (1.7)	ND (0.0)
Methylotrophs	1086	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)
Unknown	775	1 (5.5)	0.8 (7.6)	2 (4.5)	0.2 (5.1)	0.3 (4.3)	0.6 (4.8)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

Table 7.3b Distribution of BHP producing bacteria in Spanish soil samples, identified by BHPs.

Bacteria	BHPs	Casa de las Canadas			Carril de la Osa		
		2004	2005	2006	2004	2005	2006
		[BHP] (μg_{BHP} g^{-1}Dry soil) (%BHP contribution)					
Various sources	655, 714, 1000, 1002ce, 1060ce	0.5 (56.7)	3 (57.8)	5 (47.6)	2 (64.5)	7 (60.4)	2 (33.3)
Purple non sulphur bacteria	627, 712, 746, 761	0.2 (27.4)	2 (29.8)	4 (40.7)	0.5 (22.2)	3 (21.9)	3 (47.5)
[a]without adenosylhopane or hopanelactone		ND (0.0)	0.4 (7.1)	1 (13.3)	ND (0.0)	0.4 (3.6)	1 (16.5)
N_2 and NH_3 fixing bacteria	627,746, 760	0.2 (27.1)	1 (27.6)	3 (29.5)	0.6 (25.5)	3 (20.8)	2 (34.8)
[a]without adenosylhopane or hopanelactone		ND (0.0)	0.3 (4.8)	0.2 (2.1)	0.1 (3.4)	0.3 (2.5)	0.3 (3.7)
Cyanobacteria	2Me 669, 2Me 728, 941, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	0.1 (6.0)	0.2 (4.3)	0.3 (2.9)	0.1 (4.3)	1 (10.0)	0.5 (6.8)
Methanotrophs	772	ND (0.0)	0.1 (1.3)	0.2 (2.0)	0.1 (1.7)	0.1 (0.8)	0.1 (1.6)
Acetic Acid Bacteria	653,	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	0.1 (0.7)
Methylotrophs	1086	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	ND (0.0)	0.2 (2.3)
Unknown	775	0.1 (10.2)	0.1 (2.6)	0.4 (3.7)	0.1 (3.9)	0.5 (4.4)	0.3 (4.1)

[a] adenosylhopane (**1e**) and hopanelactone (**1t**) have been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

7.4 Vietnamese Paddy Field soils

7.4.1 Introduction

Paddy fields account for the release of approximately 30% of the global atmospheric methane (Bodeller et al., 2000). The methane generated in the anoxic conditions of flooded rice paddies is subsequently oxidised in the soil water interface by methanotrophs, where approximately 80% of the CH_4 is oxidised by methanotrophs (Hanson and Hanson, 1996). Type I and Type II methanotrophs are both present in paddy fields (Macalady et al., 2002) and their active population is predominantly located in the 0-2 cm layer of the paddy soil and increases dramatically during the flooded growing season increasing by up to 500% (Macalady et al., 2002). Type I are *Methylococcaceae* (gammaproteobacteria) and function at relatively low CH_4 partial

pressure and Type II are methylocystaceae (alphaproteobacteria) and function at relatively high CH_4 partial pressure (Hanson and Hanson, 1996). It would therefore be expected that during the growing season there would be high concentrations of Type II methanotrophs (Macalady et al., 2002). There are 3 BHP markers for type I and II methanotrophs; aminotriol (**1f**) which is also present in nitrogen fixing bacteria, sulphur reducing bacteria, purple non-sulphur bacteria and other bacteria (Table 2.2) and aminotetrol (**1h**) which is also present in sulphate reducing bacteria (Table 2.2) and aminopentol (**1c;** Table 2.2).

The samples were collected by Dr Florianne Clement (Newcastle University) in September 2005 from Northern Vietnam (Fig. 7.18) from 3 different paddy fields with the samples being the 0-15 cm layer of the soil and taken from the edge and 2 m from the edge of the field. The samples were frozen prior to transport to Newcastle. The samples were taken immediately after the paddies had been drained in preparation for the next growing season.

Fig. 7.18 Sample location of Vietnamese samples (Map from www.pickatrail.com/)

7.4.2 Results

The results from field F1 (Fig. 7.19) show that 5 different BHPs dominate the BHP profile; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) are the structures that tend to dominate all soils but an additional BHP is now significant contributor to the BHP profile, aminopentol (**1c**). The rise of aminopentol (**1c**) as a significant BHP is unusual as it is a major contributor in all the paddy field samples (Figs. 7.20 and 7.21) but in all other observed samples during this it is a minor contributor. The distribution of the minor BHPs is very similar to that observed in other soils, e.g. Palace Leas, but it must be noted that BHhexol (**1b**) was also present in all the paddy field samples (Figs. 7.19 to 7.21) and that this BHP is very rare in other soil samples.

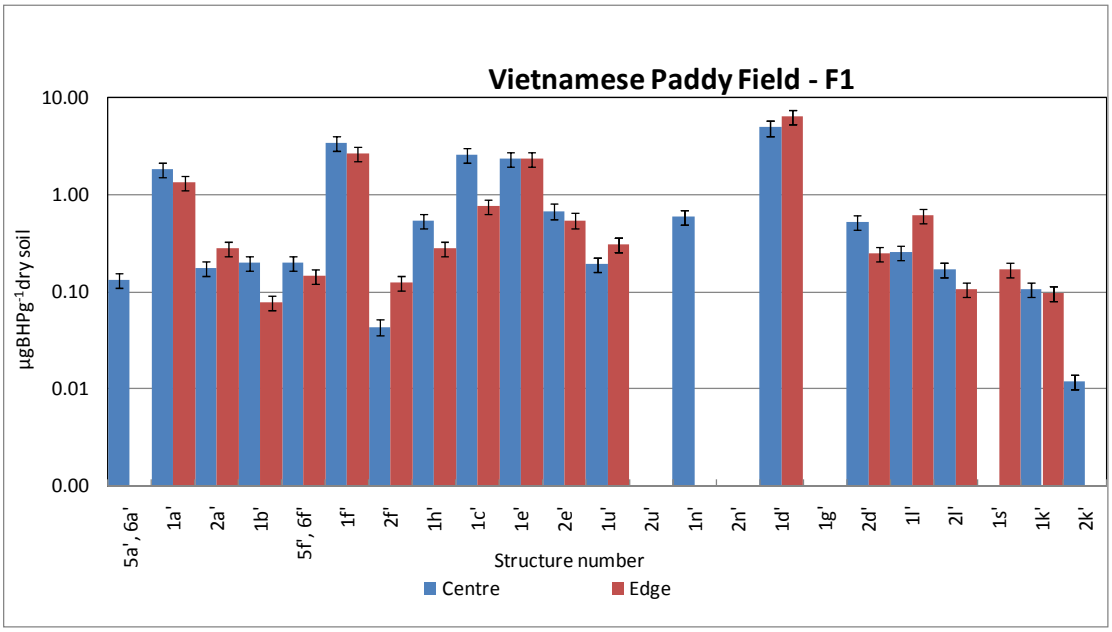


Fig 7.19 Log graph distribution of semi-conservative BHP concentration (µgBHPg^{-1} Dry Soil) for Vietnamese paddy field F1

Paddy field F2 (Fig. 7.20) differs from F1 in that the concentration of BHPs in the edge samples is much higher that that seen in the centre samples. The same BHP dominate the profile; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) with the latter being the most significant BHP in all the samples (Figs. 7.19 -7.21).

The results for F3 (Fig. 7.21) also show a significant increase in BHP concentration in the edge samples compared to the centre samples and the 2-methyl adenosylhopane (**2e**) concentration is much lower and similar to that in F1.

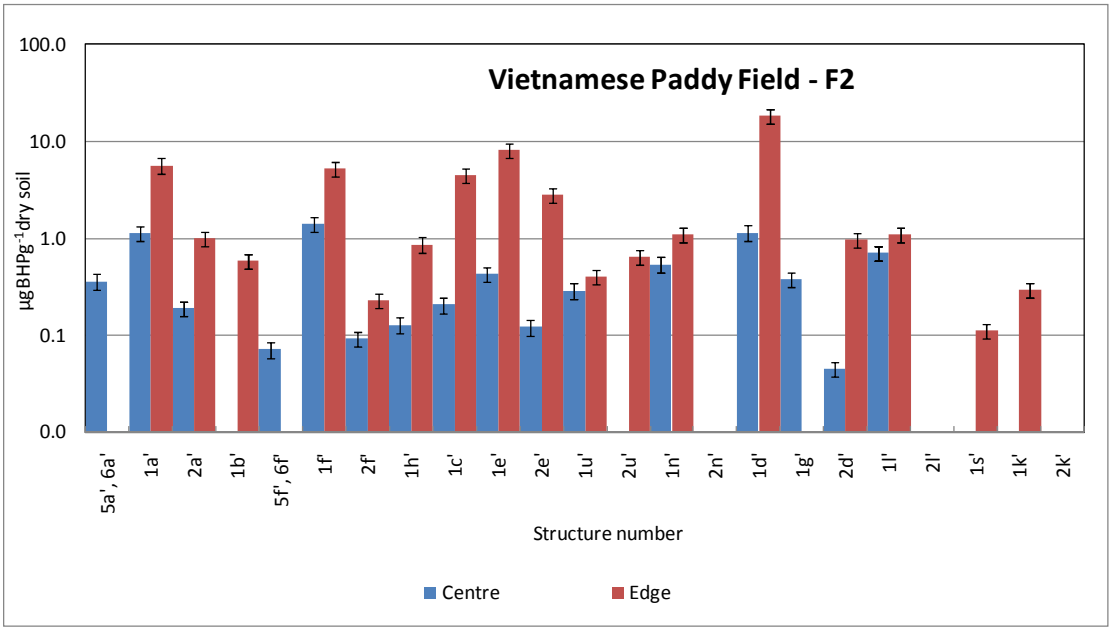


Fig 7.20 Log graph distribution of semi-quantitiative BHP concentration (µgBHPg^{-1} Dry Soil) for Vietnamese paddy field F2

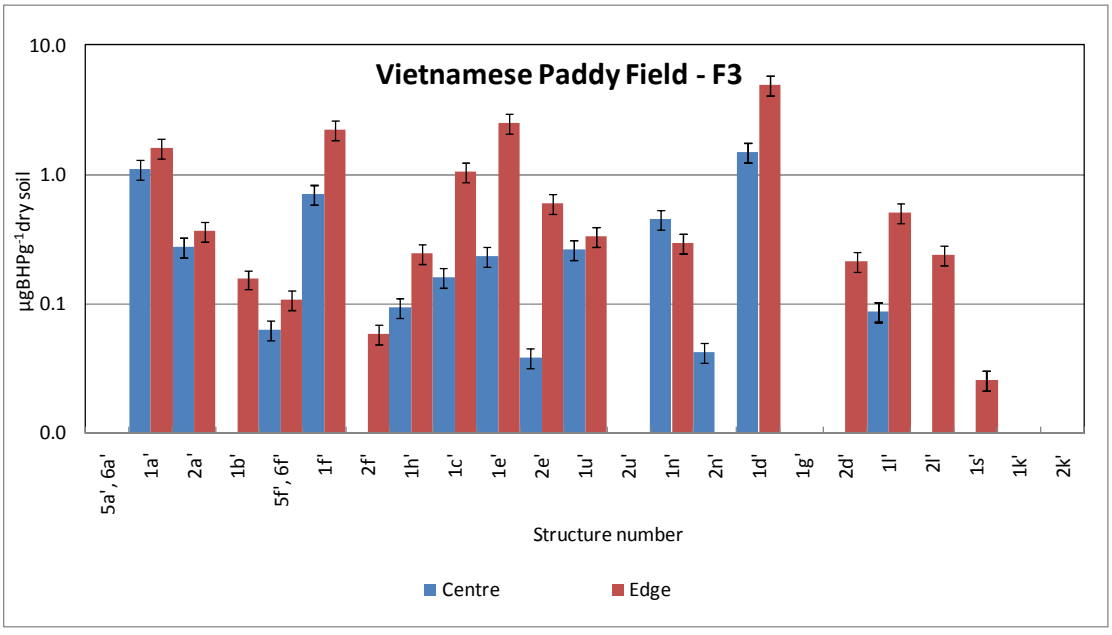


Fig 7.21 Log graph distribution of BHP semi-quantitative concentration (µgBHPg^{-1} Dry Soil) for Vietnamese paddy field F3

7.4.3 Discussion

There are 3 major differences between the paddy field samples and all other BHP profiles analysed during this research all of which imply a domination of a few species within the paddy field soil and not the more diverse populations seen in other soils. The presence of relatively high levels of aminopentol (**1c**) implies a significant population of type I methanotrophs (Neunlist and Rohmer 1985 b and c). However the overall concentration of methanotrophs (Table 7.4) is similar to that observed in other soils (e.g. Palace Leas plot 2), and this is due to the relatively low concentration of aminotetrol (**1h**). Both aminotetrol (**1h**) and aminotriol (**1f**), the type II methanotroph markers (e.g. Neunlist and Rohmer 1985 b and c) have lower concentrations than seen in other soils. The implication of this is that the population of type II methanotrophs is lower than expected from the research by Macalady et al (2002). This could be due to the sampling time and there may be a significant fall in type II population and growth of type I in is less CH_4 productive phase of the growing cycle. For this to have happened there would also have to be a significant consumption of the type II BHPs and the population changed.

The second major difference is the presence of BHhexol (**1b**) in all the paddy fields whereas they are absent in other soils. BHhexol (**1b**) has no known source bacteria but has been found in sediments from Loch Ness (Talbot and Farrimond, 2007).

The BHP producing soil bacterial population contains a much lower contribution by PNSB and NH_3 and N_2 fixing bacteria, although the level of nitrogen fixing bacteria. This is coupled with a rise in the relative contribution of cyanobacteria and methanotrophs.

Table 7.4 Distribution of BHP producing bacteria in Vietnamese paddy field soil samples, identified by BHPs.

Bacteria	BHPs	Vietnam F1		Vietnam F2		Vietnam F3	
		Int.	Ext.	Int.	Ext.	Int.	Ext.
		[BHP] ($\mu gBHPg^{-1}$ Dry Soil) (%BHP contribution)					
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	10 (43.3)	11 (66.8)	5 (66.1)	30 (42.5)	3 (67.5)	9 (59.8)
Purple non sulphur bacteria	712, 746, 761	3 (11.3)	3 (16.9)	0.8 (10.8)	9 (16.5)	0.6 (11.2)	3 (19.1)
[a]without adenosylhopane		0.4 (1.6)	0.4 (2.7)	0.4 (5.0)	0.4 (0.8)	0.3 (6.6)	0.4 (2.9)
N_2 and NH_3 fixing bacteria	746, 760	3 (12.6)	3 (17.5)	0.5 (7.5)	11 (21.1)	0.3 (0.3)	3 (20.1)
[a]without adenosylhopane		0.7 (2.8)	0.6 (3.3)	0.1 (1.7)	3 (5.4)	0.1 (0.8)	0.6 (3.9)
Cyanobacteria	2Me 669, 2Me 728, 943, 957, 2Me 1016, 2Me 1074, 2Me 1132	2 (6.3)	0.8 (4.6)	0.9 (11.9)	3.3 (4.6)	0.8 (15.4)	1 (7.6)
Methanotrophs	772, 830	3 (13.1)	1 (6.3)	0.3 (4.6)	5 (7.5)	0.3 (5.1)	1 (8.4)
Acetic Acid Bacteria	653	0.1 (0.5)	ND (0.0)	0.4 (5.0)	ND (0.0)	ND (0.0)	ND (0.0)
Methylotrophs	1086	ND (0.0)	0.2 (1.0)	ND (0.0)	0.1 (0.2)	ND (0.0)	0.1 (0.2)
Unknown	775, 1118	0.3 (1.3)	0.2 (1.1)	ND (0.0)	2 (2.1)	ND (0.0)	0.2 (1.0)

[a] adenosylhopane (**1e**) has been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

7.5 Peat

7.5.1 Introduction

Priest Pot is a highly productive lake in the English Lake District (Innes et al., 1997), (Figs. 7.22 and 7.23), that has been extensively studied for the BHP content of its sediments (Innes et al., 1997; Farrimond et al., 2000, Watson et al., 2002; Talbot et al., 2003). There is a significant decrease in the BHT (**1a**) concentration with depth (Innes et al., 1997) which is repeated for a wider range of BHPs (Talbot et al., 2003).

The lake is surrounded by peat deposits and peats are low pH, waterlogged environments that are known to have high levels of organic carbon preservation due to low levels of O_2 (White, 2006). Peat bogs and other wetlands are the largest natural source of atmospheric methane (e.g. Sinninghe-Damste et al., 2005) and therefore would be expected to have a high level of methanotroph bacteria which could be

identified using the BHP methanotroph markers aminotetrol (**1h**) and aminopentol (**1c**; e.g. Neunlist and Rohmer 1985b, 1985c). There have been many other studies in the hopanoid content of peats (e.g. Quirke et al., 1984; Ries-Kautt and Albrecht, 1989 Dehmer, 1992; Farrimond and Flanagan, 1995; Duan and Ma. 2001; McClymont et al., 2008) using GC to identify the hopanoids and therefore little information was available on the intact BHPs with only BHT (**1a**) being identified by Quirke et al. (1984). More recent work has been conducted on Bissendorfer Moor, Germany (Talbot et al., in prep) that has identified a wide range of intact BHPs in cores down to 4 m including many of the BHPs identified in soils. The implication is that may be soil derived organic matter is deposited and preserved in peats. Alternatively these BHPs could be being produced by bacteria in the peat. Analysis of the stereochemistry of the BHP will indicate if the BHPs are produced in situ or are preserved relics of soil derived BHPs.

The samples were collected by Dr Helen Talbot in August 2002. Two samples were analysed; PPC3, a 10 cm peat core from 4 m from lake edge under a stand of trees and PPC5, a 10 cm peat core from 1 m from lake edge. Both were freeze dried and analysed using the methods described in chapter 2.

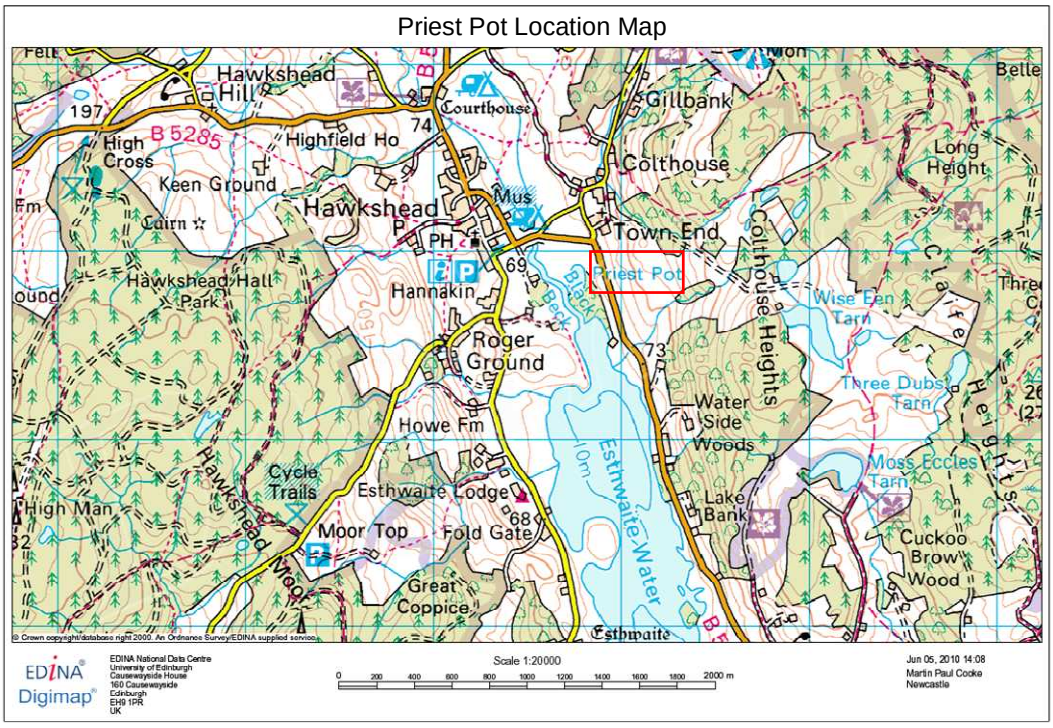


Fig. 7.22 Priest Pot location map, Cumbria, 54°22'N, 3°00'W

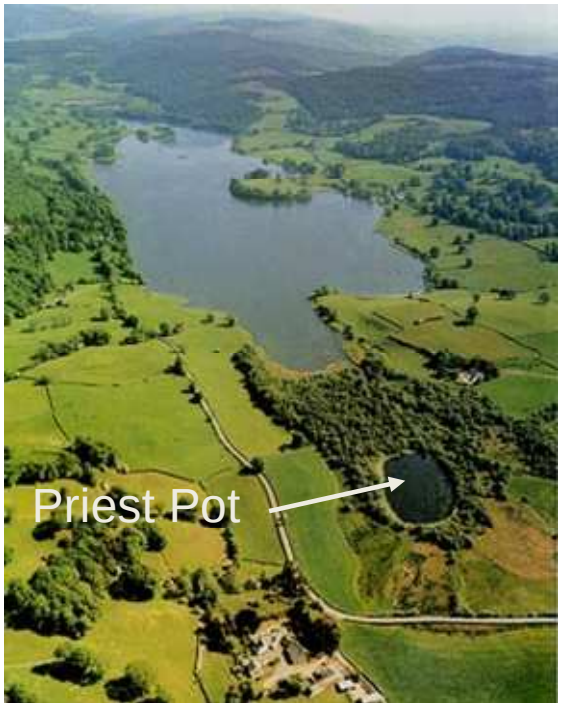


Fig 7.23 Aerial Photograph of Priest Pot (From starcentral.mbl.edu/microscope/)

7.5.2 Results

The BHP profile for Priest Pot peat sample PPC5 (Fig. 7.24) contains 18 different BHPs and is dominated by 4 structures; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) accounting for 70.5% of the total BHP contribution and BHT cyclitol ether (**1d**) being the most significant accounting for 36.3% of the total BHP concentration. The total BHP concentration is 216 $\mu g_{BHP}\ g^{-1}$ Dry Sample. Aminopentol (**1c**) accounts for 4% of total BHP concentration and for the first time unsaturated aminopentol (**5c** or **6c**) is observed in the BHP profile.

