Isolation and characterisation of organic components in sediments from an estuarine environment

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Abstract

Humic substances (humic acids and fulvic acids) and humin were isolated from sediments from an estuarine environment. The clay-size fractions from the sediment cores were also isolated to access the organic matter in its natural state (without being subjected to chemical pre-treatments or to extraction procedures). Organic carbon compositions of the sediments were low, resulting in low yields of humic substances and of humin. High concentrations of urea added to aqueous alkaline media improved the extraction yields of humic substances, and these isolates had enhanced compositions from lignin and from peptide structures. Humin was solubilised using acidified dimethyl sulfoxide, and insoluble humin was concentrated using hydrofluoric acid. These humin isolates are very similar but the insoluble humins have larger compositions of highly ordered aliphatic hydrophobic species that are responsible for the insoluble nature to these fractions. Nuclear magnetic resonance (NMR) spectroscopy of the organic matter associated with the clay-sized fraction of the sediments illustrated that the extraction solvent sequence does not alter the organic fractions. The low concentrations of organic carbon in the clay-sized fraction hinder in-depth characterisation studies, but such studies are enhanced when the clay-sized fraction has minimal pre-treatment (10 % hydrofluoric acid). High ash compositions and analyses of the elemental compositions show that silicates are selectively removed, but the remaining inorganic species concentrated in the demineralised clay-sized fraction have a negative impact on characterisation studies.

Analyses show that marine organic matter is incorporated in the humic substances whereas the humins are dominated by terrestrial organic matter. Peptides are shown to make major contributions to the humic substances and humin, and there is evidence to indicate that a large proportion of these structures are from microbial inputs. Evidence for lignin is very strong in the base of the sediment cores suggesting that Galway Bay was a terrestrial environment prior to a sea level rise. Carbohydrates are shown to persist at depth due to the presence of highly ordered cellulose. Labile carbohydrates and peptides are preserved as a result of their associations with hydrophobic domains provided by the aliphatic hydrocarbons, and inorganic colloids such as iron hydroxides.

Humic substances will eventually become re-mineralised with time whereas the humin components persist in the environment. These therefore represent a more stable sink for organic carbon. Swollen-state ^{13}C NMR show that there are regions of the organic compositions inaccessible to the solvent and components in these species are responsible for the refractory natures of the organic materials.

DECLARATION

The work presented in this thesis is the original work of the author, under the supervision of Prof. Michael H. B. Hayes and Dr. Catherine Dalton. Other sources of information, when used, have been acknowledged. No part of this thesis has been previously submitted to this or any other university.

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List of Abbreviations

C	carbon
CH_4	methane
CO_2	carbon dioxide
CPMAS	cross polarisation magic angle spinning
Da	Dalton
DCF	demineralised clay-sized fraction
DEAE	diethylaminoethyl cellulose
DMSO	dimethyl sulfoxide
DOM	dissolved organic carbon
DOSY	diffusion ordered spectroscopy
ESI	electrospray ionisation
FA	fulvic acid
FTIR	Fourier transform infrared spectroscopy
Gg C	gigagrams of carbon
GHG	green house gas
H_2SO_4	sulfuric acid
HA	humic acid
HCl	hydrochloric acid
HF	hydrofluoric acid
HFC	hydrofluorocarbons
HPSEC	high pressure size exclusion chromatography
HU	humin
IHU	insoluble humin
IHSS	International Humic Substances Society
LDI	laser desorption ionisation
MS	mass spectrometer
MW	molecular weight
N	nitrogen
N_2O	nitrous oxide
NaOH	sodium hydroxide
NHS	non humic substances
NMR	nuclear magnetic resonance spectroscopy
NOM	natural organic matter
OC	Organic carbon
OM	organic matter
PFC	perfluorocarbons
ppm	parts per million
PVP	polyvinyl pyrrolidone
SF_6	sulfur hexafluoride
SHU	soluble humin
SOM	soil organic matter