The BHP profile for PPC3 is different from that for PPC5 with a total BHP concentration of 139 $\mu g_{BHP}\ g^{-1}$ Dry Sample and a total of 17 different BHP structures identified. The BHP profile is again dominated by BHT cyclitol ether (**1d**; 43% of total BHP concentration) followed by aminotriol (**1f**), adenosylhopane type-1 (**1u**) and then adenosylhopane (**1e**). Unsaturated aminopentol (**1c**) is absent from this samples and aminopentol (**1c**) is at a much lower concentration (0.5% of total).

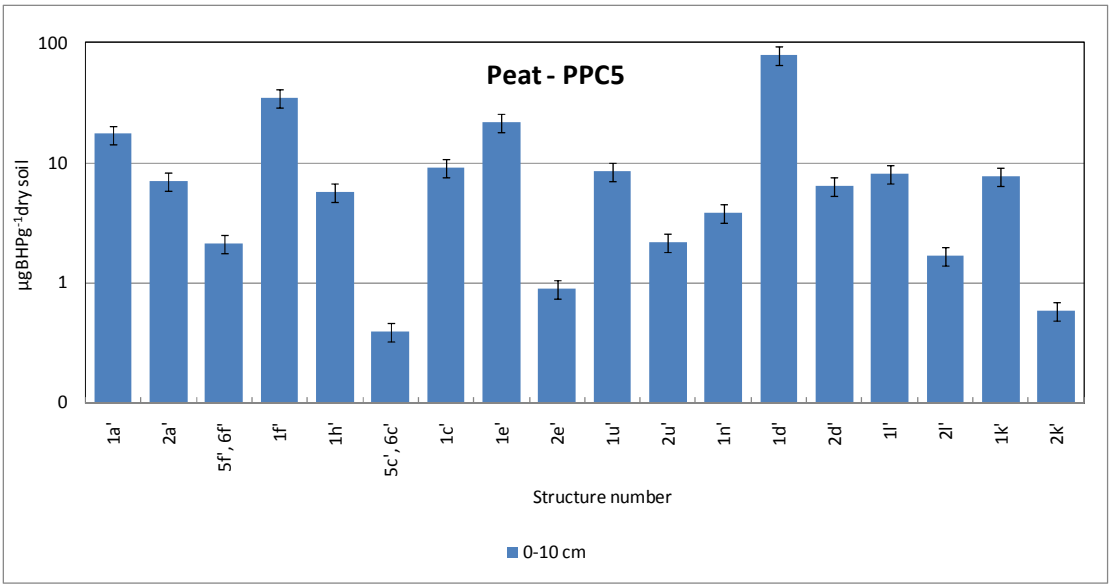


Fig 7.24 Log graph distribution of semi-quaqntitative BHP concentration (µgBHPg^{-1} Dry Soil) for Priest Pot Peat PPC5

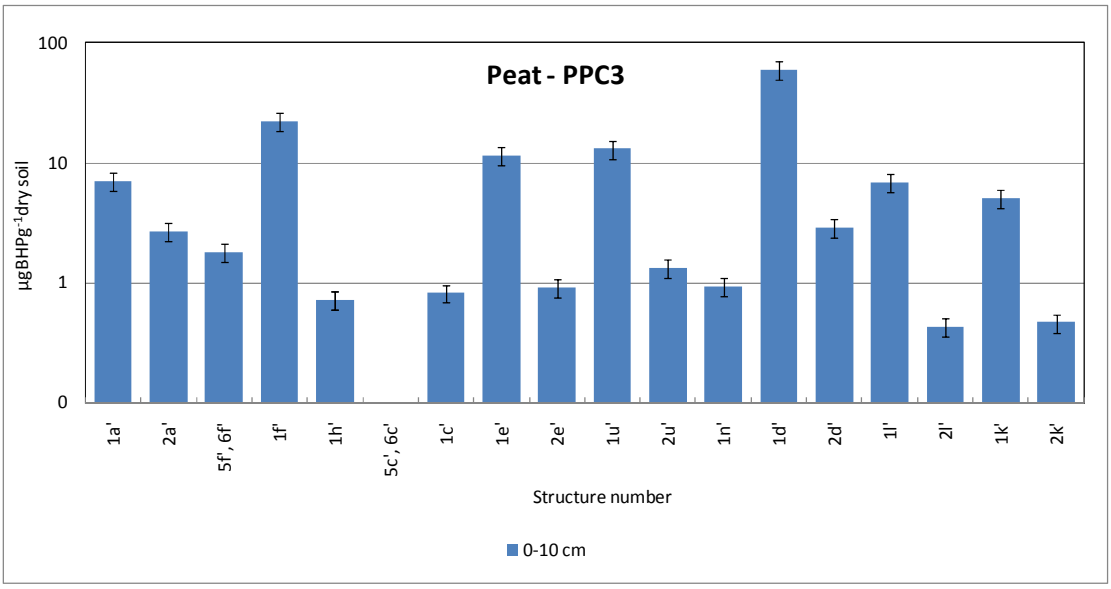


Fig 7.25 Log graph distribution of semi-quantitiative BHP concentration (µgBHPg^{-1} dry Soil) for Priest Pot Peat PPC3

7.5.3 Discussion

The relatively high concentration of BHT cyclitol ether (**1d**) in both soil samples (Figs 7.24 and 7.25) is distinctive as it has not been observed in any other soil samples. The distribution of the BHPs is otherwise similar to that seen at Palace Leas and Hack Hall farm and numerous other locations with significant levels of aminotriol (**1f**), adenosylhopane (**1e**) and adenosylhopane type-1 (**1u**).

The proportion of methanotroph derived BHPs in PPC5 is relatively high compared to other sites (7.0%, Table 7.5) as would be expected by the high level of methane production associated with peats (Sinninghe-Damste et al., 2005), but this observation is not repeated in PPC3 (Table 7.5) where methanotroph derived BHPs only account for 1% of the total BHP concentration which is less than observed in most soils. The concentration of PNSB and NH_3 oxidising bacteria is also reduced when compared to other sites and this is due to the dominance of BHT cyclitol ether (**1d**) in the BHP profile. The cyanobacterially related BHPs are present in proportions similar to that seen in other soils in this study.

The overall pattern of BHP distribution in peats is similar to that observed in soils with increases in BHT cyclitol ether (**1d**) and aminopentol (**1c**) that originate in the peat. The upper layers of peat from Russia, Canada and Estonia have been recently found to contain high quantities of the heterotrophic *Burkholderia* (Opelt and Berg, 2004; Belova et al., 2006; Opelt et al., 2007). Cvejic et al. (2000) identified that BHP production occurred in *Burkholderia*, with saturated tetrafunctionalised compounds, specifically BHT (**1a**) and BHT cyclitol ether (**1d**) dominating. This could explain the high concentration of BHT cyclitol ether (**1d**) however the concentration of BHT (**1a**) is not correspondingly high so a more complex relationship may occurring in the peat. The high levels of methanotrophs, particularly in PPC5 (Fig. 7.24 and Table 7.5) are indicated by the presence of aminopentol (**1c**) and unsaturated aminopentol (**5c** or **6c**). These BHPs are related to Type I methanotrophs such as *Methyococcus* and *Methylocaldum* (Neunlist and Rohmer, 1985b and 1985c; Cvejic et al., 2000; Talbot et al., 2001). An investigation into acidic northern wetlands found that they are mainly colonized by Type II methanotrophs (Dedysh 2009), which do not produce aminopentol (**1c**), however more recent work by Talbot et al (in prep) has indicated that Type I methanotrophs are also present in peats.

Table 7.5 Distribution of BHP producing bacteria in Priest Pot peat samples.

Bacteria	BHPs	Peat PPC5	Peat PPC3
		[BHP] ($\mu gBHPg^{-1}$ Dry Soil) (%BHP contribution)	
Various sources	655, 714, 1000, 1002ce, 1002glu, 1060ce	139 (64.2)	97 (69.4)
Purple non sulphur bacteria	712, 746, 761	33 (15.0)	26 (19.0)
[a]without adenosylhopane		11 (4.9)	15 (10.7)
N_2 and NH_3 fixing bacteria	746, 760	23 (10.5)	12 (8.9)
[a]without adenosylhopane		0.9 (0.4)	0.9 (0.7)
Cyanobacteria	2Me 669, 2Me 1016, 2Me 1074, 2Me 1132	20 (9.0)	7 (5.3)
Methanotrophs	772, 828, 830	15 (7.0)	2 (1.0)
Acetic Acid Bacteria		ND (0.0)	ND (0.0)
Methylotrophs		ND (0.0)	ND (0.0)
Unknown	775, 1118	10 (4.6)	6 (4.6)

[a] adenosylhopane (**1e**) has been used for both purple non sulphur bacteria and nitrogen and ammonia fixing bacteria as both sources are valid. ND – Not Detected

7.6 Comparison of soils

Generally the soils analysed in this chapter are very similar to those analysed in other chapters with a dominance of; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**) and BHT cyclitol ether (**1d**) and up to 20 other more minor BHPs in each samples. However there are some obvious differences that can be observed such as the relatively high concentrations of BHT cyclitol ether (**1d**) in the peat (Figs. 7.24 and 7.25) and paddy field (Figs 7.19-7.21) samples and the relatively high concentrations of aminopentol (**1c**) in the paddy field samples (Figs 7.19-7.21). These all indicate that in certain environments certain bacteria and bacterial processes can dominate the soil whereas in other locations a more diverse bacterial population exists.

The results from the Spanish soils clearly demonstrated an increase in BHP concentration in the soil in the years following a forest fire. This suggests the possibility for BHPs can be used to measure increases in bacterial populations and soil recovery following forest fires.

7.6.1 PCA

To assess the differences between the soils described in this chapter, the Canadian soils (Xu et al., 2008) and the allotment soils (Redshaw et al., 2008) analysed as part of this research the BHP producing bacterial populations were analysed using PCA.

The loading plot (Fig 7.26) clearly shows a wide separation between the various groups and the 2 components are both significant (38% and 24% respectively). There is a negative loading on PC1 for PNSB and NH_3 and N_2 fixing bacteria. These 2 components are very strongly linked to the presence of adenosylhopane (**1e**) in the soils which is classed as a marker for both bacterial groups. There is a strong positive loading for methanotrophs on PC2 and an approximately opposite loading for cyanobacteria. This seems logical as cyanobacteria would be expected to thrive in the near surface whereas the methanotrophs would be expected to occur at deeper layer where the oxic and anoxic layers meet and methane oxidation would occur. Acetic acid bacteria are located in the centre of the plot for these soils and are rare in the soils so would not be expected to have a strong influence on the loadings distribution.

The scores plot of individual samples (Fig. 7.27) clearly shows that there are demonstrable differences between the soils although there is significant overlap between the soils due to the dominance of the major BHPs. The Canadian soils (Xu et al., 2008) and the Spanish soils are the most strongly correlated with respect to PNSB and NH_3 and N_2 fixing bacteria whereas the Vietnam paddy fields are most strongly correlated with respect to methanotrophs as would be expected due to the presence of aminopentol (**1c**) in all the samples.

The Swedish samples have a correlation that is influenced by cyanobacteria and the SW England allotments (Redshaw et al., 2008) are influenced by a mix of methanotrophs, these soils were the first to be reported as having aminopentol (**1c**) in their BHP profile, and PNSB and NH_3 and N_2 fixing bacteria.

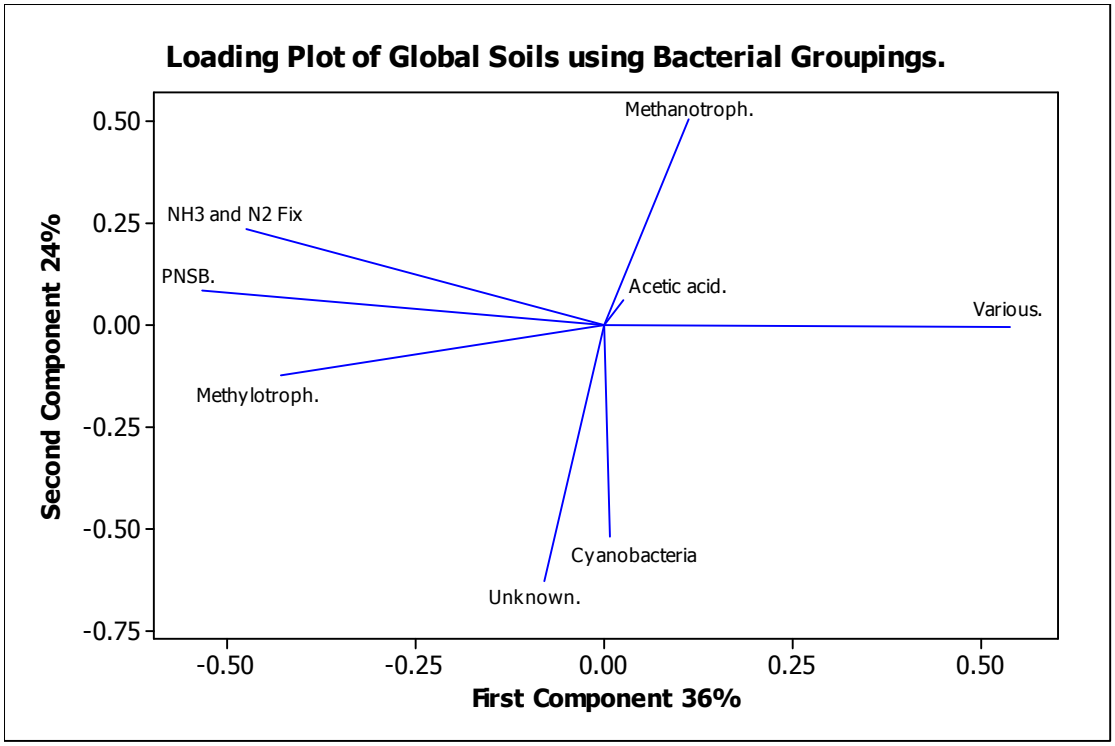


Fig. 7.26 Loading Plot for all global soils using the bacterial groupings described in Table 7.2

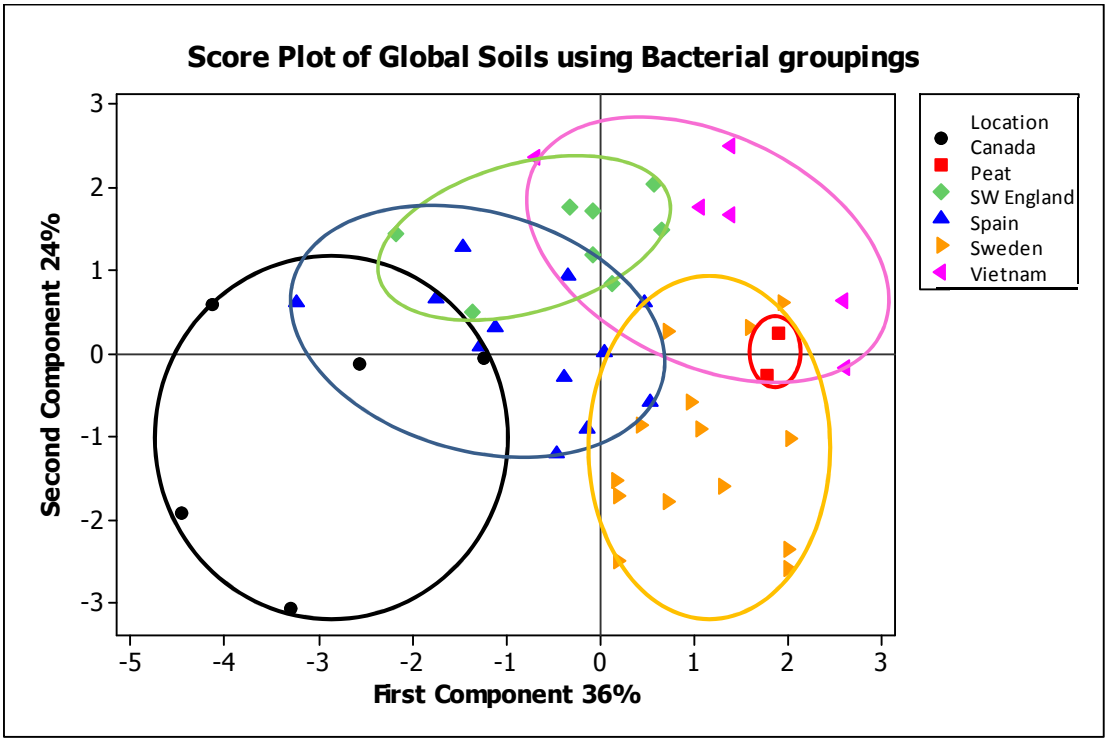


Fig. 7.27 Score Plot for all global soils using the bacterial groupings described in Table 7.2

The peat soils that were expected to be strongly influenced by methanotrophs have no obvious correlation with the methanotroph signal but are however very similar and plot closely together with a loading related to BHPs from various sources. The most

significant BHP in these peat samples is BHT cyclitol ether (**1d**) and this has been linked to *Burkholderia* (Cvejic et al. 2000) which is known to be present in peats (Opelt and Berg, 2004; Belova et al., 2006; Opelt et al., 2007).

The PCA analysis clearly demonstrates that there are clear and measurable differences between soils from around the world but that the BHP profiles of all soils are very similar with a dominance of 4 or 5 commonly occurring BHPs and a suite of up to 20 minor BHPs.

7.7 Statistical Analysis of all soils and sediments

The ability to separate soils and sediments from around using the BHP profile the world is fundamental to this thesis as it will confirm that differences in the BHP profile of different soils can be used to elucidate information on the BHP producing bacteria population. It will confirm the usefulness of BHPs as a biomarker for soil bacterial activity.

Comparison of all the soils and sediments discussed in this thesis and the Canadian (Xu et al., 2008) and SE England soils (Redshaw et al., 2008) was carried out using PCA and the bacterial groupings described throughout this report. The loading plot (Fig. 7.28) clearly shows that there are 2 distinct loadings, the methanotroph and unknown source bacteria, which correlate negatively with respect to the first component and all other bacterial sources which correlate positively to the first component. This produces 2 bands of results on the scores plot (Fig 7.28) with a band of results negatively correlated to the first component and a band positively correlated to the first component. The results from the Congo Fan and ODP 1074, Palace Leas and Hack Hall Farm are all negatively correlated with respect to the first component. The Palace Leas and Hack Hall Farm results are very closely related and indicate an influence dominated by BHPs of unknown source and methanotrophs with the other source bacteria being of less influence. The Congo results are split into 2 groups with 1a (Fig 7.29) containing the near surface core samples and group 1 the deeper core samples and the more distant surface samples. This indicates that the deeper samples are more strongly influenced by their methanotroph related signal and the near surface core samples are very similar in composition to the soils at Palace Leas and Hack Hall. This is in general agreement with the hypothesis that the Congo Fan samples are influenced by SOM.

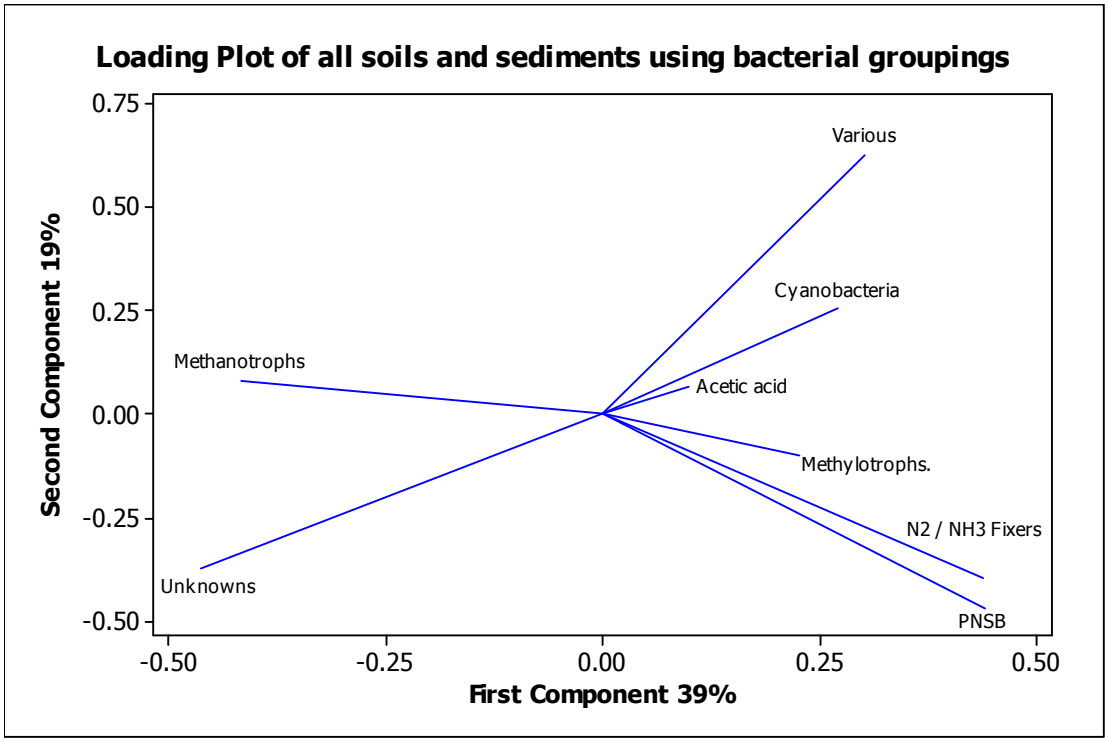


Fig. 7.28 Loading Plot for all global soils and sediments using the bacterial groupings

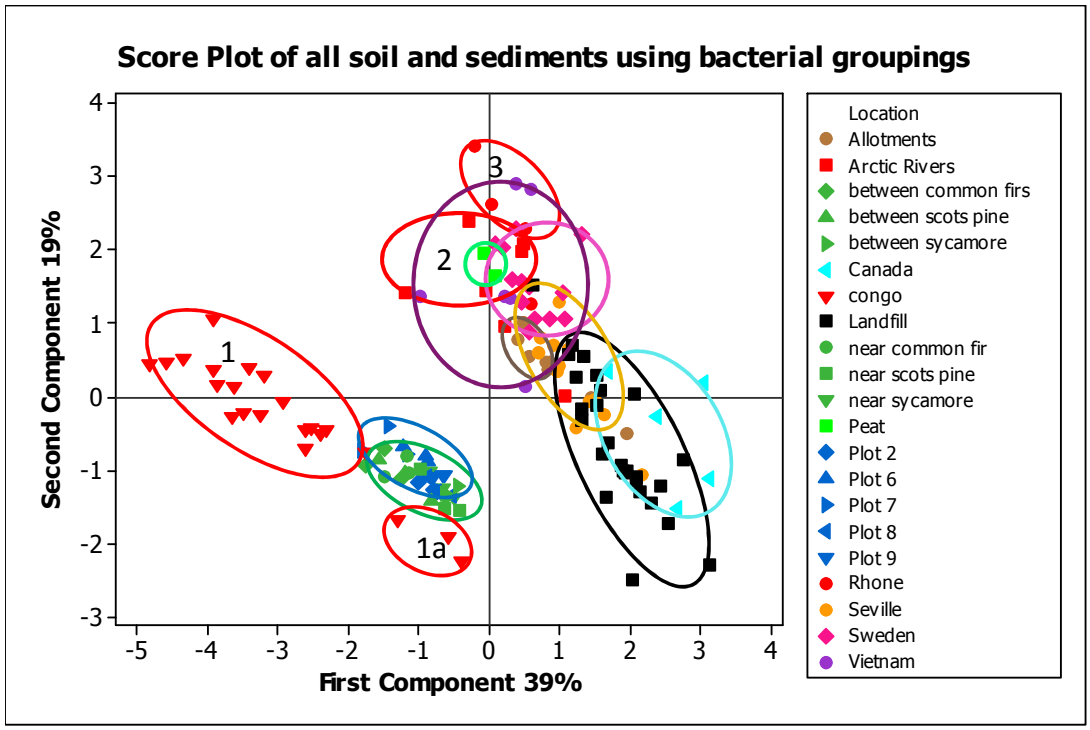


Fig. 7.29 Score Plot for all global soils and sediments using the bacterial groupings.

1 = Congo Fan ODP 1074 deep core samples, 1a = Congo Fan ODP 1074 surface core samples,

2 = Arctic river surface samples, 3 = Rhone estuary samples

The other samples all correlate positively with respect to the first component and their spread in dictated by the second component. This is due to the general low level of methanotrophs on these samples and fewer identified BHPs. The Hack Hall and Palace Leas samples contain a much wider distribution of BHPs than the other global samples and this increased number of BHP structures is often dominated by novel BHPs of unknown origin.

The landfill samples are all strongly influenced by the presence of PNSB and NH_3 and N_2 fixing bacteria as discussed in chapter 5. The Canadian samples (Xu et al., 2009) are also strongly influenced by the presence of PNSB and NH_3 and N_2 fixing bacteria although to a less extent than the landfill samples. The Seville samples are the influenced by a wide range of bacteria and represent the central grouping of all the other global samples.

The peat and Vietnam samples are strongly influenced by BHPs from various sources and have a high level of BHT cyclitol ether (**1d**) that could indicate the presence of *Burkholderia* (Cvejic et al., 2000b). These 2 sets of samples are also the most strongly influenced by methanotrophs in this positively correlated band of global soils and this can be clearly seen in the results (Tables 7.27 and 7.25 respectively).

The allotment samples from SE England (Redshaw et al., 2009) are centrally located within the scores plot indicating no clear influence from any single bacterial population.

The 2 remaining sets of estuary samples are both influenced by BHPs from various sources and methanotrophs but the low number of identified BHPs in these samples (Chapter 6) results in them being plotted away from the Congo samples. The arctic estuary samples (Fig. 7.28; 2) are especially interesting as they plot inexactly the same location as peat and the catchment of all these rivers is dominated by peat (van Dongen et al., 2008) and close to the Swedish soil samples. The implication of this is as the Congo sediment samples are strongly influenced by a soil derived BHP signal the Arctic river samples are strongly influenced by a peat derived BHP signal. These 2 sets of data confirm that the SOC BHP signal is carried down rivers to the marine environment.

7.8 Summary

The broad aim of this phase of the research was to achieve the following goal

Goal 10

To identify the distinct BHP profiles of soils from different global locations.

The PCA analysis of the soils in this chapter (Figs 7.25 and 7.26) and the wider analysis of all soils tested in this thesis (Figs 7.27 and 7.28) clearly demonstrates that there are significant differences in the BHP profiles of the soils and sediments tested. These differences can be related to specific bacterial population and the occurrence of these bacterial populations in the soils is in broad agreement with that expected from the environments. For example in peat samples the most significant BHP is BHT cyclitol ether (**1d**) and this has been linked to *Burkholderia* (Cvejic et al. 2000b) which is known to be present in peats (Opelt and Berg, 2004; Belova et al., 2006; Opelt et al., 2007).

The PCA analysis (Fig 7.28) clearly shows that terrestrially derived BHPs are transported down rivers in both the Arctic and Congo and preserved in the marine sediments.

The analysis of the BHPs in a series of soils taken after forest fires in Seville, Southern Spain, has clearly shown an increase in BHP concentration and number of BHP structures indicating the recovery of the soils bacterial population in the soil following a forest fire in agreement with Prieto-Fernandez et al., (1998) who identified a slow but significant rise in the microbial biomass in the years following a forest fire.

The paddy field samples show a lower than expected level of methanotrophs which could be related to the sampling time and few BHP structures indicating the lack of a diverse BHP producing bacteria population.

It is clear from these analyses that there are clear differences between the BHP producing bacterial populations of soils from around the world with these differences being driven by 2 main factors; the relative concentration of methanotroph derived BHPs compared to other BHP producing source bacteria and the overall number of BHP structures identified within the sample. The greater the diversity of BHPs the

greater likelihood of there being BHPs of unknown origin which can be seen by the location of, for example, Palace Leas and Hack Hall farm on the scores plot (Fig.7.28).

7.9 Conclusion

Analysis of the BHP profiles of a series of soils from around the world has shown that the soils are, similar to the soils from NE England, dominated by 5 main BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type-1 (**1u**) and BHT cyclitol ether (**1d**). There are sufficient differences between the BHP profiles with respect to type of BHP structures observed and their quantity to identify significant differences between the soils. The separation of the soils using PCA and specific bacterial groupings (Fig. 7.28) has shown that the BHP profile is in agreement with much of the current understanding of the microbial activity in these environments, e.g. *Burkholderia* in peat and to a less extent methanotrophs in paddy fields.

The BHP profile appears also give an indication of the diversity of the BHP producing bacterial population and there appears to be a link between number of structures and bacterial diversity. This link is best seen in the increase in the number of BHP structures and BHP concentration in soils from Seville that were recovering from a forest fire. However it must be remembered that in Palace Leas plot 7 the diversity of BHPs and their concentration was directly related to bacterial stress and not changes in population. This relationship between concentration of BHPs and number of observed structures and the bacterial population size and diversity is still unclear although a logical approach would be that the BHP concentration and number of structures increases with increases in the bacterial population, such as those seen in Seville, until a stable population is achieved and then the BHP concentration and number of structures varies in response to environmental stress.

Using PCA (Figs. 7.27 and 7.28) there is a clear separation between soils based on 2 factors; the concentration of methanotroph derived BHPs and the number of BHPs with unknown origin. These 2 factors generate 2 distinct clusters of locations which are further separated by the influence of adenosylhopane and its presence in PNSB and nitrogen and ammonia fixing bacteria.

This PCA analysis has also confirmed the transport of terrestrially derived BHPs by rivers in the Arctic, where a terrestrial peat signal is preserved and the Congo where a soil BHP signal is preserved.

8. Conclusions

The aim of this thesis was to assess the potential of BHPs to act as a biomarker for bacterial activity or specific environmental conditions in soils and sediments by testing 2 different hypotheses.

Hypothesis 1

The BHP fingerprint of a soil indicates the current bacterial population.

Hypothesis 2

Complex intact BHPs are preserved in ancient marine sediments.

8.1 Hypothesis 1

8.1.1 Distribution of BHPs in Soil

The analysis of all the soils in this study has shown that there is typical BHP profile that is dominated by 4 or 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type 1 (**1u**) and BHT cyclitol ether (**1d**). These BHPs accounted for >60% of the total BHP concentration in most soils. The soil samples have been shown to contain uo to 25 different BHPs at levels down to <0.5 ($\mu g_{BHP} g^{-1}_{TOC}$). In total 47 different BHPs were identified in these soils.

The distribution of BHPs in the soils appears to be driven by position of source bacteria in the soil column, soil type, environmental conditions, leaching of BHPs and bacteria down the soil column and preservation or degradation of the BHPs. These factors complicate the distribution of the BHP within the soils. The most important are soil type, oxygenation and postion in the soil column. These define the bacterial population and therefore the origin of the BHPs within the soil. High bacterial populations will produce high levels of BHPs but environmental stress on that BHP producing bacterial population will also promote the production of BHPs (Palace Leas plot 7). The environmental conditions then subsequently affect the fate of the bacterial population and therefore that of the BHPs. Decreases in bacterial population will lead

to the release of BHPs into the soil. These free BHPs can then either be consumed, degraded in aerobic conditions, for example, leached down the through the soil column to more anaerobic sites where they will be preserved, bind to clay particles or become incorporated into humin. Alternatively the bacteria can become dormant, potentially producing more BHPs to "armour" the cell, and then be leached down through the soil column. These BHPs will subsequently be released during the extraction process.

These processes can be seen in the relative increase in BHPs as a percentage of the soil total lipid extract (TLE) moving down the soil profile indicating that BHPs are relatively more recalcitrant than many other organic structures. Coupled with this relative increase as a proportion of soil TLE there is a decrease in the number of observed structures indicating that some degradation of BHPs is occurring or that there is a decrease in the BHP producing bacterial population.

8.1.2 Relationship between BHPs and Source Bacteria

There is a strong link between source bacteria and BHPs, highlighted by the decrease in the occurrence of cyanobacterially related BHT pentose (**1n**) and its homologues (**2n**; **5n** or **6n**) with the depth in the Palace Leas and Hack Hall Farm plots, which indicates movement and degradation of the BHPs from the expected near surface source of cyanobacteria derived BHPs.

Serveral other long term fertiliser projects have been undertaken across the globe, for example, Hutsch (1998) reported that the addition of ammonium fertiliser strongly inhibited the activity of methanotrophs by up to 90%. Therefore an ammonium treated soil would be expected to contain little of no evidence of BHP methanotroph markers as seen in the BHP profile of Paalce Leas plot 7.

Seghers at el., (2003) compared the effect of organic fertilisers and nitrate fertilisers on methanotroph populations and demonstrated that using organic fertilisers, manure, the number of methanotrophs, were higher than in sites treated with mineral fertilisers (nitrate, phosphate and potassium]. Therefore, a site treated with manure would be expected to be higher in BHP methanotroph markers than other sites. This is seen at Paalce Leas where the manured plot 2 contains higher levels of the methanotroph marker aminopentol (**1c**).

The BHP profile appears to also give an indication of the diversity of the BHP producing bacterial population and there appears to be a link between number of structures and bacterial diversity. This link is best seen in the increase in the number of BHP structures and semi-quantitative BHP in soils from Seville that were recovering from a forest fire. However it must be remembered that in Palace Leas plot 7 the diversity of BHPs and their concentration was directly related to bacterial stress and not changes in population. This relationship between concentration of BHPs an number of observed structures and the bacterial population size and diversity is sill unclear although a logical approach would be that the BHP concentration and the number of structures increases with increases in the bacterial population, such as those seen in Seville, until a stable population is achieved and then the BHP concentration and number of structures varies in response to environmental stress.
The paddy field samples show a lower than expected level of methanotrophs which could be related to the sampling time and few BHP structures indicating the lack of a diverse BHP producing bacterial population.

8.1.3 Differences between Soil BHP profiles.

Using BHP profile analysis differences between the Palace Leas plots BHP producing bacteria as a result of fertiliser reimes can be ascertained. The differences can be simple (such as the presence oif significantly more aminopentol (1c) in plot 2 manure treatment) or more complex and require the use of PCA to separate the plot characteristics (Fig. 3.37). Using the 6 different bacterial groups that can be identified using BHP analysis; methanotrophs, cyanobacteria, purple non-sulphur bacteria, nitrogen fixers and ammonia oxidisers, acetic acid bacteria and methylotrophs (in addition to groups for various and unknown source bacteria) and using these groupings in the PCA analysis the separation between the plots is clear (Fig. 3.38). The differences between the plots are variable with plots 2 and 7 being the most changed when compared to control, plot 6. This indicates that the different fertilisers have different effect on the soil BHP profile. In some cases thre are small, (plots 8 and 9), and indicates that the soil make upo coulds be a significant force in determining the BHP profile, and therefore the bacterial population.
This separation is repeated in the Hack Hall Farm investigation. The PCA analysis (Figs 4.15 and 4.16) shows that the 0-5 cm layers of all samples are more strongly

influenced by cyanobacteria than the 10-15 cm layers which are more strongly influenced by the presence of methanotrophs. This is in agreement with the expected distribution of bacteria in the soil and it again appears to confirm the relationship between sourc bacteria in the soil and it again appears to confirm that the relationship between source bacteria and specific BHPs is robust. The sycamore samples are more strongly influenced by cyanobacteria than the other samples, as would be expected fromthe more sunlight received by these samples. There are differences between the BHP profiles that enable a separation to be observed between the different tree species with common firs samples being most strongly influence by methanotrophs and the sycamore samples influenced by cyanobactria (Fig. 4.16).

The BHP profiles of landfill cover soils are distinct from other sites investigated due to the very low BHP concentrations and the low number of different BHP structures identified. There is no evidence from the BHP results from any of the landfill cover soils that there is an increase in the population of methanotrophs and when compared to other sites (Palace Leas and Hack Hall Farm) the level of methanotrophs is slightly reduced. As all the sites, except Chicken Road, are known to have high elevated CH_4 levels this result was somewhat unexpected. The enhanced gas collection and control methods found in a modern, well operated landfill such as Seghill may account for the the very low levels of methanotrophs at the site. The low levels of methanotroph markers at the much more poorly constrained Chicken Road, Octavia Court and West Allotment sites may be due to the use of till in the cover soil. The BHP profiles indicate that the cover soil BHP producing bacteria population is dominated by PNSB, N_2 fixing and NH_3 oxidising bacteria in accordance with the observations of Yu et al., 2009 (NH_3 oxidising bacteria only).

Using PCA (Figs 7.27 and 7.28) there is a clear separation between soils based on 2 factors; the concentration of methanotrophs and BHPs with unknown origin, analogous to the number of observed BHPs. These 2 factors generate 2 distinct clusters of locations which are further separated by the influence of adenosylhopane and its presence in PNSB and nitrogenand ammonia fixing bacteria.

It is clear from these analyses that there are clear differences between the BHP producing bacterial populations of soils from around the world driven by the relative concentration of methanotroph derived BHPs and the overall number of BHP structures identified in the samples.

8.1.4 Temporal Difference in BHP population

The analysis of Palace Leas and Hack Hall Farm showed no significant variation in the BHP distribution within the locations. The implication of this is that the types of BHP producing bacteria in the bacterial population do not change significantly. Within the observed year ther is, however, a significant change in the total concentration of BHPs in the plots with rises in the summer and autumn months. This implies that there is either an increase in total population of BHP producing bacteria in the soil or that the bacteria are producing more BHPs in response to environmental change. Logic suggests that it is the former as during the colder wetter months a reduced bacterial population will be less active. It appears that, as previously discussed, intact BHPs can be quickly degraded in aerobic soil or incorporated into humin with only a small fraction surviving inact that is amenable to extraction using the methods used here. Further study is required in this area as it is difficult to draw firm conclusions on annual cycles from only one year.

8.2 Hypothesis 2

Complex intact BHPs are preserved in ancient marine sediments.

Analysis of ODP 1075 shows a remarkable preservation of intact BHP structures to an age of 1Ma which contradicts previous studies suggesting rapid degradation due to diagenesis (Talbot et al., 2003) and the subsequent formation of geohopanoids (e.g. Rohmer et al., 1980; Quirk et al., 1984; Bissaret et al., 1997; Innes et al., 1997; Rodier et al., 1999; Tritz et al., 1999; Watson and Farrimond, 2000), or the rapid incorporation into kerogen (Farrimond et al., 2003). A total of 15 different structures were identified and adenosylhopane (**1e**), aminotetrol (**1h**), aminopentol (**1c**), BHpentol cyclitol ether (**1l**) and BHhexol cyclitol ether (**1k**) were all identified continually down the core (Fig. 6.5), indicating that there is significant preservation of intact BHPs. There is no clear trend in total BHP concentration with depth, possibly indicating that the peaks in BHP concentration are related to peaks organic matter input into the sediment.

The identification of aminopentol (**1c**), a marker for aerobic methane oxidation at all depths in the ODP1075 core (Fig. 6.5) indicates that complex terrestrial or water

column derived BHP structures are preserved intact during burial to an age of at least 1 million years in marine sediments. There is also an underlying diagenetic trend of reduction in concentration of BHPs, including the soil markers, over time together with a corresponding increase in the possible adenosylhopane (**1e**) diagenetic product anhydroBHT (**1j**; Schaeffer et al., 2008).

The identification of proposed soil specific BHP marker compounds adenosylhopane (**1e**), 2-Me adenosylhopane (**2e**), adenosylhopane type-1 (**1u**) and 2-Me adenosylhopane type-1 (**2u**) in all the estuary sample and the obvious decrease in concentration with distance from river mouth in the Congo and Rhone samples supports these recalcitrant molecules to be used as proxies for the movement and burial of SOC by rivers. The decrease in concentration with distance from river mouth indicates progressive dilution by marine derived organic matter or diagenesis in the water column.

Arctic estuary surface sediments contain soil-specific BHP marker compounds at levels comparable to those in soils themselves, indicating a significant soil input to the sediment. This is in agreement with earlier studies suggesting a high flux of fluvially transported SOM to the Arctic shelf systems.

Overall these results clearly demonstrate the potential for BHPs to be used as a biomarker when investigating the transport of TOM via rivers to marine environments and agree with the results obtained using the BIT index for the Congo Fan and West Africa (Hopmans et al., 2004) and the Rhone (Kim et al., 2010). These global results will enable soil marker BHPs to be correlated with the BIT index and will provide an additional in sights in to the fate of TOM thereby enabling a fuller understanding its sources and fate.

8.3 Summary

This research has achieved its aim of assessing the potential of BHPs to act as a biomarker for bacterial activity or specific environmental conditions in soils and sediments. The following points summarise the research

- The BHP profiles of soils are dominated by 4 or 5 BHPs; BHT (**1a**), aminotriol (**1f**), adenosylhopane (**1e**), adenosylhopane type 1 and BHT cyclitol ether (**1d**).

- 44 different BHPs have been identified during this research including the tentative identification of 10 novel BHPs.
- The presence of specific BHPs has been linked to known source bacteria and confirmed by comparison with other research (Palace Leas, Hack Hall Farm, Landfills and global soils).
- A clear differentiation between different environments can be observed in the BHP profile by using PCA (All soils).
- BHPs appear to be labile in oxic soils with clear cycling in concentrations being observed annually (Palace Leas, Hack Hall).
- BHPs appear to be expressed at increased concentrations when bacteria are stressed (Palace Leas)
- The total number of structures and concentration of BHPs can be used to compare changes in bacterial population (Palace Leas, Hack Hall Farm, and Seville).
- BHPs appear to be recalcitrant in anoxic marine sediments (Congo)
- The BHP profile has been linked to the occurrence of other biomarkers (Arctic).
- A group of soil marker BHPs have been identified that can be used to trace SOM to marine environments (Congo, Rhone and Arctic river estuaries). However there is some apparent degradation of these soil marker BHPs during diagenesis so comparisons are difficult between different river systems.

8.4 Future Research Areas

It is clear from this research that BHPs are a useful biomarker for bacterial populations and activity in modern soils and in ancient and modern sediments but many questions still exist about the source, role and fate of BHPs in the environment.

8.4.1 Investigation into other environments

This research has focussed on soils from the northern hemisphere especially NE England. The soils in this area are post glacial till derived soils from a temperate environment and although other global soils have been investigated there is a need to

analyse soils from a much more diverse locations. Primary investigation sites should be the soils from the catchment areas of the river estuaries investigated in this thesis; Congo, Rhone, the 5 GRARs and the 2 Alaskan rivers. Although the evidence from these rivers indicates the transport of SOM due to the presence of the soil marker BHPs in the sediments, a full investigation into the BHP profile of the soils of the river catchments and the river sediments will confirm this hypothesis and enable a fuller understanding of the fate of BHPs in sediments including sites of degradation and accumulation.