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Chapter 1 Introduction

1.1 General Introduction

Increased awareness about the accumulation of carbon (C) in the atmosphere has triggered an escalation in research aimed at understanding C cycling in the environment in greater detail. Emphasis is being placed on the need to lower carbon dioxide (CO_2) emissions and to sequester C because of international concerns about the level of greenhouse gas (GHG) emissions, the consequences of global warming, and the sustainability of life as we know it. Human activities have led to considerable anthropogenic GHG emissions. The main consequences of climate change include: increased global temperatures; melting ice and rising seas; extreme weather (heat waves, droughts, heavy rainfall, storms and floods); as well as repercussions to human health, to national economies, and to wildlife (European Commission Climate Action, 2010). These concerns are highlighted by the Kyoto Protocol which aims to limit GHG emissions from developed countries (Conference of the Parties, 1997). The Kyoto Protocol set a legally binding target for Ireland to limit its GHG emissions to 13% above 1990 levels in the period 2008-2012 (EPA, 2006). Ireland complied with the Kyoto Protocol limit during this 5 year reporting period (total GHG emissions – (offsets from approved forest sinks + surrender of purchased Kyoto Protocol carbon credits)) as the emissions were below the limits (EPA, 2013a). The reduction in GHG emissions during this period are related to the downturn in the economy resulting in reductions in energy emissions, transport emissions, industry and commercial emissions, as well as reductions in the agriculture sector due a decrease in the livestock population, and in the use of fertilisers (EPA, 2013a). However, there were elevated emissions from agriculture in 2012 due to an increase in the sheep and cattle population (EPA, 2013a). By 2020, Ireland has agreed to meet an EU target of a 20% reduction in GHG emissions compared to the 2005 levels (EPA, 2013b). The gases which are listed as greenhouse gases (GHG) include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), as well as thirteen hydrofluorocarbons (HFC), seven perfluorocarbons (PFC), and sulfur hexafluoride (SF_6) (McGettigan et al., 2010). Both CH_4 and N_2O are very potent GHGs, but CO_2 receives much greater attention because its emissions are much higher, and it has a longer residency time in the atmosphere. It takes 100-300

years for CO_2 to move from the atmosphere to permanent residency in the deep ocean compared to 10 years required for CH_4 (Bala, 2013).

Prior to the industrial revolution, CO_2 concentrations in the atmosphere were 280 ppm (Siegenthaler and Sarmiento, 1993); this value has recently reached 400 ppm, recorded in Mauna Loa, Hawaii in 2013 (Bala, 2013). If CO_2 concentrations continue to rise, levels will reach those reported for the Cretaceous period (150–66 million years ago) when tropical moist conditions and vast flooding of the continents prevailed (Bala, 2013). Effects of global warming are already evident, i.e. increased temperatures, changes in weather patterns and the melting of the polar ice caps. The chemistry of the global oceans has been altered, due to dissolution of CO_2 in the water, resulting in acidification and disruptions to marine life (Bala, 2013). In the ocean the carbonate equilibrium is the pH controlled distribution of dissolved inorganic C species (i.e. CO_2 (aqueous), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions). The increased CO_2 concentrations in seawater will increase the total dissolved inorganic C species and increase the H^+ concentration resulting in a lowering of the pH and a shift in the relative proportions of each dissolved inorganic species (Koch et al., 2013). In addition to ocean acidification, the changes in the proportion and concentration of dissolved inorganic C species has effects in relation to global warming, photosynthesis, and calcification in marine macroalgae which depend on $CaCO_3$ for their growth and the thalli (vegetative tissue) structure (Koch et al., 2013).

Sediments and soils are regarded as important C sinks. Under article 3.3 of the Kyoto protocol, C sinks (anything that absorbs more C than it releases) are recognised as valuable resources for offsetting CO_2 accumulation in the atmosphere. There are five principal global C sinks namely: the ocean; the geologic pool; the terrestrial pool; the vegetation pool; and the atmospheric pool. Oceans are the largest global C pool and are estimated to hold approximately 38,000 Gg C (gigagrams of carbon, Mahli, 2002). The oceanic pool consists of dissolved inorganic C including CO_2, HCO_3^- and CO_3^{2-} (Siegenthaler and Sarmiento, 1993; Kock, 2013), as well as C in retained organic matter (OM), and sedimentary rocks. OM is ubiquitous in the environment and its reactivity is critical as its transformations directly influence the production of GHG. A comprehension of these transformation processes is essential to understand our present climate and how it may react to anthropogenic changes

(Lal, 2004; Simpson et al., 2011). Two Gt C are taken up by the ocean annually (Siegenthaler and Sarmiento, 1993), oceanic sediments contain 150 Gt C (in the form of OM) in surface sediments, and carbonate in surface sediments is estimated at 2,500 Gt C (Ridgwell and Edwards, 2007). The modern concerns about climate change are focusing attention on the importance of sediment and soil OM as C sinks. Therefore, it is imperative that we understand the processes that lead to the conservation of C in the environment, and to obtain a better awareness of the structures and the interactions of the organic components.

The overall objective of the research reported in this thesis is to provide more detailed information about the nature and the associations of OM in sediments from an estuarine environment. In order to study the compositions of OM it is necessary to isolate the OM components and to fractionate these into entities that have reasonable degrees of compositional homogeneity. These fractions include: fulvic acids, humic acids and humin materials. Humic substances (HSs) have been studied for decades and the volume of research is increasing continuously. Despite this, the structures of HSs still remains unclear, and the compositions vary depending on the environment that these are extracted from. Analytical techniques are continually improving, and bringing additional compositional information into the field of OM research.