Further study into environments with known bacterial activities, such are the work on peats in this thesis (Chapter 7), can enable the strengthening of the link between BHPs and source bacteria.

This research will enable BHPs to be used to investigate palaeo-environments and potentially provide detailed information of the bacterial activity and environmental conditions in the source location of the organic content of sedimentary material.

8.4.2 Investigation into the relationship between BHPs and source bacteria

This research has clearly established a link between source bacteria and BHPs in the environment by comparing the BHP profile with the work of other researchers on similar environments (e.g. Seghers et al., 2003 and the relationship between methanotrophs and soils treated with manure). Other linkages between BHPs and source bacteria have been made by comparing the location of BHPs with the expected location of bacteria in soil (e.g. cyanobacteria marker BHPs are predominantly found in the surface soil) by comparing the relationships of different bacterial groups using PCA. The influences of cyanobacteria derived BHPs and methanotroph derived BHPs are opposite on most PCA plots (e.g. Fig. 7.28) and these BHPs would be expected to inhabit different locations in the soil with the methanotrophs at deeper levels than the cyanobacteria.

Whilst not a contradiction to some current thinking there are some issues with using BHP – source bacteria linkages. Rashby et al., (2007) and Doughty et al., (2009) have both linked 2 methyl BHP production to other sources in addition to cyanobacteria. Bradley et al., (2010) place adenosylhopane (1e) at the centre of the addition of the side chain to the triterpenoid ring system. If this is the case then the relationship of adenosylhopane to source bacteria must be at doubt. However landfill sites are rich in

adenosylhopane (Chapter 5) and this result is in agreement with other research (Yu et al., 2009) and sediments do not appear to contain high levels of adenosylhopane.

These questions will continue to dominate research of BHPs and research must be undertaken that simultaneously analyses BHP profiles and bacteria activity using the primer for *shc*. This will improve the link between the BHP profile and bacterial content.

8.4.3 Investigation into the role BHPs in bacteria

Whilst the link between BHP and source bacteria and environment is vital to understanding the significance of the BHP profile it does identify their role in bacteria.

The link between BHPs and membrane stability is relatively well understood (Ourisson and Rohmer, 1982; Rohmer et al., 1984; Ourisson et al., 1987, Saenz et al., 2010) this role alone, and evolutionary change, cannot explain the extreme diversity of BHPs and BHPs must have other more specific or different roles in bacteria. The wide range of BHP side chains that will protrude from the membrane could have a role in enzyme stability and activation. The association of a specific BHP with an enzyme may activate or deactivate it by a structural relationship of by feedback inhibition (Moreau et al., 1997).

Different BHPs may be produced by bacteria in response to environmental change (Welander et al., 2009) and research must be conduction using microcosms to identify the response of bacterial communities to environmental change. A fuller understanding of the fate of intact BHPs would lead to an increased understanding of the turnover of organic carbon in soil which will improve the understanding of the carbon cycle.

8.4.4. Fate of BHPs in the environment

The distribution and concentration of BHPs in the soil profile is influenced by soil type, source bacteria, soil oxygenation and leaching rate. It is vital to understand the effect that each has on the fate of the BHPs to understand the source, age and role of BHPs within the soil bacterial community.

This thesis has used solvent extraction techniques and HPLC-MS^n to identify the intact BHP composition of the soil. These techniques do not identify BHPs bound to humin, acid hydrolysis is needed, nor do they readily identify geohopanoids, where GC-MS is required. These techniques should be used to conduct a fuller investigation into the fate of BHPs in the soils. The main reason the GC-MS was not used is that adenosylhopane and its homologues are not amenable to GC-MS and constitute a significant proportion of the BHP profile.

It is important to understand the diagenetic fate of the BHPs to assess how the concentration varies during the annual cycling that is observed in the Palace Leas and Hack Hall sites. Are the BHPs reabsorbed by the bacteria, consumed by other bacteria, incorporated into humin or degraded to geohopanoids? Is the degradation to geohopanoids conducted biotically or abiotically.

This investigation would be carried out using $\delta^{13}C$ labelled catabolites that could be traced through the food chain and $\delta^{13}C$ BHPs that could be tracked either through the bacterial population or allowed to degrade in sterilised soils, for example.

8.4.5 Investigation into BHP structures

The tentative identification of 10 different novel BHPs during this research has been one of the most exciting and frustrating aspects of this research. New structures present the opportunity to identify new bacterial sources and new processes but the inability to identify them and link to bacteria is a hurdle to the complete understanding of the BHP profile.

The ability it identify the novel structures discovered during this research, specifically Adenosylhopane types 1, 2, 3 and 4, is essential and requires a high resolution mass spectrometer, to obtain accurate structure masses, prep HPLC to obtain pure samples and an NMR to identify the exact structure.

FIN

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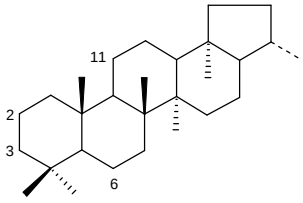
Appendix 1. BHP structure names, identifying base peak ion, structure number and abbreviated name.

Name	**Base Peak *m/z***	**Structure Number**	*Abbreviated name*
32,35 Anhydrobacteiohopanetetrol	**613**	**1j'**	AnhydroBHT
Ribonylhopane	**627**	**1t'**	Hopane lactone
Δ^{6}- Bacteriohopane-32,33,34,35-tetrol	**653**	**5a'**	$\Delta^{6\text{ or }11}$-BHT, unsaturated BHT
Δ^{11}- Bacteriohopane-32,33,34,35-tetrol	**653**	**6a'**	$\Delta^{6\ 11}$-BHT, unsaturated BHT
Bacteriohopane-32,33,34,35-tetrol	**655**	**1a'**	BHT
2-methyl Bacteriohopane-32,33,34,35-tetrol	**669**	**2a'**	2-me BHT
3-methyl Bacteriohopane-32,33,34,35-tetrol	**669**	**3a'**	3-me BHT
12-methyl Bacteriohopane-32,33,34,35-tetrol	**669**	**4a'**	12-me BHT
$\Delta^{6\text{ or }11}$ 35-aminobacteriohopane-32,33,34-triol	**712**	**5f', 6f'**	Unsaturated aminotriol
Bacteriohopane-31,32,33,34,35-pentol	**713**	**1m'**	BHpentol
35-aminobacteriohopane-23,33,34-triol	**714**	**1f'**	Aminotriol
2 methyl 35-aminobacteriohopane-32,33,34-triol	**728**	**2f'**	2-me aminotriol
3 methyl 35-aminobacteriohopane-32,33,34-triol	**728**	**3f'**	3-me aminotriol
30-(5'-adenosyl)hopane	**746**	**1e'**	Adenosylhopane
2 methyl 30-(5'-adenosyl)hopane	**760**	**2e'**	2-me adenosylhopane
'*Adenosylhopane type- 1'*[a]	**761**	**1u'**	'Adenosylhopane type- 1'
Bacteriohopane-30,31,32,33,34,35-hexol	**771**	**1b'**	BHhexol
35-aminobacteriohopane-31,32,33,34-tetrol	**772**	**1h'**	Aminotetrol
'2-me adenosylhopane type-1'	**775**	**2u'**	'2-me adenosylhopane type-1'
'Adenosylhopane type-2'	**802**	**1v'**	*'Adenosylhopane type-2'*
'2-me adenosylhopane type-2'	**816**	**2v'**	*'2-me adenosylhopane type-2'*
'2-me adenosylhopane type-3'	**818**	**2w'**	*'2-me adenosylhopane type-3'*
'Adenosylhopane type-4'	**818**	**1x'**	*'Adenosylhopane type-4'*
$\Delta^{6\text{ or }11}$ 35-aminobacteriohopane-30,31,32,33,34-pentol	**828**	**5c', 6c'**	*Unsaturated Aminopentol*
35-aminobacteriohopane-30,31,32,33,34-pentol	**830**	**1c'**	*Aminopentol*
3 methyl 35-aminobacteriohopane-30,31,32,33,34-pentol	**844**	**2c'**	*3-me aminopentol*
$\Delta^{6\text{ or }11}$ 35, pentose Bacteriohopane-32,33,34-tetrol	**941**	**5n', 6n'**	*Unsaturated BHT pentose*
35, pentose Bacteriohopane-32,33,34-tetrol	**943**	**1n'**	*BHT pentose*
2 methyl 35, pentose Bacteriohopane-32,33,34-tetrol	**957**	**2n'**	2-me BHT pentose
$\Delta^{6\text{ or }11}$ Bacteriohopanetetrol carbopseudopentose ether	**1000**	**5d', 6d'**	Unsaturated BHT ce
35, pentose Bacteriohopane-31,32,33,34-pentol	**1001**	**1o'**	BHpentol pentose
Bacteriohopanetetrol carbopseudopentose ether	**1002**	**1d'**	BHT ce
Bacteriohopanetetrol glycoside	**1002**	**1g'**	BHT glu
$\Delta^{6\text{ or }11}$ 2 methyl Bacteriohopanetetrol carbopseudopentose ether	**1014**	**7d'**	Unsaturated 2-me BHT ce
$\Delta^{6\text{ or }11}$ 2 methyl Bacteriohopanetetrol carbopseudohexose ether	**1014**	**5p'**	Unsaturated BHT ce methylated on sugar
2 methyl Bacteriohopanetetrol carbopseudopentose ether	**1016**	**2d'**	2-me BHT ce
2 methyl Bacteriohopanetetrol carbopseudopentose ether	**1016**	**3d'**	3-me BHT ce
'Bacteriohopanetetrol carbopseudohexose ether'	**1016**	**1p'**	'BHT ce methylated on sugar'

Name	**Base Peak *m/z***	**Structure Number**	*Abbreviated name*
$\Delta^{6 \text{ or } 11}$ Bacteriohopanepentol carbopseudopentose ether	**1058**	**5l', 6l'**	Unsaturated BHpentol ce
Bacteriohopanepentol carbopseudopentose ether	**1060**	**1l'**	BHpentol ce
Bacteriohopanepentol glycoside	**1060**	**1q'**	BHpentol glu
2 methyl Bacteriohopanepentol carbopseudopentose ether	**1074**	**2l'**	2-me BHpentol cyclitol ether
3 methyl Bacteriohopanepentol carbopseudopentose ether	**1074**	**3l'**	3-me BHpentol ce
Guanidine-substituted Bacteriohopanetetrol carbopseudopentose	**1086**	**1s'**	Guanidine substituted BHT ce
Bacteriohopanehexol carbopseudopentose ether	**1118**	**1k'**	BHhexol ce
2 methyl Bacteriohopanehexol carbopseudopentose ether	**1132**	**2k'**	2-me BHhexol ce
3 methyl Bacteriohopanehexol carbopseudopentose ether	**1132**	**3k'**	3-me BHhexol ce
'Bacteriohopanehexol carbopseudohexose ether'	**1132**	**1r'**	'BHhexol ce methylated on sugar'
C_{32} hopanol		**1y**	C_{32} hopanol
C_{31} hopanol		**1z**	C_{31} hopanol
C_{30} hopanol		**1aa**	C_{30} hopanol

Appendix 2. BHP structures



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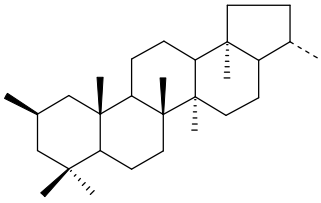

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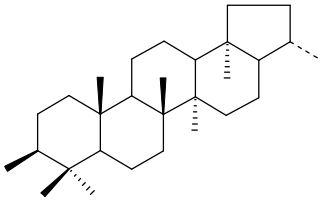
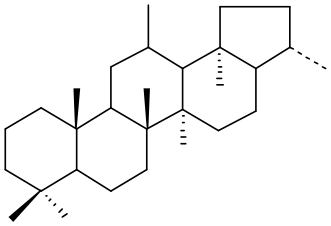


3

4: Δ^6; **5**: Δ^{11}; **6** $\Delta^{6,11}$

7: Δ^6; **8**: Δ^{11}; **9**: $\Delta^{6,11}$



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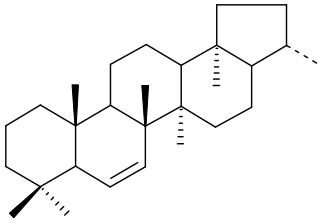

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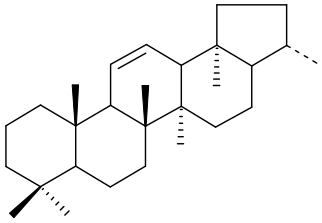

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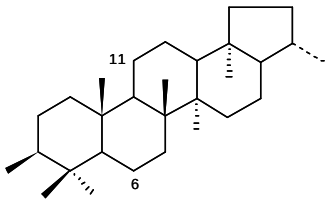

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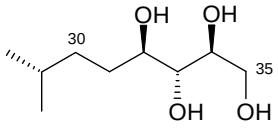

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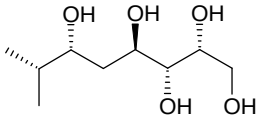

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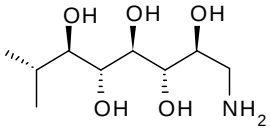

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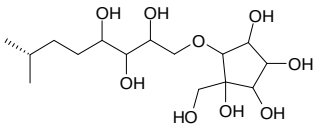

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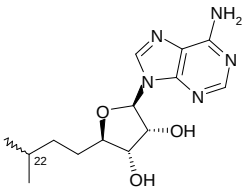
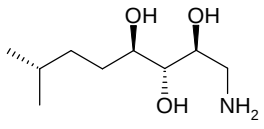
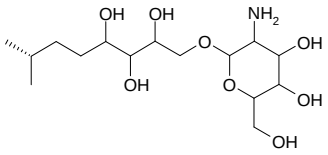
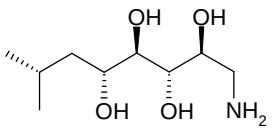


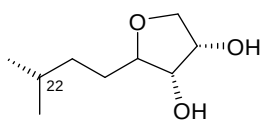
e, e’ = di-acetate
e’’ = tri-acetate
e’’’ = tetra-acetate



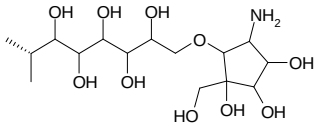
f, f’ = tetra-acetate,



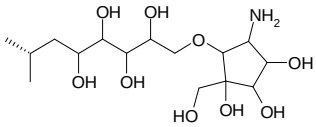




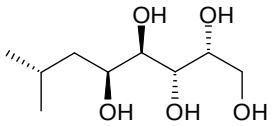
g, g’ = hepta-acetate



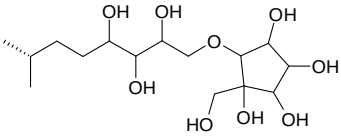
h, h’ = penta-acetate



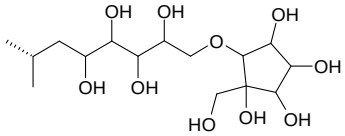
j, j’ = di-acetate



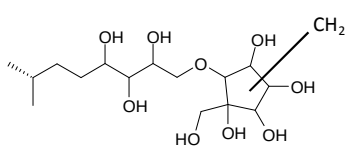
k, k’ = nona-acetate



l, l’ = octa-acetate



m, m’ = penta-acetate

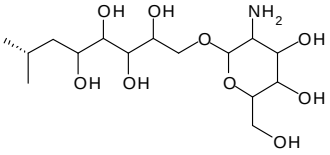


n, n’ = hepta-acetate
n’’ = octa-acetate

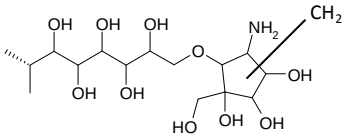
o, o’ = octa-acetate

p, p’ = hepta-acetate

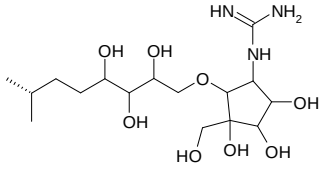
q, octa-acetate



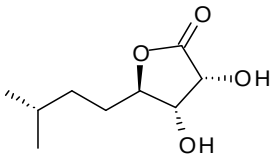
r



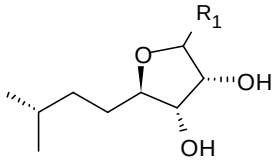
s, s’ = octa-acetate



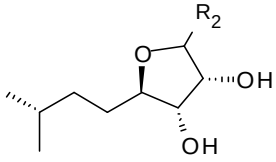
t, t’ = diacetate



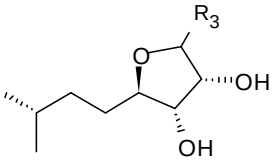
u, u’ = acetylated



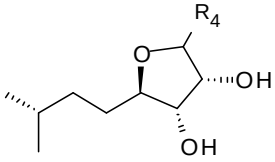
v, v’ = acetylated



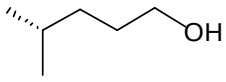
w, w’ = acetylated



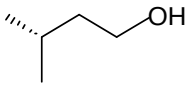
x, x’ = acetylated



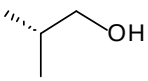
y



z



aa



Appendix 3. BHP Concentrations.

A1 Palace Leas

A1(i) Palace Leas Plot 2 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 2 Core 1 September 2004			Palace Leas Plot 2 Core 2 September 2004			Palace Leas Plot 2 Core 2 September 2004 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	40	30	23	34	25	22	46	39	8
2 Me 669	**2a'**	12	15	11	12	15	15	12	21	4
712	**5f', 6f'**	2	6	7	3	6	22	6	28	7
714	**1f'**	93	131	141	74	159	191	83	159	75
2 Me 728	**2f'**	1	2	ND	1	1	3	ND	ND	1
746	**1e'**	73	88	86	60	63	79	63	100	27
760	**2e'**	20	26	20	14	15	19	12	22	6
761	**1u'**	56	59	56	34	49	65	44	72	18
772	**1h'**	5	3	5	4	8	8	5	6	3
775	**2u'**	2	6	3	2	4	4	3	4	ND
830	**1c'**	ND	2	2	6	3	3	8	5	0
943	**1n'**	ND	ND	ND	ND	ND	ND	7	6	ND
957	**2n'**	ND	ND	ND	ND	ND	ND	3	ND	ND
1000	**5d', 6d'**	ND	ND	ND	ND	ND	ND	ND	3	ND
1002 ce	**1d'**	108	122	149	47	131	124	85	148	40
1002 glu	**1g'**	ND	ND	ND	8	ND	ND	11	ND	ND
2 Me 1016	**2d'**	12	15	16	6	11	11	7	19	4
1060 ce	**1l'**	28	35	39	15	3	48	16	51	16
2 Me 1074	**2l'**	2	4	3	ND	4	ND	3	6	1
1086	**1s'**	3	ND	ND	1	1	ND	3	ND	ND
1118	**1k'**	7	11	10	6	13	13	8	19	3
2 Me 1132	**2k'**	ND	ND	ND	ND	ND	ND	1	3	0
Total		464	554	572	327	510	628	426	708	214
TOC (%)		7.3	4.5	3.5	10.0	5.7	4.2	10.0	5.7	4.2

A1(ii) Palace Leas Plot 2 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 2 Core 1 January 2005				Palace Leas Plot 2 Core 2 January 2005			Palace Leas Plot 2 Core 2 January 2005 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm repeat	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	31	27	28	36	36	26	23	34	29	20
2 Me 669	**2a'**	10	9	8	9	10	11	9	10	1	9
3 Me 669	**3a'**	1	2	2	3	ND	2	2	ND	2	6
712	**5f', 6f'**	10	16	20	10	12	16	15	9	16	17
714	**1f'**	119	123	123	111	147	134	112	131	134	134
2 Me 728	**2f'**	ND	2	3	2	2	2	2	ND	3	3
3 Me 728	**3f'**	2	2	3	1	1	3	2	ND	3	4
746	**1e'**	95	71	73	93	106	63	74	132	121	114
760	**2e'**	24	20	21	26	23	17	20	27	31	31
761	**1u'**	45	55	60	53	43	54	51	45	49	49
772	**1h'**	6	7	5	7	9	5	4	10	6	6
775	**2u'**	3	2	2	4	4	2	2	ND	ND	ND
830	**1c'**	7	5	3	7	11	3	2	9	2	1
943	**1n'**	ND	ND	ND	ND	5	ND	ND	3	ND	ND
1002 ce	**1d'**	86	83	96	91	3	87	93	132	151	145
2 Me 1016	**2d'**	7	6	6	7	9	4	4	11	16	13
1058	**5l', 6l'**	3	9	ND	5	ND	ND	ND	ND	ND	ND
1060 ce	**1l'**	14	13	15	18	14	13	16	43	52	47
2 Me 1074	**2l'**	1	2	ND	ND	4	ND	ND	ND	ND	3
3 Me 1074	**3l'**	2	2	1	1	2	1	3	ND	ND	ND
1118	**1k'**	5	3	5	4	7	3	3	7	7	3
Total		464	443	467	480	444	438	429	604	617	595
TOC (%)		7.6	6.2	4.6	7.7	7.7	5.5	4.1	7.7	5.5	4.1