Studies concerning the sources of OM and processes within the estuaries will provide more insight into the mechanisms that control the global C cycle because 80% of the global OM burial and 90% of the global sedimentary mineralisation are believed to be concentrated in the coastal zone (Pernetta and Milliman, 1995; van Heemst et al., 2000). OM from an estuarine environment will be analysed and studied in detail in this project. The humic substances will be extracted and characterised in detail. There will also be a focus on soluble and insoluble humins in order to study the most recalcitrant materials in the C sink. These materials are precursors to the genesis of fossil C, i.e. kerogen. Unextracted OM will be studied to assess the total OM in the environment, and to ascertain the components that degrade more readily, as well as assessing if the solvents used in extraction have an impact on the materials extracted.

1.2 Aims of the Project

This investigation into the chemical composition of HSs and recalcitrant OM in sediments from an estuarine environment will provide additional information about C sinks. New information gained about the compositions of the OM fractions from estuarine environments will be applicable to studies of OM from other environments and help to understand the compositions and associations of recalcitrant OM in greater detail. OM in this environment has received much less attention, compared to soils. Of particular interest is the humin fraction, which is an intermediate between fresh OM and fossil C. This fraction is the least studied and can provide insights into the long term stability of C in the environment.

Surface sediments and sediment core material will be analysed in this study by:

- Assessment of the suitability of the sequential solvent extraction method for the extraction of OM from sediments;
- Extraction and in-depth characterisation of HSs, humin and insoluble humin from the two sediment cores at different depths;
- Isolation of clay-sized fraction and a demineralised clay-sized fraction to characterise the total OM; and
- Assessments of HF treatments on clay-sized fraction by determination of ash contents and the inorganic compositions of the clay-sized fraction using SEM-EDX.

The use of an alkaline-urea solvent has not previously been used for the extraction of HSs from sediments. For the first time also, the DMSO-acid solvent has been used for the extraction of humin from sediments. The compositions of the OM from the top and base of the sediment cores will be compared to assess: (1), if there are different organic inputs over time; and (2), if the retained components change with time. The sequestration of OM in the estuarine environment depends on the amounts and compositions of the mineral colloids, such as the clays and the (hydrous)oxides, and the associations of OM with these colloids. Therefore, the mineralogy of the sediments will also be assessed.

This thesis is composed of an introduction chapter (Ch. 1) to put the research in context. There are two literature review chapters (Ch.2 and Ch. 3) to discuss recalcitrant organic components in the environment and their major compositions. There is a discussion of the methodologies used for the extraction of humic substances and humin (Ch. 4). Background information is provided on the main spectroscopic methods (Ch. 5) for the analysis of humic substances and humin. The methods utilised to isolate and characterise the humic substances and humin are outlined in Ch. 6. There are four results chapters (Ch. 7 – 10) that discuss the isolation and characterisation of the organic components. These results chapters are presented in research paper format (introduction, methods, results, discussion, conclusion). The data in chapters 8 and 9 have been submitted for publication, and are under review. The main conclusions of the study are outlined in Ch. 11. Relevant references are provided at the end of each chapter. Appendices are located at the end of the thesis.

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Chapter 2 Organic Carbon in the Environment: The Recalcitrant Compounds and their Association with Sediments

Studies have shown that humic substances (HSs) and humin (HU) are derived from biomolecules such as carbohydrate, protein, lipids and other biomass derived molecules such as lignin, and long-chain aliphatic hydrocarbons. Inevitably the HSs will retain many of the structural features of the biomolecules of origin (Hatcher et al., 2001). Many of these molecules exhibit aqueous solubility but can also retain some hydrophobicity in portions of their structural make-up. Hydrophobic interactions are one of the main forces responsible for the maintenance of macromolecules in their ordered structures (Huque, 1989). Some of these species are intimately associated, or covalently bonded with the humic fraction and are difficult to separate (Sutton and Sposito, 2005). Oxygen containing functional groups (carboxylic acids, phenols, alcohols, ketones, quinones, esters, and ethers) are responsible for the majority of reactions with HSs. In an estuarine environment there is a mixed source of organic matter (OM) inputs to the humification process. The origin of the OM will influence the compositions and properties of the HSs and HU. Analytical techniques utilised to characterise the organic molecules present in HSs and HU include: nuclear magnetic resonance (NMR) spectroscopy; pyrolysis gas chromatography mass spectrometry (pyGCMS) and chromatography.