A1(iii) Palace Leas Plot 2 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 2 Core 1 January 2006			Palace Leas Plot 2 Core 2 January 2006			Palace Leas Plot 2 Core 1 May 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	3	4	ND	ND	ND	ND	ND	ND
655	**1a'**	31	20	20	19	17	12	46	37	10
2 Me 669	**2a'**	12	10	18	8	9	1	14	22	4
3 Me 669	**3a'**	ND	ND	3	ND	ND	ND	ND	ND	ND
712	**5f', 6f'**	7	17	28	2	4	23	15	26	26
714	**1f'**	79	151	166	30	34	143	155	194	188
2 Me 728	**2f'**	1	2	2	0.3	1	4	2	1	5
3 Me 728	**3f'**	ND	ND	ND	0.3	1	2	3	3	4
746	**1e'**	73	70	93	28	41	84	106	118	58
760	**2e'**	20	21	23	9	16	27	27	26	17
761	**1u'**	46	47	49	9	31	41	47	91	23
772	**1h'**	5	6	6	1	1	5	10	9	10
775	**2u'**	2	3	4	1	1	ND	ND	5	ND
816	**2v'**	ND	1	ND	ND	ND	ND	ND	ND	ND
830	**1c'**	10	ND	ND	1	1	2	5	ND	ND
943	**1n'**	ND	ND	ND	3	1	ND	ND	ND	ND
1002 ce	**1d'**	80	91	119	40	57	130	119	137	67
2 Me 1016	**2d'**	8	9	9	5	7	14	12	16	4
3 Me 1016	**3d'**	0	0	0	0	0	0	0	0	0
1058	**5l', 6l'**	ND	ND	ND	2	ND	ND	ND	ND	ND
1060 ce	**1l'**	16	20	31	7	16	26	19	16	9
2 Me 1074	**2l'**	3	2	3	1	1	3	3	8	2
3 Me 1074	**3l'**	ND	ND	ND	0.3	2	4	ND	ND	ND
1086	**1s'**	5	ND	ND	1	ND	ND	ND	ND	ND
1118	**1k'**	5	5	5	1	2	0	3	4	0
2 Me 1132	**2k'**	ND	1	1	0.3	1	ND	ND	2	ND
Total		404	479	582	167	240	515	586	710	423
TOC (%)		8.6	5.4	3.5	10.4	7.1	4.2	8.0	5.7	3.1

A1(iv) Palace Leas Plot 2 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 2 Core 1 May 2006 repeat			Palace Leas Plot 2 Core 2 May 2006			Palace Leas Plot 2 Core 1 October 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
613	**1j'**	ND	ND	ND	ND	ND	ND	1	ND	ND
655	**1a'**	35	32	18	73	91	15	44	57	43
2 Me 669	**2a'**	8	22	8	22	45	6	22	33	18
712	**5f', 6f'**	7	25	21	6	42	38	31	93	73
714	**1f'**	80	186	127	140	374	212	265	687	670
2 Me 728	**2f'**	2	1	3	ND	6	3	4	9	13
3 Me 728	**3f'**	1	4	2	ND	ND	4	3	6	12
746	**1e'**	63	117	32	116	211	119	120	244	164
760	**2e'**	15	0.1	9	34	62	30	29	67	47
761	**1u'**	34	77	52	51	119	40	71	114	63
772	**1h'**	8	6	6	20	25	9	11	30	24
775	**2u'**	0	6	2	2	5	3	4	11	2
830	**1c'**	3	ND	ND	46	3	ND	4	5	9
844	**2c'**	ND	ND	ND	8	18	ND	ND	ND	ND
1002 ce	**1d'**	69	164	65	149	413	144	187	415	391
1002 glu	**1g'**	ND	ND	ND	2	3	ND	ND	ND	ND
2 Me 1016	**2d'**	7	10	4	1	33	11	18	50	45
3 Me 1016	**3d'**	ND	ND	ND	1	ND	ND	ND	ND	ND
1060 ce	**1l'**	4	24	11	14	49	19	23	76	76
2 Me 1074	**2l'**	0.4	1	2	2	8	2	3	10	7
3 Me 1074	**3l'**	ND	3	ND	3	14	7	4	15	9
1086	**1s'**	ND	ND	ND	ND	ND	2	6	ND	ND
1118	**1k'**	2	ND	ND	5	31	2	9	32	27
2 Me 1132	**2k'**	2	ND	ND	2	5	ND	ND	2	ND
3 Me 1132	**3k'**	ND	ND	ND	ND	ND	ND	ND	2	ND
Total		339	671	358	692	1543	655	852	1935	1674
TOC (%)		8.0	5.7	3.1	2.8	8.8	5.4	3.4	5.8	4.5

A1(v) Palace Leas Plot 2 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 2 Core 2 October 2006			Palace Leas Plot 2 Core 1 January 2007			Palace Leas Plot 2 Core 1 January 2007		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	102	38	16	23	23	15	76	43	51
2 Me 669	**2a'**	25	18	7	18	20	15	34	35	34
3 Me 669	**3a'**	8	ND	ND	ND	ND	ND	ND	4	ND
712	**5f', 6f'**	23	55	24	32	7	15	29	43	50
714	**1f'**	349	403	213	174	77	89	271	261	286
2 Me 728	**2f'**	4	6	4	2	1	3	4	3	6
3 Me 728	**3f'**	4	6	4	1	ND	ND	3	ND	ND
746	**1e'**	112	214	53	70	89	52	109	130	119
760	**2e'**	29	65	16	12	20	10	26	34	28
761	**1u'**	72	59	69	53	51	42	121	111	99
772	**1h'**	22	21	6	5	2	2	15	10	12
775	**2u'**	ND	2	3	5	6	4	8	8	7
802	**1v'**	2	ND	ND	ND	ND	ND	ND	ND	ND
816	**2v'**	4	ND	ND	ND	ND	ND	ND	ND	ND
830	**1c'**	15	ND	1	0.3	ND	ND	3	6	ND
844	**2c'**	3	ND	ND	ND	ND	ND	ND	ND	ND
943	**1n'**	ND	ND	ND	ND	7	6	ND	ND	ND
957	**2n'**	ND	ND	ND	4	ND	ND	ND	ND	ND
1002 ce	**1d'**	212	369	137	72	102	85	214	212	284
1002 glu	**1g'**	2	ND	ND	ND	1	1	ND	ND	ND
2 Me 1016	**2d'**	15	29	17	7	9	7	22	14	23
3 Me 1016	**3d'**	ND	ND	ND	1	ND	ND	ND	ND	ND
1060 ce	**1l'**	21	74	18	11	18	23	26	25	40
2 Me 1074	**2l'**	ND	7	ND	2	3	ND	6	4	9
3 Me 1074	**3l'**	ND	ND	ND	ND	ND	ND	7	4	8
1118	**1k'**	13	22	1	8	9	11	9	6	11
2 Me 1132	**2k'**	3	ND	ND	ND	1	0.2	3	ND	2
Total		1023	1382	586	499	445	377	975	945	1062
TOC (%)		7.4	4.9	2.8	5.9	4.2	3.5	6.5	4.6	3.9

A1(vi) Palace Leas Plot 2 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 2 Core 1 January 2007	Palace Leas Plot 2 Core 2 January 2007		
		10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	4	ND	ND
653	**5a', 6[a]'**	ND	ND	3	ND
655	**1a'**	5	44	37	46
2 Me 669	**2a'**	25	14	25	26
3 Me 669	**3a'**	5	ND	ND	ND
712	**5f', 6f'**	28	26	38	63
714	**1f'**	280	206	253	361
2 Me 728	**2f'**	4	3	4	4
3 Me 728	**3f'**	3	3	2	6
746	**1e'**	165	114	80	59
760	**2e'**	24	25	32	17
761	**1u'**	13	7	101	88
772	**1h'**	16	7	9	9
775	**2u'**	8	3	6	8
830	**1c'**	6	ND	ND	ND
1002 ce	**1d'**	217	149	221	164
2 Me 1016	**2d'**	16	15	16	13
3 Me 1016	**3d'**	ND	ND	2	ND
1060 ce	**1l'**	37	17	43	25
2 Me 1074	**2l'**	5	4	4	3
3 Me 1074	**3l'**	4	2	ND	ND
1118	**1k'**	11	6	11	4
2 Me 1132	**2k'**	3	2	2	3
Total		863	646	886	894
TOC (%)		3.9	6.5	4.6	3.9

A1(vii) Palace Leas Plot 6 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 6 Core 1 September 2004			Palace Leas Plot 6 Core 2 September 2004			Palace Leas Plot 6 Core 1 January 2005		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	15	19	20	16	13	4	5	13	13
653	**5a', 6[a]'**	7	ND	ND	ND	ND	ND	ND	ND	ND
655	**1a'**	169	73	54	85	66	42	44	39	30
2 Me 669	**2a'**	43	48	38	52	48	42	22	25	25
712	**5f', 6f'**	24	20	30	18	21	21	20	34	20
714	**1f'**	238	225	266	175	168	169	224	313	148
2 Me 728	**2f'**	ND	5	8	ND	ND	ND	3	7	3
3 Me 728	**3f'**	ND	ND	ND	ND	1	ND	ND	ND	ND
746	**1e'**	390	352	284	208	191	196	178	243	206
760	**2e'**	24	23	27	21	18	20	15	24	22
761	**1u'**	188	274	184	133	172	197	107	168	146
772	**1h'**	ND	4	4	4	3	5	7	5	4
775	**2u'**	18	20	10	15	17	15	8	12	9
941	**5n', 6n'**	34	ND	ND	ND	ND	ND	ND	ND	ND
943	**1n'**	102	13	ND	42	13	ND	14	6	ND
957	**2n'**	ND	ND	ND	ND	ND	ND	22	27	ND
1000	**5d', 6d'**	ND	ND	ND	ND	ND	ND	5	4	ND
1001	**1o'**	34	ND	ND	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	570	432	524	422	328	377	189	253	182
1002 glu	**1g'**	ND	ND	ND	ND	ND	ND	22	ND	ND
2 Me 1016	**2d'**	22	32	24	39	21	23	7	6	13
3 Me 1016	**3d'**	11	ND	ND	ND	ND	ND	ND	ND	ND
1058	**5l', 6l'**	ND	ND	ND	ND	ND	ND	3	3	ND
1060 ce	**1l'**	79	76	90	66	53	57	29	33	20
2 Me 1074	**2l'**	ND	18	33	6	8	6	10	4	ND
3 Me 1074	**3l'**	ND	ND	ND	ND	15	19	ND	13	14
1086	**1s'**	8	ND	ND	ND	29	4	ND	ND	ND
1118	**1k'**	75	23	30	48	ND	37	17	21	14
2 Me 1132	**2k'**	ND	2	ND	3	2	ND	1	3	ND
Total		2006	1659	1624	1351	1172	1213	943	1234	855
TOC (%)		6.6	3.1	2.5	4.5	2.7	2.5	6.8	3.3	2.4

A1(viii) Palace Leas Plot 6 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 6 Core 2 January 2005			Palace Leas Plot 6 Core 1 January 2006			Palace Leas Plot 6 Core 2 January 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	15	12	12	ND	ND	ND	ND	ND	ND
653	**5a', 6[a]'**	ND	ND	ND	2	ND	ND	ND	ND	ND
655	**1a'**	81	41	25	44	28	74	56	26	24
2 Me 669	**2a'**	45	29	18	22	26	9	27	16	15
712	**5f', 6f'**	28	23	25	44	44	5	21	5	7
714	**1f'**	296	212	253	448	415	507	242	68	53
2 Me 728	**2f'**	3	6	5	4	7	3	2	1	1
3 Me 728	**3f'**	ND	ND	2	ND	ND	ND	ND	ND	ND
746	**1e'**	277	169	154	101	102	95	118	53	42
760	**2e'**	20	13	19	10	13	8	4	6	5
761	**1u'**	109	118	106	61	99	0	65	75	68
772	**1h'**	10	4	5	7	7	15	3	ND	ND
775	**2u'**	9	8	6	8	9	ND	ND	4	5
941	**5n', 6n'**	22	11	9	ND	ND	ND	ND	ND	ND
943	**1n'**	37	59	46	20	ND	29	22	ND	3
957	**2n'**	ND	ND	ND	5	11	40	ND	ND	ND
1000	**5d', 6d'**	ND	ND	ND	11	8	ND	ND	ND	ND
1002 ce	**1d'**	296	275	186	204	199	227	167	91	90
1002 glu	**1g'**	37	46	21	44	ND	ND	5	2	0
2 Me 1016	**2d'**	16	21	14	22	29	17	12	12	10
1060 ce	**1l'**	45	40	33	36	35	17	19	22	16
2 Me 1074	**2l'**	15	29	12	2	3	3	ND	ND	ND
3 Me 1074	**3l'**	5	ND	ND	ND	ND	ND	ND	ND	ND
1086	**1s'**	ND	ND	ND	2	ND	23	ND	ND	ND
1118	**1k'**	18	26	18	40	24	23	34	10	6
2 Me 1132	**2k'**	ND	ND	ND	5	3	4	3	1	0.3
Total		1378	1142	966	1133	1055	1100	800	394	344
TOC (%)		5.1	3.3	2.5	6.0	4.8	3.2	6.1	3.3	2.5

A1(ix) Palace Leas Plot 6 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 6 Core 1 May 2006			Palace Leas Plot 6 Core 2 May 2006			Palace Leas Plot 6 Core 2 May 2006 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	58	56	33	431	26	20	41	22	29
2 Me 669	**2a'**	28	63	40	70	24	28	20	13	30
712	**5f', 6f'**	35	14	23	56	49	49	64	30	34
714	**1f'**	355	288	282	3317	430	442	636	425	231
2 Me 728	**2f'**	4	9	5	13	12	ND	4	7	6
3 Me 728	**3f'**	2	ND	ND	ND	ND	ND	5	ND	ND
746	**1e'**	120	159	126	433	12	96	47	71	70
760	**2e'**	12	18	20	42	15	15	3	10	8
761	**1u'**	80	97	168	ND	77	127	44	52	140
772	**1h'**	7	4	5	113	4	3	8	5	2
775	**2u'**	9	10	15	ND	4	14	3	2	7
943	**1n'**	28	ND	ND	191	8	ND	19	ND	ND
1002 ce	**1d'**	302	222	351	1117	295	388	175	252	297
1002 glu	**1g'**	6	10	11	62	2	ND	ND	ND	ND
1014	**7d'**	2	3	2	ND	ND	ND	ND	ND	ND
2 Me 1016	**2d'**	19	21	25	72	46	40	25	22	40
1060 ce	**1l'**	34	23	43	119	39	58	19	34	21
2 Me 1074	**2l'**	3	1	4	9	4	20	ND	3	ND
3 Me 1074	**3l'**	5	9	12	ND	10	ND	ND	5	ND
1086	**1s'**	ND	ND	ND	ND	ND	ND	1	0	ND
1118	**1k'**	31	17	9	149	21	15	5	21	8
2 Me 1132	**2k'**	1	ND	3	19	1	2	ND	2	4
3 Me 1132	**3k'**	2	ND	ND	ND	ND	ND	ND	2	ND
Total		1131	1012	1163	6213	1069	1317	1113	970	926
TOC (%)		6.3	3.8	3.0	3.9	6.4	4.9	3.0	6.4	4.9

A1(x) Palace Leas Plot 6 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 6 Core 1 October 2006			Palace Leas Plot 6 Core 2 October 2006			Palace Leas Plot 6 Core 1 January 2007		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	72	115	76	67	35	21	91	no data	25
2 Me 669	**2a'**	48	57	61	48	31	21	53		19
712	**5f', 6f'**	54	89	78	65	19	29	167		7
714	**1f'**	658	867	532	631	309	203	1255		50
2 Me 728	**2f'**	10	14	23	7	4	5	10		1
3 Me 728	**3f'**	3	5	ND	6	ND	2	6		ND
746	**1e'**	181	368	251	193	70	50	154		77
760	**2e'**	27	71	49	26	13	7	29		4
761	**1u'**	145	231	308	110	82	98	205		95
772	**1h'**	8	17	16	10	5	3	31		0
775	**2u'**	19	25	18	14	12	6	14		6
943	**1n'**	ND	ND	ND	24	ND	ND	38		6
1002 ce	**1d'**	423	712	424	481	234	107	1241		95
1002 glu	**1g'**	2	ND	9	4	9	2	ND		ND
2 Me 1016	**2d'**	27	46	27	52	26	14	57		17
3 Me 1016	**3d'**	ND	ND	ND	14	ND	ND	ND		ND
1060 ce	**1l'**	42	58	34	67	34	19	141		22
2 Me 1074	**2l'**	1	ND	ND	6	3	3	14		ND
3 Me 1074	**3l'**	ND	14	7	11	9	6	35		ND
1086	**1s'**	ND	ND	ND	ND	ND	ND	16		ND
1118	**1k'**	72	71	33	67	14	12	131		15
2 Me 1132	**2k'**	3	5	3	1	ND	1	3		2
3 Me 1132	**3k'**	7	9	ND	ND	ND	ND	ND		ND
Total		1792	2747	1941	1875	900	600	3649		439
TOC (%)		2.9	5.0	3.8	5.2	4.0	2.9	5.0		2.6

A1(xi) Palace Leas Plot 6 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 6 Core 2 January 2007		
		0-5 cm	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	ND	3
655	**1a'**	27	25	11
2 Me 669	**2a'**	19	20	10
712	**5f', 6f'**	35	29	38
714	**1f'**	290	234	235
2 Me 728	**2f'**	4	2	6
3 Me 728	**3f'**	ND	ND	3
746	**1e'**	61	72	45
760	**2e'**	6	7	8
761	**1u'**	75	114	108
772	**1h'**	4	1	3
775	**2u'**	7	8	2
957	**2n'**	3	ND	ND
1002 ce	**1d'**	124	120	105
1014	**7d'**	2	ND	3
2 Me 1016	**2d'**	20	21	12
3 Me 1016	**3d'**	ND	4	2
1060 ce	**1l'**	13	24	21
2 Me 1074	**2l'**	2	2	5
3 Me 1074	**3l'**	ND	5	ND
1118	**1k'**	20	10	9
2 Me 1132	**2k'**	2	1	1
Total		710	689	621
TOC (%)		5.4	3.8	2.7

A1(xii) Palace Leas Plot 7 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 7 Core 1 September 2004			Palace Leas Plot 7 Core 1 September 2004 repeat injection			Palace Leas Plot 7 Core 1 September 2004 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	ND	ND	ND	ND	ND	ND	ND	1
653	**5a', 6[a]'**	9	ND	ND	12	ND	ND	ND	ND	ND
655	**1a'**	152	50	19	162	123	20	131	37	17
2 Me 669	**2a'**	26	16	5	32	32	7	22	10	6
712	**5f', 6f'**	3	35	13	ND	21	3	3	6	3
714	**1f'**	271	491	113	271	351	29	218	113	24
2 Me 728	**2f'**	2	7	ND	36	4	ND	ND	ND	ND
746	**1e'**	192	216	108	230	387	94	204	123	80
760	**2e'**	32	25	11	54	47	9	31	12	7
761	**1u'**	1	28	26	14	67	26	12	19	21
772	**1h'**	4	5	ND	5	5	ND	3	ND	ND
775	**2u'**	8	3	0.1	8	10	3	1	3	3
941	**5n', 6n'**	20	28	7	17	25	3	ND	ND	ND
943	**1n'**	23	18	ND	51	13	ND	25	3	ND
957	**2n'**	28	23	ND	58	25	2	51	5	1
1002 ce	**1d'**	184	424	94	277	314	37	235	114	35
1014	**7d'**	6	4	ND	11	3	ND	ND	ND	ND
2 Me 1016	**2d'**	15	30	8	9	20	4	21	7	4
1060 ce	**1l'**	10	23	7	19	2	4	17	5	4
2 Me 1074	**2l'**	ND	1	ND	ND	ND	ND	ND	ND	ND
1118	**1k'**	15	33	1	3	24	3	19	7	2
2 Me 1132	**2k'**	1	2	ND	4	ND	ND	3	ND	ND
Total		997	1458	411	1262	1469	245	995	465	208
TOC (%)		10.7	2.9	1.7	10.7	2.9	1.7	10.7	2.9	1.7

A1(xiii) Palace Leas Plot 7 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 7 Core 2 September 2004			Palace Leas Plot 7 Core 2 September 2004 repeat injection			Palace Leas Plot 7 Core 1 January 2005		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	16	11	no data	16	9	no data	ND	ND	2
655	**1a'**	265	117		367	132		99	156	30
2 Me 669	**2a'**	47	35		64	23		28	34	11
712	**5f', 6f'**	10	35		18	23		14	3	3
714	**1f'**	585	489		703	609		441	219	106
2 Me 728	**2f'**	10	4		5	2		172	ND	ND
3 Me 728	**3f'**	ND	ND		ND	ND		26	ND	ND
746	**1e'**	231	345		372	298		26	262	114
760	**2e'**	25	39		69	43		26	33	7
761	**1u'**	ND	92		21	19		10	26	26
772	**1h'**	41	12		39	13		27	5	3
775	**2u'**	19	8		30	4		ND	ND	3
941	**5n', 6n'**	82	17		97	45		13	0	0
943	**1n'**	139	61		82	36		ND	8	11
957	**2n'**	177	ND		186	95		ND	11	20
1001	**1o'**	ND	ND		ND	ND		13	ND	ND
1002 ce	**1d'**	854	610		680	580		508	83	71
1002 glu	**1g'**	ND	ND		122	ND		ND	ND	ND
1014	**7d'**	ND	ND		ND	ND		9	ND	ND
2 Me 1016	**2d'**	7	13		8	13		46	9	9
1058	**5l', 6l'**	15	4		26	12		ND	ND	ND
1060 ce	**1l'**	79	28		53	24		33	3	3
2 Me 1074	**2l'**	9	ND		ND	ND		ND	ND	2
1118	**1k'**	116	32		106	59		54	5	1
2 Me 1132	**2k'**	15	2		10	3		6	ND	ND
Total		2727	1948		3048	2029		1503	857	424
TOC (%)		23.8	3.4		23.8	3.4		30.7	4.5	2.2

A1(xix) Palace Leas Plot 7 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 7 Core 1 January 2005 repeat			Palace Leas Plot 7 Core 2 January 2005			Palace Leas Plot 7 Core 1 January 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	64	69	34	117	57	no data	25	53	37
2 Me 669	**2a'**	16	15	12	9	7		18	77	8
712	**5f', 6f'**	2	3	4	3	29		44	4	36
714	**1f'**	109	153	102	377	434		428	387	465
2 Me 728	**2f'**	ND	ND	1	ND	4		9	3	3
746	**1e'**	48	107	133	188	289		97	87	130
760	**2e'**	8	14	44	28	31		13	11	16
761	**1u'**	2	12	30	6	65		100	4	31
772	**1h'**	4	4	2	14	8		6	11	8
775	**2u'**	1	4	2	ND	6		9	12	3
941	**5n', 6n'**	ND	ND	ND	18	ND		ND	ND	ND
943	**1n'**	3	10	ND	48	11		7	19	14
957	**2n'**	9	17	12	49	33		11	23	9
1002 ce	**1d'**	48	65	89	345	397		208	191	216
1002 glu	**1g'**	ND	ND	ND	8	7		ND	ND	7
2 Me 1016	**2d'**	5	6	4	14	11		26	7	ND
1060 ce	**1l'**	3	3	5	20	19		35	13	ND
2 Me 1074	**2l'**	ND	ND	1	ND	ND		ND	ND	ND
1086	**1s'**	ND	ND	1	ND	ND		ND	ND	ND
1118	**1k'**	4	2	3	47	47		25	22	22
2 Me 1132	**2k'**	0.1	ND	ND	4	2		3	2	1
Total		327	484	481	1295	1456		1063	926	1004
TOC (%)		30.7	4.5	2.2	16.1	3.4		34.8	8.8	2.1

A1(xx) Palace Leas Plot 7 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 7 Core 2 January 2006			Palace Leas Plot 7 Core 1 May 2006			Palace Leas Plot 7 Core 2 May 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
613	**1j'**	2	3	ND	32	16	no data	ND	ND	no data
653	**5a', 6[a]'**	2	ND	ND	ND	ND		ND	ND	
655	**1a'**	80	74	42	348	400		130	172	
2 Me 669	**2a'**	12	11	8	57	59		27	32	
712	**5f', 6f'**	2	8	6	48	17		13	31	
714	**1f'**	280	250	129	2322	1201		910	903	
2 Me 728	**2f'**	1	2	1	11	5		5	4	
746	**1e'**	64	140	90	185	378		167	294	
760	**2e'**	8	20	9	8	62		20	45	
761	**1u'**	2	11	17	ND	22		ND	38	
772	**1h'**	8	7	3	47	19		27	24	
775	**2u'**	ND	ND	ND	ND	ND		ND	7	
941	**5n', 6n'**	17	ND	ND	86	47		10	ND	
943	**1n'**	14	19	16	87	77		64	ND	
957	**2n'**	9	21	36	ND	ND		82	90	
1000	**5d', 6d'**	0	0	0	0	0		21	ND	
1001	**1o'**	ND	ND	ND	ND	ND		10	ND	
1002 ce	**1d'**	129	239	174	935	760		458	448	
1002 glu	**1g'**	3	ND	ND	22	22		11	15	
1014	**7d'**	ND	ND	ND	10	6		ND	ND	
2 Me 1016	**2d'**	4	10	7	71	40		37	42	
1058	**5l', 6l'**	ND	ND	ND	5	ND		6	4	
1060 ce	**1l'**	12	9	9	54	28		42	23	
2 Me 1074	**2l'**	ND	ND	ND	5	ND		ND	3	
1118	**1k'**	19	23	18	99	92		66	45	
2 Me 1132	**2k'**	1	2	1	7	2		7	4	
Total		670	849	566	4423	3247		2074	2222	
TOC (%)		16.5	4.8	2.9	36.4	11.4		3.0	29.6	

A1(xxi) Palace Leas Plot 7 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 7 Core 1 October 2006			Palace Leas Plot 7 Core 1 October 2006 repeat			Palace Leas Plot 7 Core 2 October 2006 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	182	85	no data	170	52	182	402	245	66
2 Me 669	**2a'**	30	37		33	13	30	81	46	27
712	**5f', 6f'**	28	29		9	29	28	29	39	44
714	**1f'**	711	744		704	481	711	1332	984	855
2 Me 728	**2f'**	5	7		7	3	5	9	4	6
746	**1e'**	134	262		161	121	134	155	268	261
760	**2e'**	17	20		19	16	17	20	50	38
761	**1u'**	ND	55		ND	33	ND	ND	ND	62
772	**1h'**	40	31		35	16	40	43	32	19
775	**2u'**	ND	3		ND	3	ND	ND	ND	4
830	**1c'**	ND	ND		ND	ND	ND	4	ND	ND
941	**5n', 6n'**	10	ND		ND	ND	10	19	20	ND
943	**1n'**	82	22		100	10	82	78	69	47
957	**2n'**	187	93		217	37	187	213	132	143
1001	**1o'**	10	ND		ND	ND	10	19	20	ND
1002 ce	**1d'**	574	508		442	243	574	956	689	912
1002 glu	**1g'**	11	12		15	ND	11	ND	ND	ND
1014	**7d'**	ND	ND		ND	ND	ND	14	14	5
2 Me 1016	**2d'**	24	15		17	9	24	28	26	24
1058	**5l', 6l'**	ND	ND		ND	ND	ND	11	10	10
1060 ce	**1l'**	30	17		37	13	30	129	49	42
2 Me 1074	**2l'**	3	ND		2	ND	3	5	5	ND
1118	**1k'**	73	39		62	11	73	108	94	35
2 Me 1132	**2k'**	ND	ND		ND	ND	ND	18	6	0
3 Me 1132	**3k'**	8	ND		11	ND	8	ND	ND	ND
Total		2142	1981		2030	1090	2142	3628	2757	2585
TOC (%)		2.7	16.6	3.6	16.6	3.6	2.7	32.4	9.1	3.0

A1(xxii) Palace Leas Plot 7 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 7 Core 1 January 2006			Palace Leas Plot 7 Core 1 January 2006 repeat			
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	10-15 cm (RI)
653	**5a', 6[a]'**	ND	no data	ND	3	ND	ND	3
655	**1a'**	157		41	112	72	23	24
2 Me 669	**2a'**	20		14	16	13	11	17
712	**5f', 6f'**	32		106	23	31	54	58
714	**1f'**	1344		920	923	591	452	450
2 Me 728	**2f'**	5		11	5	5	5	4
3 Me 728	**3f'**	ND		ND	2	ND	2	1
746	**1e'**	253		320	168	124	129	94
760	**2e'**	31		19	17	12	10	1
761	**1u'**	10		30	13	30	33	36
772	**1h'**	41		20	41	20	4	6
775	**2u'**	ND		ND	2	4	3	3
941	**5n', 6n'**	9		ND	28	ND	ND	ND
943	**1n'**	69		17	27	13	ND	ND
957	**2n'**	115		ND	ND	ND	ND	ND
1001	**1o'**	9		ND	ND	ND	ND	ND
1002 ce	**1d'**	488		420	307	281	172	181
1002 glu	**1g'**	20		11	16	ND	4	2
2 Me 1016	**2d'**	22		18	13	15	12	13
3 Me 1016	**3d'**	ND		ND	ND	2	ND	ND
1060 ce	**1l'**	33		26	19	15	11	15
2 Me 1074	**2l'**	ND		10	2	ND	2	2
1118	**1k'**	66		30	44	29	1	9
2 Me 1132	**2k'**	9		2	3	1	ND	1
Total		2726		2015	1783	1256	927	921
TOC (%)		25.2		2.5	18.0	5.3	2.4	2.4

A1(xxiii) Palace Leas Plot 8 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 8 Core 1 September 2004			Palace Leas Plot 8 Core 2 September 2004			Palace Leas Plot 8 Core 1 January 2005		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	5	3	4	ND	ND	ND	ND	ND
655	**1a'**	112	35	33	37	25	24	27	22	18
2 Me 669	**2a'**	69	14	8	16	16	15	5	14	12
3 Me 669	**3a'**	ND	ND	ND	ND	2	ND	ND	ND	ND
712	**5f', 6f'**	34	7	34	32	34	29	30	36	18
714	**1f'**	248	53	172	196	181	163	188	257	103
2 Me 728	**2f'**	ND	ND	7	5	7	7	5	6	4
3 Me 728	**3f'**	ND	ND	5	4	3	3	ND	ND	2
746	**1e'**	480	111	39	69	82	27	139	115	100
760	**2e'**	183	51	10	19	28	7	39	41	35
761	**1u'**	460	106	82	81	78	70	72	85	73
772	**1h'**	ND	2	4	3	3	3	3	5	1
775	**2u'**	25	3	3	8	6	5	4	5	6
802	**1v'**	ND	ND	1	ND	ND	ND	ND	ND	ND
816	**2v'**	ND	ND	2	ND	ND	ND	2	ND	2
943	**1n'**	17	6	3	3	2	ND	6	ND	ND
957	**2n'**	0	0	0	0	0	0	0	0	0
1000	**5d', 6d'**	0	0	0	0	0	0	0	0	0
1001	**1o'**	0	0	0	0	0	0	0	0	0
1002 ce	**1d'**	647	220	76	106	134	70	113	154	102
2 Me 1016	**2d'**	85	22	3	9	12	4	9	24	10
3 Me 1016	**3d'**	ND	ND	ND	ND	ND	ND	1	ND	ND
1058	**5l', 6l'**	ND	ND	ND	ND	ND	ND	ND	ND	1
1060 ce	**1l'**	172	39	2	19	32	9	38	40	29
2 Me 1074	**2l'**	16	3	ND	2	4	ND	2	3	3
3 Me 1074	**3l'**	ND	7	ND	ND	5	0.1	5	ND	3
1086	**1s'**	ND	3	ND	ND	0	ND	ND	ND	ND
1118	**1k'**	35	7	3	9	8	12	13	15	8
2 Me 1132	**2k'**	8	ND	ND	ND	1	ND	2	1	1
Total		2592	687	484	619	652	445	695	823	525
TOC (%)		4.1	3.8	2.9	4.5	3.2	2.9	3.7	3.0	3.4

A1(xxiv) Palace Leas Plot 8 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 8 Core 2 January 2005			Palace Leas Plot 8 Core 1 January 2006			Palace Leas Plot 8 Core 1 January 2006 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	49	22	26	94	50	28	73	39	27
2 Me 669	**2a'**	16	15	3	18	20	11	30	17	13
3 Me 669	**3a'**	ND	ND	ND	ND	ND	ND	4	3	ND
712	**5f', 6f'**	27	23	2	15	18	17	43	16	18
714	**1f'**	185	144	19	420	239	157	444	182	193
2 Me 728	**2f'**	3	5	1	7	5	9	9	7	9
3 Me 728	**3f'**	3	2	ND	ND	ND	ND	6	ND	ND
746	**1e'**	143	43	40	183	140	118	142	134	130
760	**2e'**	34	14	13	36	43	38	40	36	33
761	**1u'**	101	62	29	63	105	75	71	98	84
772	**1h'**	5	2	1	9	10	5	19	4	4
775	**2u'**	4	5	1	2	2	3	3	2	2
941	**5n', 6n'**	ND	3	ND	ND	ND	ND	ND	ND	ND
943	**1n'**	ND	8	2	27	8	ND	13	ND	ND
957	**2n'**	ND	ND	ND	30	ND	ND	ND	4	4
1002 ce	**1d'**	110	87	37	277	145	164	393	175	158
1002 glu	**1g'**	ND	ND	ND	ND	30	ND	ND	ND	ND
2 Me 1016	**2d'**	9	9	6	15	16	16	28	14	16
1060 ce	**1l'**	36	12	11	29	36	34	35	34	28
2 Me 1074	**2l'**	4	2	ND	ND	2	2	6	2	6
3 Me 1074	**3l'**	3	2	ND	ND	ND	ND	ND	ND	ND
1086	**1s'**	ND	ND	ND	2	3	2	ND	ND	5
1118	**1k'**	13	2	1	20	12	6	11	8	5
2 Me 1132	**2k'**	ND	1	1	3	ND	2	3	2	2
3 Me 1132	**3k'**	ND	ND	ND	3	ND	2	ND	ND	ND
Total		738	460	193	1249	885	685	1364	775	739
TOC (%)		5.5	3.3	3.0	5.8	4.2	2.9	5.8	4.2	2.9

A1(xxv) Palace Leas Plot 8 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 8 Core 2 January 2006			Palace Leas Plot 8 Core 1 May 2006			Palace Leas Plot 8 Core 2 May 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	3	ND	ND	ND	ND	ND	ND	ND	ND
655	**1a'**	18	28	17	49	13	9	99	22	13
2 Me 669	**2a'**	8	15	9	28	6	6	33	19	6
3 Me 669	**3a'**	1	2	2	ND	ND	ND	ND	ND	ND
712	**5f', 6f'**	12	39	32	33	25	37	59	97	44
714	**1f'**	93	251	190	186	141	223	567	303	247
2 Me 728	**2f'**	2	8	9	7	6	12	3	9	11
3 Me 728	**3f'**	1	3	ND	2	3	ND	4	4	6
746	**1e'**	67	178	12	138	31	34	294	143	43
760	**2e'**	19	59	37	31	10	13	69	51	20
761	**1u'**	42	108	67	85	33	16	108	59	22
772	**1h'**	3	9	4	7	2	3	12	13	6
775	**2u'**	ND	4	3	3	2	1	8	ND	ND
943	**1n'**	5	3	ND	ND	ND	ND	24	ND	ND
957	**2n'**	5	10	ND	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	96	192	173	293	57	76	479	383	61
1014	**7d'**	ND	ND	ND	5	ND	ND	ND	ND	ND
2 Me 1016	**2d'**	9	18	15	27	5	7	37	31	9
1060 ce	**1l'**	22	49	46	31	18	4	56	30	6
2 Me 1074	**2l'**	ND	5	1	4	0	1	5	3	ND
3 Me 1074	**3l'**	ND	ND	ND	7	ND	ND	8	8	ND
1086	**1s'**	ND	ND	ND	ND	ND	1	4	2	ND
1118	**1k'**	4	4	6	9	0	2	15	13	ND
2 Me 1132	**2k'**	ND	ND	ND	5	ND	ND	ND	ND	ND
Total		408	980	620	936	350	445	1870	1177	488
TOC (%)		5.4	3.3	2.6	5.8	2.9	2.9		6.3	4.6

A1(xxvi) Palace Leas Plot 8 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 8 Core 2 May 2006 repeat			Palace Leas Plot 8 Core 1 October 2006			Palace Leas Plot 8 Core 2 October 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	50	38	13	64	80	26	61	21	ND
2 Me 669	**2a'**	16	17	6	26	48	10	42	12	ND
3 Me 669	**3a'**	ND	ND	ND	ND	13	ND	ND	ND	ND
712	**5f', 6f'**	23	59	44	48	111	41	55	71	13
714	**1f'**	194	314	247	351	537	225	322	360	69
2 Me 728	**2f'**	5	12	11	6	12	11	10	15	ND
3 Me 728	**3f'**	2	5	6	2	8	7	6	9	ND
746	**1e'**	119	119	43	151	332	105	64	98	309
760	**2e'**	27	38	20	47	123	37	28	53	98
761	**1u'**	91	93	22	142	149	48	175	81	121
772	**1h'**	5	9	6	7	11	3	8	5	ND
775	**2u'**	4	3	ND	5	ND	4	3	3	ND
943	**1n'**	10	ND	ND	13	ND	ND	14	ND	ND
957	**2n'**	ND	ND	ND	ND	ND	ND	ND	6	ND
1002 ce	**1d'**	145	270	61	255	573	138	285	212	67
2 Me 1016	**2d'**	11	23	9	20	56	13	25	22	11
1058	**5l', 6l'**	3	ND	ND	ND	ND	ND	ND	ND	ND
1060 ce	**1l'**	10	18	6	30	70	25	27	22	0
2 Me 1074	**2l'**	1	3	ND	4	11	2	5	ND	ND
3 Me 1074	**3l'**	2	5	ND	5	17	5	ND	ND	ND
1086	**1s'**	3	2	ND	ND	ND	ND	ND	ND	ND
1118	**1k'**	1	11	ND	7	15	8	3	5	ND
2 Me 1132	**2k'**	ND	ND	ND	2	4	1	4	3	ND
Total		716	1030	488	1177	2135	698	1130	988	688
TOC (%)		2.8	6.3	4.0	6.0	5.1	2.5	4.8	3.3	3.4

A1(xxvii) Palace Leas Plot 8 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 8 Core 1 January 2006			Palace Leas Plot 8 Core 2 January 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	ND	ND	no data	6	9
655	**1a'**	32	9	13		71	64
2 Me 669	**2a'**	12	7	7		26	19
3 Me 669	**3a'**	ND	ND	ND		10	ND
712	**5f', 6f'**	37	6	19		49	76
714	**1f'**	223	31	92		281	462
2 Me 728	**2f'**	2	ND	2		5	18
3 Me 728	**3f'**	3	ND	1		5	4
746	**1e'**	68	45	44		120	67
760	**2e'**	19	13	13		25	20
761	**1u'**	110	37	48		207	168
772	**1h'**	4	ND	2		2	8
775	**2u'**	5	3	ND		16	9
1002 ce	**1d'**	93	42	63		353	247
2 Me 1016	**2d'**	11	5	9		21	21
3 Me 1016	**3d'**	ND	ND	ND		3	ND
1060 ce	**1l'**	25	11	20		53	31
2 Me 1074	**2l'**	2	ND	1		4	3
3 Me 1074	**3l'**	2	ND	3		13	8
1118	**1k'**	6	3	4		8	5
2 Me 1132	**2k'**	2	1	1		ND	ND
3 Me 1132	**3k'**	1	ND	ND		ND	ND
Total		651	213	337		1249	1227
TOC (%)		5.7	7.4	2.5		4.3	3.0

A1(xxviii) Palace Leas Plot 9 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 9 Core 1 September 2004			Palace Leas Plot 9 Core 1 September 2004 repeat			Palace Leas Plot 9 Core 2 September 2004		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	3	3	ND	5	4	5	10	11	8
655	**1a'**	39	28	44	28	22	38	73	41	33
2 Me 669	**2a'**	19	21	22	15	17	18	33	35	25
712	**5f', 6f'**	10	10	24	4	4	8	23	18	25
714	**1f'**	159	143	284	51	63	122	271	257	255
2 Me 728	**2f'**	3	3	2	ND	1	1	3	3	7
746	**1e'**	90	114	113	62	63	72	158	164	120
760	**2e'**	6	7	6	4	7	5	10	9	8
761	**1u'**	85	89	62	58	72	40	111	182	131
772	**1h'**	3	2	4	1	1	3	5	4	3
775	**2u'**	12	14	9	8	9	7	14	18	12
941	**5n', 6n'**	ND	ND	ND	ND	ND	ND	39	16	8
943	**1n'**	ND	11	8	3	0	13	57	23	35
957	**2n'**	8	14	4	5	3	4	ND	ND	ND
1002 ce	**1d'**	143	239	211	67	85	90	296	395	334
1002 glu	**1g'**	ND	ND	ND	ND	ND	ND	7	ND	ND
2 Me 1016	**2d'**	12	22	17	7	10	7	2	ND	ND
1058	**5l', 6l'**	ND	ND	ND	ND	ND	ND	8	8	6
1060 ce	**1l'**	11	26	20	8	11	7	31	31	31
2 Me 1074	**2l'**	2	5	ND	ND	2	2	ND	8	9
3 Me 1074	**3l'**	ND	ND	ND	ND	ND	ND	ND	4	ND
1118	**1k'**	13	24	15	7	9	10	65	34	23
2 Me 1132	**2k'**	1	2	ND	1	1	1	6	5	4
Total		619	779	847	334	381	455	1214	1256	1071
TOC (%)		3.9	3.0	5.7	3.9	3.0	5.7	8.1	3.3	2.3

A1(xxix) Palace Leas Plot 9 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 9 Core 1 January 2005			Palace Leas Plot 9 Core 1 January 2005 repeat			Palace Leas Plot 9 Core 2 January 2005		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	ND	ND	4	2	5	17	14	12
655	**1a'**	46	18	39	40	19	23	110	73	39
2 Me 669	**2a'**	21	14	31	18	15	18	44	36	26
712	**5f', 6f'**	12	12	20	8	2	3	41	31	28
714	**1f'**	193	129	202	131	50	50	567	415	325
2 Me 728	**2f'**	ND	2	8	2	2	1	6	10	8
746	**1e'**	147	144	58	105	134	85	266	213	195
760	**2e'**	8	7	2	5	7	6	14	15	13
761	**1u'**	82	78	158	6	76	89	118	134	124
772	**1h'**	4	2	4	3	ND	1	9	8	6
775	**2u'**	9	5	12	7	5	7	14	12	11
941	**5n', 6n'**	ND	ND	ND	ND	ND	ND	57	21	6
943	**1n'**	9	ND	ND	10	ND	ND	81	70	44
957	**2n'**	10	6	ND	9	3	2	ND	ND	ND
1002 ce	**1d'**	150	60	116	104	12	43	463	453	273
1002 glu	**1g'**	ND	ND	ND	ND	ND	ND	14	7	3
2 Me 1016	**2d'**	11	3	10	6	1	3	11	24	9
1060 ce	**1l'**	15	8	15	9	5	8	46	40	32
2 Me 1074	**2l'**	ND	2	ND	1	ND	1	6	6	3
1086	**1s'**	1	ND	2	ND	ND	ND	ND	ND	ND
1118	**1k'**	17	7	9	14	3	3	53	40	17
2 Me 1132	**2k'**	1	1	ND	1	ND	1	3	4	ND
Total		736	499	685	485	336	350	1938	1626	1173
TOC (%)		5.3	2.7	2.6	5.3	2.7	2.6	6.4	3.3	2.5

A1(xxx) Palace Leas Plot 9 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 9 Core 1 January 2006			Palace Leas Plot 9 Core 2 January 2006			Palace Leas Plot 9 Core 2 January 2006 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	ND	ND	ND	ND	ND	2	ND	ND
655	**1a'**	63	45	40	15	93	25	39	25	21
2 Me 669	**2a'**	28	26	30	16	49	28	23	21	21
712	**5f', 6f'**	35	36	31	13	47	28	15	19	5
714	**1f'**	32	352	232	94	296	154	94	127	47
2 Me 728	**2f'**	4	7	7	4	8	6	1	5	2
3 Me 728	**3f'**	2	3	2	ND	5	ND	ND	ND	ND
746	**1e'**	157	158	186	121	317	186	101	120	66
760	**2e'**	7	4	12	16	43	20	10	15	7
761	**1u'**	77	102	124	114	372	164	125	132	90
772	**1h'**	8	7	4	1	6	2	1	2	ND
775	**2u'**	13	12	12	8	30	13	8	8	7
943	**1n'**	50	13	9	5	31	ND	9	ND	3
957	**2n'**	ND	22	22	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	251	243	222	154	465	262	127	15	94
1002 glu	**1g'**	ND	ND	ND	ND	ND	ND	2	1	ND
2 Me 1016	**2d'**	17	10	10	13	28	17	10	8	8
1060 ce	**1l'**	33	31	34	30	54	31	17	30	13
2 Me 1074	**2l'**	ND	3	2	3	ND	14	ND	ND	ND
3 Me 1074	**3l'**	ND	6	8	ND	ND	ND	ND	ND	ND
1118	**1k'**	36	16	18	12	45	15	14	16	7
2 Me 1132	**2k'**	ND	ND	ND	ND	ND	ND	1	1	1
Total		812	1086	994	620	1884	965	600	546	392
TOC (%)		8.5	4.1	3.1	6.4	3.2	2.5	6.4	3.2	2.5

A1(xxxi) Palace Leas Plot 9 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Palace Leas Plot 9 Core 1 May 2006			Palace Leas Plot 9 Core 2 May 2006			Palace Leas Plot 9 Core 1 October 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	125	113	104	88	411	87	22	55	26
2 Me 669	**2a'**	37	61	77	33	254	44	9	32	20
712	**5f', 6f'**	46	48	49	4	152	46	21	80	66
714	**1f'**	584	571	535	327	1008	342	147	742	440
2 Me 728	**2f'**	4	10	10	1	31	15	2	10	11
3 Me 728	**3f'**	3	4	3	ND	ND	ND	ND	3	4
746	**1e'**	189	294	230	64	742	10	54	194	173
760	**2e'**	9	16	13	4	16	7	4	16	16
761	**1u'**	69	148	157	54	596	154	41	77	87
772	**1h'**	13	15	9	6	37	6	3	12	7
775	**2u'**	8	18	18	13	51	17	5	9	8
941	**5n', 6n'**	11	ND	ND	ND	ND	ND	ND	ND	ND
943	**1n'**	69	ND	ND	30	ND	ND	8	18	7
1001	**1o'**	11	ND	ND	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	355	654	490	343	1372	253	105	357	271
1002 glu	**1g'**	20	10	13	ND	ND	ND	ND	8	ND
1014	**7d'**	2	ND	ND	ND	ND	ND	ND	ND	ND
2 Me 1016	**2d'**	36	53	42	8	36	ND	3	32	17
3 Me 1016	**3d'**	ND	ND	6	ND	ND	ND	ND	11	6
1058	**5l', 6l'**	ND	ND	ND	13	27	ND	ND	ND	ND
1060 ce	**1l'**	25	6	26	18	77	15	5	25	19
2 Me 1074	**2l'**	ND	11	6	2	13	ND	ND	ND	ND
3 Me 1074	**3l'**	ND	12	6	ND	18	ND	ND	ND	ND
1086	**1s'**	2	ND	7	ND	ND	ND	ND	ND	ND
1118	**1k'**	65	106	39	39	159	4	7	42	14
2 Me 1132	**2k'**	5	8	2	2	9	ND	ND	3	4
3 Me 1132	**3k'**	7	10	7	2	ND	ND	ND	5	ND
Total		1671	2139	1828	1035	4966	1000	436	1711	1187
TOC (%)		8.8	3.9	3.9	2.8	8.0	4.3	8.6	5.2	3.9

A1(xxxii) Palace Leas Plot 9 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Palace Leas Plot 9 Core 1 October 2006			Palace Leas Plot 9 Core 1 January 2007			Palace Leas Plot 9 Core 1 January 2007 repeat		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	80	59	29	60	36	25	158	31	51
2 Me 669	**2a'**	24	41	19	22	20	18	66	21	17
712	**5f', 6f'**	65	22	66	43	46	80	51	40	82
714	**1f'**	540	520	521	489	284	509	613	277	482
2 Me 728	**2f'**	8	11	11	5	8	16	3	8	13
3 Me 728	**3f'**	5	5	4	3	2	6	4	2	5
746	**1e'**	176	179	120	157	91	41	299	97	83
760	**2e'**	13	11	10	16	10	7	69	11	11
761	**1u'**	80	126	98	74	126	76	238	124	111
772	**1h'**	13	11	11	8	5	16	14	9	16
775	**2u'**	8	21	12	5	15	7	12	11	8
941	**5n', 6n'**	ND	ND	ND	4	ND	ND	ND	ND	ND
943	**1n'**	28	ND	ND	20	ND	ND	ND	ND	ND
957	**2n'**	12	43	5	39	ND	ND	ND	ND	ND
1002 ce	**1d'**	330	405	227	296	109	109	384	113	171
1002 glu	**1g'**	5	12	3	6	ND	ND	ND	3	ND
2 Me 1016	**2d'**	20	34	23	24	11	6	20	12	11
3 Me 1016	**3d'**	ND	ND	ND	7	ND	ND	ND	ND	ND
1060 ce	**1l'**	22	27	17	29	20	9	38	19	16
1060 glu	**1q'**									
2 Me 1074	**2l'**	2	6	ND	ND	3	2	4	4	6
3 Me 1074	**3l'**	ND	7	ND	ND	1	ND	6	ND	ND
1086	**1s'**	0	0	0	0	0	0	0	0	0
1118	**1k'**	24	42	9	33	13	4	12	14	12
2 Me 1132	**2k'**	3	3	2	2	1	2	6	1	ND
3 Me 1132	**3k'**	ND	4	ND	ND	ND	ND	ND	ND	ND
Total		1454	1573	1183	1332	799	927	1987	792	1092
TOC (%)		8.2	4.1	3.5	5.9	3.9	2.8	5.9	3.9	2.8

A1(xxxiii) Palace Leas Plot 9 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Palace Leas Plot 9 Core 2 January 2007		
		0-5 cm	5-10 cm	10-15 cm
627	**1t'**	5	4	4
655	**1^{a}'**	54	13	15
2 Me 669	**2a'**	26	14	10
712	**5f', 6f'**	47	34	44
714	**1f'**	499	275	255
2 Me 728	**2f'**	4	5	9
3 Me 728	**3f'**	3	ND	ND
746	**1e'**	112	47	61
760	**2e'**	15	7	11
761	**1u'**	68	96	73
772	**1h'**	14	4	9
775	**2u'**	7	9	6
943	**1n'**	35	10	8
1002 ce	**1d'**	243	80	90
1002 glu	**1g'**	4	1	1
2 Me 1016	**2d'**	21	13	7
3 Me 1016	**3d'**	7	1	ND
1060 ce	**1l'**	28	10	8
2 Me 1074	**2l'**	ND	4	4
3 Me 1074	**3l'**	3	1	ND
1118	**1k'**	19	8	8
2 Me 1132	**2k'**	2	1	1
Total		1201	633	623
TOC (%)		6.3	5.1	3.6

A2 Hack Hall Farm

A2(i) Hack Hall Farm Near Scots Pine BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm near Scots Pine May 2006			Hack Hall Farm near Scots Pine August 2006			Hack Hall Farm near Scots Pine December 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	ND	ND	8	ND	ND	2	ND	ND
655	**1a'**	65	35	40	37	36	65	27	21	19
2 Me 669	**2a'**	12	10	9	10	4	21	7	7	6
3 Me 669	**3a'**	3	ND	ND	ND	ND	ND	ND	ND	ND
712	**5f', 6f'**	1	6	3	8	ND	12	18	6	3
714	**1f'**	59	49	44	125	22	186	130	43	27
2 Me 728	**2f'**	1	ND	ND	1	ND	5	ND	ND	ND
746	**1e'**	51	48	49	55	42	146	33	38	23
760	**2e'**	3	4	6	5	ND	16	1	3	1
761	**1u'**	16	18	17	21	14	101	22	14	22
772	**1h'**	4	4	3	6	ND	4	3	1	1
775	**2u'**	1	ND	ND	ND	2	ND	ND	2	ND
830	**1c'**	5	10	5	5	5	ND	1	ND	3
941	**5n', 6n'**	ND	ND	ND	ND	ND	5	ND	ND	ND
943	**1n'**	2	ND	ND	ND	ND	13	ND	ND	ND
1002 ce	**1d'**	50	33	39	44	19	167	48	28	18
1002 glu	**1g'**	ND	ND	ND	14	ND	ND	1	ND	ND
2 Me 1016	**2d'**	2	2	4	3	ND	10	4	3	ND
1058	**5l', 6l'**	ND	ND	ND	3	ND	ND	ND	ND	ND
1060 ce	**1l'**	19	10	16	14	7	34	12	11	9
2 Me 1074	**2l'**	ND	ND	2	ND	ND	6	2	ND	ND
1118	**1k'**	5	2	2	16	4	4	8	2	2
2 Me 1132	**2k'**	ND	ND	ND	2	ND	5	ND	ND	ND
Total		297	231	239	374	155	809	319	179	134
TOC (%)		8.0	4.5	3.7	6.3	3.2	3.5	5.9	5.0	2.9

A2(ii) Hack Hall Farm Near Scots Pine BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Hack Hall Farm near Scots Pine March 2007		
		0-5 cm	5-10 cm	10-15 cm
613	**1j'**	ND	ND	5
653	**5a', 6[a]'**	4	ND	ND
655	**1a'**	29	21	31
2 Me 669	**2a'**	1	6	9
712	**5f', 6f'**	4	9	ND
714	**1f'**	31	62	20
746	**1e'**	31	32	50
760	**2e'**	ND	1	4
761	**1u'**	9	11	21
772	**1h'**	ND	2	ND
775	**2u'**	ND	2	ND
830	**1c'**	ND	0.4	ND
941	**5n', 6n'**	10	ND	ND
943	**1n'**	2	3	ND
1002 ce	**1d'**	18	26	29
2 Me 1016	**2d'**	ND	1	ND
1060 ce	**1l'**	8	5	9
2 Me 1074	**2l'**	ND	5	ND
1118	**1k'**	5	1	2
Total		151	189	181
TOC (%)		9.0	5.7	3.9

A2(iii) Hack Hall Farm Between Scots Pine BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm between Scots Pine May 2006			Hack Hall Farm between Scots Pine August 2006			Hack Hall Farm between Scots Pine December 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	72	32	76	68	47	47	43	42	33
2 Me 669	**2a'**	13	11	18	14	15	3	10	9	12
712	**5f', 6f'**	19	11	63	15	7	16	16	19	22
714	**1f'**	194	92	424	185	58	169	148	119	128
2 Me 728	**2f'**	1	ND	7	ND	ND	2	ND	ND	ND
3 Me 728	**3f'**	1	1	4	ND	ND	1	ND	ND	ND
746	**1e'**	148	120	187	97	78	77	61	59	66
760	**2e'**	9	6	ND	6	3	3	4	4	5
761	**1u'**	31	26	35	19	25	24	25	27	35
772	**1h'**	7	5	22	6	4	8	3	3	5
775	**2u'**	3	ND	ND	ND	ND	ND	3	ND	ND
830	**1c'**	4	24	0	3	4	12	1	2	24
844	**2c'**	0	0	0	0	0	0	0	0	0
941	**5n', 6n'**	8	ND	ND	15	5	3	ND	ND	ND
943	**1n'**	13	ND	ND	17	11	16	ND	ND	ND
1002 ce	**1d'**	245	136	329	142	76	87	34	26	27
1002 glu	**1g'**	ND	ND	ND	ND	ND	ND	11	16	15
2 Me 1016	**2d'**	9	7	15	6	4	7	2	ND	ND
3 Me 1016	**3d'**	ND	ND	ND	ND	ND	2	ND	ND	ND
1058	**5l', 6l'**	ND	ND	ND	ND	ND	ND	1	ND	ND
1060 ce	**1l'**	49	27	40	18	10	18	10	6	9
2 Me 1074	**2l'**	3	ND	4	2	2	2	ND	ND	1
3 Me 1074	**3l'**	ND	ND	7	ND	ND	ND	ND	ND	ND
1086	**1s'**	0	0	0	0	0	0	0	0	0
1118	**1k'**	22	3	6	29	5	8	12	6	5
2 Me 1132	**2k'**	3	1	ND	6	1	1	1	ND	ND
Total		848	481	1250	647	355	502	382	337	364
TOC (%)		4.8	3.7	3.5	6.5	3.9	4.4	6.1	5.2	5.2

A2(iv) Hack Hall Farm Between Scots Pine BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm between Scots Pine March 2007		
		0-5 cm	5-10 cm	10-15 cm
655	**1a'**	28	40	8
2 Me 669	**2a'**	7	9	2
712	**5f', 6f'**	24	9	3
714	**1f'**	138	63	34
2 Me 728	**2f'**	1	ND	ND
746	**1e'**	36	71	8
760	**2e'**	ND	6	ND
761	**1u'**	19	34	9
772	**1h'**	4	ND	1
775	**2u'**	1	5	ND
830	**1c'**	1	ND	2
943	**1n'**	5	7	2
1002 ce	**1d'**	65	69	13
1002 glu	**1g'**	1	ND	ND
2 Me 1016	**2d'**	4	4	1
1060 ce	**1l'**	21	15	2
2 Me 1074	**2l'**	1	ND	ND
1118	**1k'**	8	2	ND
Total		365	334	85
TOC (%)		6.3	5.1	3.7

A2(v) Hack Hall Farm Near Common Fir BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm near Common Fir May 2006			Hack Hall Farm near Common Fir August 2006			Hack Hall Farm near Common Fir December 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	80	65	39	no data	no data	105	74	29	33
2 Me 669	**2a'**	30	26	19			32	11	9	15
712	**5f', 6f'**	24	20	20			37	19	6	7
714	**1f'**	218	166	140			264	244	57	65
2 Me 728	**2f'**	2	ND	1			ND	ND	ND	ND
3 Me 728	**3f'**	ND	ND	1			ND	ND	ND	ND
746	**1e'**	120	109	51			179	89	54	61
760	**2e'**	10	4	3			13	8	2	3
761	**1u'**	10	17	20			86	13	15	28
772	**1h'**	7	5	7			11	14	2	0
775	**2u'**	5	3	ND			ND	ND	2	3
830	**1c'**	ND	4	11			12	ND	1	7
941	**5n', 6n'**	5	ND	ND			ND	ND	ND	ND
943	**1n'**	20	ND	ND			ND	53	5	2
1002 ce	**1d'**	271	202	144			306	313	61	78
1002 glu	**1g'**	2	3	3			ND	ND	ND	ND
2 Me 1016	**2d'**	8	12	9			18	17	4	6
1060 ce	**1l'**	36	42	28			35	42	25	29
2 Me 1074	**2l'**	10	6	3			ND	3	1	2
3 Me 1074	**3l'**	ND	2	2			ND	ND	ND	ND
1118	**1k'**	11	5	6			12	10	11	6
2 Me 1132	**2k'**	ND	ND	ND			ND	ND	ND	1
Total		869	689	504			1108	935	283	340
TOC (%)		5.1	4.7	6.9			4.5	4.8	6.5	3.9

A2(vi) Hack Hall Farm Near Common Fir BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Hack Hall Farm near Common Fir March 2007		
		0-5 cm	5-10 cm	10-15 cm
613	**1j'**	5	1	ND
653	**5a', 6[a]'**	5	ND	ND
655	**1a'**	41	41	35
2 Me 669	**2a'**	11	14	8
712	**5f', 6f'**	7	8	31
714	**1f'**	39	67	173
746	**1e'**	10	72	45
760	**2e'**	2	4	3
761	**1u'**	6	23	24
772	**1h'**	ND	2	5
775	**2u'**	ND	ND	2
830	**1c'**	1	1	8
941	**5n', 6n'**	ND	4	ND
943	**1n'**	ND	4	6
1002 ce	**1d'**	63	80	79
2 Me 1016	**2d'**	ND	6	5
1060 ce	**1l'**	18	25	27
1118	**1k'**	9	12	10
2 Me 1132	**2k'**	1	1	ND
Total		217	366	454
TOC (%)		4.8	4.8	3.8

A2(vii) Hack Hall Farm Between Common Fir BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Hack Hall Farm between Common Fir May 2006			Hack Hall Farm between Common Fir August 2006			Hack Hall Farm between Common Fir December 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	ND	ND	ND	ND	no data	ND	6	ND
655	**1a'**	89	304	109	37	34		16	79	48
2 Me 669	**2a'**	24	40	32	14	11		7	12	10
712	**5f', 6f'**	18	123	50	21	13		14	9	10
714	**1f'**	290	1179	430	178	115		118	186	135
2 Me 728	**2f'**	3	7	3	ND	ND		ND	1	2
3 Me 728	**3f'**	ND	4	ND	ND	ND		ND	ND	ND
746	**1e'**	124	371	83	78	52		37	89	97
760	**2e'**	9	14	ND	6	4		4	4	4
761	**1u'**	18	44	41	37	51		16	7	12
772	**1h'**	11	55	32	6	1		4	4	6
775	**2u'**	3	ND	ND	2	2		2	12	0
830	**1c'**	9	84	42	3	5		1	9	6
943	**1n'**	21	ND	ND	6	15		ND	2	ND
1000	**5d', 6d'**	ND	ND	ND	ND	ND		ND	12	ND
1002 ce	**1d'**	299	987	353	136	118		62	156	126
1002 glu	**1g'**	ND	32	ND	4	ND		ND	ND	ND
2 Me 1016	**2d'**	9	35	22	8	9		6	6	7
1058	**5l', 6l'**	3	ND	ND	ND	ND		ND	ND	ND
1060 ce	**1l'**	59	128	6	29	34		14	21	22
2 Me 1074	**2l'**	1	15	5	ND	3		ND	ND	ND
3 Me 1074	**3l'**	2	ND	7	2	4		ND	ND	ND
1118	**1k'**	27	98	19	18	7		2	41	21
2 Me 1132	**2k'**	1	8	1	ND	ND		ND	2	1
3 Me 1132	**3k'**	1	5	1	ND	ND		ND	ND	ND
Total		1015	3526	1227	582	473		307	637	511
TOC (%)		5.5	3.9	3.7	5.6	4.9		3.0	6.7	3.4

A2(viii) Hack Hall Farm Between Common Fir BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm between Common Fir March 2007		
		0-5 cm	5-10 cm	10-15 cm
613	**1j'**	ND	8	ND
655	**1a'**	22	37	14
2 Me 669	**2a'**	8	8	5
712	**5f', 6f'**	11	4	9
714	**1f'**	95	37	84
2 Me 728	**2f'**	1	ND	ND
746	**1e'**	18	50	38
760	**2e'**	ND	3	3
761	**1u'**	16	25	19
772	**1h'**	3	2	5
775	**2u'**	1	ND	1
830	**1c'**	5	5	14
941	**5n', 6n'**	2	ND	ND
943	**1n'**	9	1	3
1002 ce	**1d'**	49	40	57
2 Me 1016	**2d'**	3	3	2
3 Me 1016	**3d'**	ND	ND	2
1060 ce	**1l'**	12	11	15
1118	**1k'**	2	2	2
2 Me 1132	**2k'**	ND	ND	1
Total		258	236	272
TOC (%)		4.8	2.8	2.8

A2(ix) Hack Hall Farm Near Sycamore BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm near sycamore May 2006			Hack Hall Farm near sycamore August 2006			
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	0-5 cm repeat	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	ND	3	ND	ND	ND	5
655	**1a'**	84	88	62	76	76	94	53
2 Me 669	**2a'**	19	18	21	34	37	40	18
3 Me 669	**3a'**	19	ND	ND	ND	ND	ND	ND
712	**5f', 6f'**	35	24	11	11	25	43	9
714	**1f'**	363	277	131	253	217	322	50
2 Me 728	**2f'**	3	ND	ND	4	3	9	ND
746	**1e'**	229	337	213	194	150	285	40
760	**2e'**	8	13	12	14	10	11	5
761	**1u'**	38	50	50	128	98	200	113
772	**1h'**	14	14	5	6	5	5	1
775	**2u'**	3	ND	3	7	4	7	6
830	**1c'**	3	12	ND	ND	ND	ND	ND
941	**5n', 6n'**	11	15	5	3	5	ND	ND
943	**1n'**	52	63	ND	12	10	ND	ND
1002 ce	**1d'**	304	391	205	330	300	465	83
1002 glu	**1g'**	47	13	9	4	3	ND	ND
2 Me 1016	**2d'**	15	29	15	13	7	20	2
1060 ce	**1l'**	36	35	15	33	40	17	7
2 Me 1074	**2l'**	2	ND	3	7	9	ND	1
3 Me 1074	**3l'**	2	ND	ND	6	4	ND	2
1118	**1k'**	30	17	8	15	16	6	3
2 Me 1132	**2k'**	2	ND	ND	2	2	ND	2
Total		1298	1395	771	1145	1016	1526	398
TOC (%)		7.5	5.1	4.9	5.5	5.5	5.4	3.7

A2(x) Hack Hall Farm Near Sycamore BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm near sycamore December 2006			Hack Hall Farm near sycamore March 2007		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	ND	ND	ND	no data	24	25
2 Me 669	**2a'**	30	20	28		11	11
712	**5f', 6f'**	13	8	8		6	8
714	**1f'**	ND	ND	ND		48	59
2 Me 728	**2f'**	11	13	10		1	2
746	**1e'**	115	105	86		52	52
760	**2e'**	ND	1	3		2	5
761	**1u'**	49	25	30		28	54
772	**1h'**	4	1	3		1	1
775	**2u'**	25	25	42		4	2
830	**1c'**	3	3	2		1	ND
943	**1n'**	6	3	1		5	ND
1002 ce	**1d'**	ND	ND	2		67	73
2 Me 1016	**2d'**	ND	ND	ND		6	5
1060 ce	**1l'**	6	ND	ND		13	11
2 Me 1074	**2l'**	76	49	51		ND	1
1118	**1k'**	ND	ND	ND		6	3
2 Me 1132	**2k'**	7	4	7		1	1
Total		20	9	11		275	313
TOC (%)		ND	1	ND		5.6	4.3
		ND	ND	ND			
		9	4	2			
		1	1	1			
		373	272	288			
		6.4	5.2	3.2			

A2(xi) Hack Hall Farm Between Sycamore BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	**Structure Number**	Hack Hall Farm between sycamore May 2006			Hack Hall Farm between sycamore August 2006			Hack Hall Farm between sycamore December 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	ND	ND	ND	3	ND	ND	2	ND
655	**1a'**	96	68	32	17	26	28	34	16	28
2 Me 669	**2a'**	18	20	13	6	5	4	23	10	4
3 Me 669	**3a'**	ND	ND	ND	ND	ND	ND	4	10	ND
712	**5f', 6f'**	22	20	7	5	ND	ND	11	13	7
714	**1f'**	264	170	82	28	20	7	76	99	55
2 Me 728	**2f'**	2	2	2	1	ND	ND	1	2	3
746	**1e'**	135	101	63	27	30	19	65	56	37
760	**2e'**	7	6	6	3	1	0	7	3	5
761	**1u'**	26	41	56	40	31	17	113	85	70
772	**1h'**	6	7	1	ND	ND	2	2	2	0
775	**2u'**	2	13	2	2	ND	ND	5	3	4
830	**1c'**	ND	ND	ND	ND	ND	1	ND	1	4
941	**5n', 6n'**	11	15	ND	ND	ND	ND	ND	ND	ND
943	**1n'**	20	30	ND	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	243	230	131	37	25	6	85	46	43
1002 glu	**1g'**	17	8	ND	ND	ND	ND	ND	3	ND
2 Me 1016	**2d'**	14	16	5	3	ND	ND	7	4	7
1060 ce	**1l'**	35	23	12	6	7	5	25	13	10
2 Me 1074	**2l'**	1	2	1	ND	ND	ND	ND	ND	ND
3 Me 1074	**3l'**	ND	ND	1	ND	ND	ND	ND	ND	ND
1118	**1k'**	31	16	4	2	ND	ND	3	3	2
2 Me 1132	**2k'**	4	1	ND	1	ND	ND	ND	1	1
Total		954	788	417	178	151	88	457	362	280
TOC (%)		5.7	5.2	3.7	7.0	2.5	1.9	4.1	3.4	2.2

A2(xii) Hack Hall Farm Between Sycamore BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Hack Hall Farm between sycamore March 2007		
		0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	ND	2
655	**1a'**	39	27	10
2 Me 669	**2a'**	ND	13	6
712	**5f', 6f'**	3	9	14
714	**1f'**	28	69	115
2 Me 728	**2f'**	ND	ND	3
3 Me 728	**3f'**	ND	ND	2
746	**1e'**	51	56	31
760	**2e'**	2	4	3
761	**1u'**	28	43	34
772	**1h'**	ND	1	1
775	**2u'**	3	4	2
830	**1c'**	ND	ND	1
1002 ce	**1d'**	71	87	68
1002 glu	**1g'**	ND	ND	1
2 Me 1016	**2d'**	6	10	9
1060 ce	**1l'**	11	19	10
2 Me 1074	**2l'**	ND	ND	2
1118	**1k'**	3	4	3
2 Me 1132	**2k'**	ND	1	1
Total		245	348	325
TOC (%)		6.1	4.7	4.4

A3 Landfills

A3(i) West Allotment BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	West allotment BH 3/4 June 2006			West allotment BH 4/1 June 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	32	29	no data	19	7	no data
2 Me 669	**2a'**	5	3		3	1	
714	**1f'**	1	7		2	6	
746	**1e'**	15	12		16	6	
760	**2e'**	2	ND		4	1	
761	**1u'**	11	ND		12	4	
772	**1h'**	ND	ND		ND	0.4	
775	**2u'**	1	ND		2	1	
1002 ce	**1d'**	2	12		2	5	
2 Me 1016	**2d'**	ND	ND		ND	1	
1060 ce	**1l'**	ND	2		1	ND	
1086	**1s'**	1	ND		1	ND	
Total		70	66		64	32	
TOC (%)		6.3	7.6		8.9	9.0	

A3(ii) Octavia Court BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Octavia Court BH8 June 2006			Octavia Court BH6 June 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
653	**5^{a}', 6^{a}'**	ND	ND	no data	2	ND	no data
655	**1^{a}'**	21	11		2	27	
2 Me 669	**2a'**	4	ND		6	4	
714	**1f'**	2	ND		5	21	
746	**1e'**	22	9		29	30	
760	**2e'**	2	1		6	4	
761	**1u'**	22	ND		18	16	
772	**1h'**	ND	ND		ND	3	
775	**2u'**	ND	1		3	1	
1002 ce	**1d'**	2	ND		2	13	
1060 ce	**1l'**	ND	ND		1	2	
1086	**1s'**	2	ND		1	2	
1118	**1k'**	ND	ND		ND	1	
2 Me 1132	**2k'**	ND	ND		ND	1	
Total	**1j'**	77	22		75	124	
TOC (%)		7.6	5.0		9.6	7.0	

A3(iii) Chicken Road BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Chicken Road BH5/1 June 2006			Chicken Road BH1 June 2006		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
627	**1t'**	ND	2	ND	ND	ND	no data
655	**1a'**	15	8	10	22	19	
2 Me 669	**2a'**	4	2	1	7	4	
712	**5f', 6f'**	ND	ND	ND	3	ND	
714	**1f'**	28	2	1	68	6	
2 Me 728	**2f'**	2	ND	ND	2	0	
746	**1e'**	24	14	11	45	29	
760	**2e'**	6	3	4	9	7	
761	**1u'**	14	7	6	21	14	
772	**1h'**	2	0.4	ND	3	ND	
775	**2u'**	ND	1	1	2	2	
941	**5n', 6n'**	ND	ND	ND	1	0	
1002 ce	**1d'**	13	3	3	27	5	
2 Me 1016	**2d'**	2	1	1	4	2	
1060 ce	**1l'**	1	2	1	6	1	
1086	**1s'**	ND	1	0.3	ND	3	
1118	**1k'**	ND	ND	ND	2	ND	
Total		113	45	40	224	92	
TOC (%)		8.2	7.8	8.0	6.9	4.6	

A3(iv) Seghill BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Seghill LWP4A May 2008			Seghill wet of topsoil mound May 2008			Seghill LMP 2.01 May 2008		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
653	**5a', 6[a]'**	ND	7	ND	ND	ND	ND	ND	ND	no data
655	**1a'**	22	11	8	49	42	19	68	54	
2 Me 669	**2a'**	5	7	ND	8	9	4	8	9	
712	**5f', 6f'**	2	1	ND	ND	2	ND	ND	1	
714	**1f'**	2	4	ND	30	31	4	18	19	
746	**1e'**	35	28	15	71	53	38	81	61	
760	**2e'**	8	8	4	10	9	6	ND	8	
761	**1u'**	15	12	11	22	24	9	18	21	
772	**1h'**	ND	ND	ND	2	ND	ND	ND	ND	
775	**2u'**	ND	4	2	3	5	ND	12	5	
1002 ce	**1d'**	13	5	3	46	33	9	24	19	
2 Me 1016	**2d'**	ND	ND	ND	6	ND	ND	ND	ND	
1060 ce	**1l'**	2	ND	ND	5	4	ND	ND	ND	
Total		103	88	43	252	211	89	230	197	
TOC (%)		4.6	3.7	4.7	2.9	2.5	3.2	2.7	2.5	

A3(v) Seghill BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Seghill LWP4B May 2008			Seghill LMP 2.02 May 2008		
		0-5 cm	5-10 cm	10-15 cm	0-5 cm	5-10 cm	10-15 cm
655	**1a'**	55	22	71	64	41	47
2 Me 669	**2a'**	9	3	7	8	7	9
712	**5f', 6f'**	4	ND	ND	ND	ND	ND
714	**1f'**	15	2	6	10	6	9
746	**1e'**	66	10	44	58	51	38
760	**2e'**	21	4	ND	6	7	ND
761	**1u'**	24	5	ND	31	20	ND
1002 ce	**1d'**	20	4	8	ND	11	ND
2 Me 1016	**2d'**	ND	1	ND	ND	ND	ND
Total		214	51	136	177	144	102
TOC (%)		3.4	2.7	1.3	2.9	2.4	2.1

A4 Congo Fan

A4(i) Congo ODP1074 BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Congo ODP 1074								
		2H2W 6567	2H6W 65-67	3H5W 75-77	4H1W 65-67	4H5W 65-67	5H3W 115-117	6H1W 115117	6H4W 115-117	7H1W 1517
613	**1j'**	19	11	11	9	18	11	45	30	35
655	**1[a]'**	39	48	29	55	30	75	80	74	64
2 Me 669	**2a'**	ND	ND	ND	ND	ND	5	4	ND	ND
714	**1f'**	14	15	50	11	49	53	20	85	19
746	**1e'**	73	69	14	42	19	53	58	39	27
772	**1h'**	4	ND	7	3	10	6	5	13	4
830	**1c'**	11	ND	33	ND	39	35	14	71	12
1000	**5d', 6d'**	ND	ND	ND	ND	ND	ND	ND	5	ND
1002 ce	**1d'**	26	28	32	21	49	77	64	82	55
1002 glu	**1g'**	ND	ND	ND	ND	ND	ND	ND	ND	3
1060 ce	**1l'**	4	5	6	3	4	8	3	10	6
1118	**1k'**	6	4	3	3	6	11	2	10	4
Total		196	180	184	147	223	334	296	419	227
TOC (%)		3.1	2.2	2.4	2.1	2.1	2.0	2.7	1.8	2.0

A4(ii) Congo ODP1074 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Congo ODP 1074								
		7H3W 75-77	7H5W 15-17	7H7W 6567	8H2 W 15-17	8H5W 11511 7	9H1W 105-107	9H4W 105-107	9H6W 65-67	10H2 W 6567
613	**1j'**	14	25	38	22	31	15	15	24	31
655	**1a'**	36	67	75	54	39	34	50	77	57
714	**1f'**	88	186	145	90	124	8	6	13	60
746	**1e'**	5	ND	6	28	ND	8	10	17	6
772	**1h'**	14	36	24	14	18	ND	ND	5	10
830	**1c'**	60	150	118	41	88	3	3	7	43
1000	**5d', 6d'**	11	12	6	11	31	ND	ND	ND	ND
1002 ce	**1d'**	79	145	80	85	61	13	16	30	48
2 Me 1016	**2d'**	ND	7	ND	4	ND	ND	ND	ND	ND
1060 ce	**1l'**	8	15	11	9	5	1	2	3	4
1118	**1k'**	8	20	7	7	7	ND	2	5	4
Total		321	664	546	364	425	82	104	180	263
TOC (%)		2.7	2.3	1.7	2.6	2.5	2.0	1.8	1.6	2.0

A4(iii) Congo ODP1074 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	Congo ODP 1074			
		10H7 W151 7,0	11H2 W115 117	11H6 W115 117	12H4 H656 7
613	**1j'**	60	21	33	28
655	**1a'**	110	43	19	71
714	**1f'**	7	4	ND	ND
746	**1e'**	8	5	ND	21
772	**1h'**	8	5	ND	21
830	**1c'**	20	15	16	89
1002 ce	**1d'**	42	28	33	43
1060 ce	**1l'**	7	2	ND	ND
1118	**1k'**	4	3	ND	ND
Total		285	154	152	374
TOC (%)		1.0	2.3	2.5	1.0

A4(iv) Congo ODP1074 BHP concentration ($\mu g_{BHP}\ g^{-1}_{TOC}$)

Base Peak *(m/z)*	Structure Number	West African Coastal Surface samples			
		4901	4909	4913	4917
655	**1a'**	35	65	6	76
714	**1f'**	ND	13	34	5
746	**1e'**	ND	5	47	ND
1002 ce	**1d'**	ND	13	16	ND
Total		35	96	103	81

A5 Rhone Delta BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Rhone Delta surface samples			
		kB16 pro-delta	MT09 X shelf	MT02 outer shelf	MT04 canyon
655	**1ª'**	68	140	61	28
2 Me 669	**2a'**	6	18	7	3
714	**1f'**	9	10	5	2
746	**1e'**	23	22	ND	ND
761	**1u'**	9	8	11	0
775	**2u'**	2	3	1	ND
1002 ce	**1d'**	21	11	ND	ND
1060 ce	**1l'**	5	ND	ND	ND
Total		144	211	84	33

A6 Arctic River Estuaries BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{TOC}$)

Base Peak ***(m/z)***	**Structure Number**	Arctic River estuaries surface samples						
		Ob river	Yenisey River	Lena River	Indikirka River	Kolyma River	Yukon River	Mackenzie River
613	**1j'**	ND	4	ND	ND	ND	ND	0.1
653	**5ª', 6ª'**	ND	ND	7	ND	ND	ND	ND
655	**1ª'**	142	223	91	137	233	159	28
2 Me 669	**2a'**	9	15	11	9	17	6	2
714	**1f'**	24	18	63	14	32	81	9
746	**1e'**	29	50	65	84	120	107	10
760	**2e'**	2	3	2	3	4	4	1
761	**1u'**	8	21	20	35	60	22	3
772	**1h'**	4	1	9	2	ND	16	1
775	**2u'**	ND	3	3	7	6	ND	ND
830	**1c'**	1	1	6	1	7	4	1
1002 ce	**1d'**	14	10	15	47	103	132	26
2 Me 1016	**2d'**	ND	ND	ND	3	6	ND	0.1
1060 ce	**1l'**	ND	ND	3	4	10	15	8
1118	**1k'**	2	1	1	2	9	6	1
2 Me 1132	**2k'**	1	1	ND	2	7	3	1
Total		236	352	296	349	615	555	91

A7 Sweden

A7(i) Sweden BHP concentration ($\mu g_{BHP}\ g^{-1}_{DRY\ SOIL}$)

Base Peak *(m/z)*	Structure Number	Sweden 1		Sweden 2		Sweden 3		Sweden 4	
		a	b	a	b	a	b	a	b
627	**1t'**	5	9	2	1	ND	ND	6	ND
653	**5a', 6[a]'**	7	ND	ND	ND	ND	ND	ND	ND
655	**1a'**	46	86	8	2	14	1	74	1
2 Me 669	**2a'**	10	18	3	1	4	ND	15	ND
712	**5f', 6f'**	2	7	ND	ND	2	ND	27	ND
714	**1f'**	126	162	21	9	65	8	259	4
2 Me 728	**2f'**	1	ND	ND	ND	ND	ND	ND	ND
746	**1e'**	85	116	16	6	40	5	68	2
760	**2e'**	4	7	1	ND	3	1	8	ND
761	**1u'**	7	11	10	4	6	1	17	1
772	**1h'**	4	1	ND	ND	1	ND	5	ND
775	**2u'**	2	5	2	1	3	1	6	ND
941	**5n', 6n'**	13	ND	ND	ND	ND	ND	ND	ND
943	**1n'**	14	16	5	2	8	1	45	1
1001	**1o'**	4	ND	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	148	194	26	11	48	6	205	3
1002 glu	**1g'**	ND	ND	ND	ND	1	ND	8	0
2 Me 1016	**2d'**	3	7	1	1	1	ND	5	ND
1060 ce	**1l'**	23	30	5	3	6	1	29	1
1118	**1k'**	35	39	9	4	2	2	39	1
2 Me 1132	**2k'**	2	3	ND	ND	1	ND	4	ND
Total		535	709	113	46	206	28	819	13

A7(ii) Sweden BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{DRY\ SOIL}$)

Base Peak *(m/z)*	Structure Number	Sweden 5		Sweden 6		Sweden 7	
		a	b	a	b	a	b
627	**1t'**	ND	ND	ND	ND	12	ND
653	**5a', 6[a]'**	ND	ND	ND	ND	26	ND
655	**1a'**	27	1	39	2	67	1
2 Me 669	**2a'**	6	1	11	1	26	1
714	**1f'**	94	4	206	8	199	7
2 Me 728	**2f'**	ND	ND	ND	ND	6	ND
746	**1e'**	28	2	43	6	101	2
760	**2e'**	2	ND	7	ND	28	ND
761	**1u'**	ND	2	6	4	ND	2
772	**1h'**	ND	ND	ND	ND	11	ND
941	**5n', 6n'**	ND	ND	ND	ND	20	ND
943	**1n'**	17	1	47	1	22	ND
957	**2n'**	ND	1	ND	ND	ND	ND
1002 ce	**1d'**	67	3	90	6	166	4
2 Me 1016	**2d'**	2	ND	ND	1	ND	ND
1058	**5l', 6l'**	ND	ND	ND	ND	39	ND
1060 ce	**1l'**	18	1	ND	3	19	1
2 Me 1074	**2l'**	3	ND	ND	1	ND	ND
1118	**1k'**	27	1	37	2	20	1
2 Me 1132	**2k'**	13	ND	11	ND	10	0
Total		304	18	498	36	732	21

A8 Seville

A8(i) Seville BHP concentration ($\mu g_{BHP}\ g^{-1}{}_{DRY\ SOIL}$)

Base Peak *(m/z)*	Structure Number	Seville, Barranca de los Laureles			Seville, Arroyo de las Canadas			Seville, Casa de las Canadas		
		2004	2005	2006	2004	2005	2006	2004	2005	2006
655	**1a'**	1	ND	4	1	1	1	ND	ND	1
2 Me 669	**2a'**	ND	ND	1	ND	ND	ND	ND	ND	ND
712	**5f', 6f'**	1	ND	ND	ND	ND	ND	ND	ND	ND
714	**1f'**	7	3	13	1	1	3	ND	2	3
746	**1e'**	4	2	13	1	2	3	ND	1	3
760	**2e'**	1	ND	1	ND	ND	ND	ND	ND	ND
761	**1u'**	2	ND	3	ND	1	1	0	0	1
943	**1n'**	ND	ND	1	ND	ND	ND	ND	ND	ND
1002 ce	**1d'**	5	2	4	1	1	2	ND	1	1
2 Me 1016	**2d'**	ND	ND	1	ND	ND	ND	ND	ND	ND
1060 ce	**1l'**	1	1	1	ND	ND	1	ND	ND	ND
1118	**1k'**	1	1	2	ND	ND	1	ND	ND	ND
Total		22	10	44	4	7	12	1	5	10

A8(ii) Seville BHP concentration ($\mu g_{BHP}\ g^{-1}_{DRY\ SOIL}$)

Base Peak *(m/z)*	Structure Number	Seville, Carril de la Osa		
		2004	2005	2006
655	**1a'**	ND	1	1
714	**1f'**	1	4	1
746	**1e'**	1	2	2
761	**1u'**	ND	ND	1
1002 ce	**1d'**	1	2	ND
1060 ce	**1l'**	ND	1	ND
Total		2	12	7

A9 Paddy Fields BHP concentration ($\mu g_{BHP}\ g^{-1}_{DRY\ SOIL}$)

Base Peak *(m/z)*	Structure Number	Vietnam F1		Vietnam F2		Vietnam F3	
		I	E	I	E	I	E
655	**1a'**	2	1	1	6	1	2
2 Me 669	**2a'**	ND	ND	ND	1	ND	ND
713	**1m'**	5	ND	ND	ND	ND	ND
714	**1f'**	3	3	1	11	1	2
746	**1e'**	2	2	ND	8	ND	3
760	**2e'**	1	1	ND	3	ND	1
771	**1b'**	ND	ND	ND	1	ND	ND
772	**1h'**	1	ND	ND	1	ND	ND
775	**2u'**	ND	ND	ND	1	ND	ND
830	**1c'**	3	1	ND	5	ND	1
943	**1n'**	1	ND	1	1	ND	ND
1002 ce	**1d'**	5	6	1	18	1	5
2 Me 1016	**2d'**	1	ND	ND	1	ND	ND
1060 ce	**1l'**	ND	1	1	1	ND	1
Total		19	17	7	57	5	15

A10 Priest Pot BHP concentration ($\mu g_{BHP}\ g^{-1}_{DRY\ SOIL}$)

Base Peak ***(m/z)***	**Structure Number**	Priest Pot PPC5	Priest Pot PPC3
655	**1a'**	17	7
2 Me 669	**2a'**	7	3
712	**5f', 6f'**	2	2
714	**1f'**	35	22
746	**1e'**	22	11
760	**2e'**	1	1
761	**1u'**	9	13
772	**1h'**	6	1
775	**2u'**	2	1
830	**1c'**	9	1
943	**1n'**	4	1
1002 ce	**1d'**	79	60
2 Me 1016	**2d'**	6	3
1060 ce	**1l'**	8	7
2 Me 1074	**2l'**	2	ND
1118	**1k'**	8	5
2 Me 1132	**2k'**	1	ND
Total		217	139