2.1 Sediment – Formation and Composition

Sediments are composed of solid particles of inorganic or organic material and they can be transported by wind, water, or ice. The inorganic material is composed of clastic fractions of sand, silt and clay. The silt and clay particles can interact with organic materials, sorbing and binding them. Marine sediments also include (as well as the clastic components) the debris from marine organisms, materials of submarine volcanism, seawater precipitates, and materials from outer space (e.g. meteorites) that are deposited and accumulate on the seafloor (*Encyclopaedia Britannica*, 2013). Sediments can be classified (genetic classification) based on their origin. The four main genetic categories of sediments are: terrigenous/lithogenous sediments that are derived from the land; biogenous sediments that are derived from biological processes; hydrogenous sediments (inorganic) that are seawater precipitates; and

cosmogenous sediments (inorganic) that are materials from outer space (Futterer, 2006).

Terrigenous/Lithogenous sediments are formed from the break-down of continental rocks resulting from exposure to physical and chemical weathering, and biological activity. River transport is the main source of terrigenous sediments to the ocean. The majority of sediments transported by rivers are deposited on continental shelves or estuaries, and only a small proportion of the riverine load will actually reach the deep sea floor. Clay minerals account for up to 50% of terrigenous sediment due to weathering of igneous and metamorphic rocks (Futterer, 2006).

Biogenous sediments are formed from the remains and shells of marine organisms creating sediments composed of calcareous, siliceous and phosphatic particles (Futterer, 2006).

Hydrogenous sediments are widely distributed but generally present only in insignificant quantities in the oceans (Futterer, 2006).

Cosmogenous sediments are inorganic materials from space (e.g. fine cosmic dust) but these sediments are not common (Futterer, 2006).

When describing sediments by their grain size one should refer to the Wentworth scale (Table 2.1). Clay is the finest clastic fraction of sediments and nearly all clastic materials of this grade are composed largely of hydrous aluminium silicates and the material is always more or less plastic (Wentworth, 1922).

Table 2.1. Wentworth scale for grain size classification.

Sediment	Type	Grain diameter (mm)
	Boulder	>256
Gravel	Cobble	65-256
	Pebble	4-65
	Granule	2-4
Sand	Very coarse	1-2
	Coarse	0.5-1
	Medium	0.25-0.5
	Fine	0.125-0.5
	Very fine	0.0625-0.125
Mud	Silt	0.039-0.0625
	Clay	0.002-0.039
Colloid		<0.0002

Organic carbon (OC) concentrations are low in sediments due to the predominance of inorganic silicate from the clastic fractions. The silt and clay fractions contain most of the organic materials, due to interactions between clay minerals, and sorption on these particles that have large surface areas. Estuarine sediments have higher clay contents than marine sediments, as well as higher productivity. Therefore, greater quantities of HSs and recalcitrant OM can be isolated from this aquatic environment. HU materials (Song et al., 2011) and kerogen concentrate in these fine clay particles (Durand, 1980).

2.2 Carbon Cycle

The C cycle is described as the fluxes of C among four main reservoirs, namely: the atmosphere; the terrestrial biosphere; the oceans; and fossil C (Schimel, 1995). C continuously moves (fluxes) from one pool to another; for example carbon dioxide (CO_2) released during the burning of fossil fuels (C moving from the fossil C sink to the atmosphere), and CO_2 is absorbed by forests during photosynthesis and is stored in the biomass (C moving from the atmosphere to the terrestrial biosphere). Globally, attempts can be made to manage sources of CO_2 i.e. restricted use of fossil fuel combustion, and improved land management, e.g. no tillage/minimum tillage practices. Management of CO_2 emissions can be mediated through international agreements like the Kyoto Protocol and the EU Commissions Climate and Energy Package that aim to limit CO_2 emissions from fossil fuels and promote the expansion of C sinks. Management of farming practices can be implemented through programmes such as the Common Agricultural Policy (CAP) in the European Union. However, a number of CO_2 sources cannot be managed as they are natural processes, e.g. CO_2 release from volcanoes, the ocean, and the soil.

CO_2 is an essential component of the earth's atmosphere and helps maintain the temperature of the planet. However, as the concentration of CO_2 increases, the global temperatures increase. Prior to the 1800's the CO_2 budget was almost at equilibrium; however, since that time there has been a large increase in CO_2 emissions due to human activities (e.g. combustion of fossil fuels, cement production, and deforestation; Cleugh et al., 2011). C sinks can sequester some of this additional CO_2; however, the excess causes an increase in temperatures. The terrestrial biosphere absorbs CO_2 through photosynthesis, and its synthesis products

i.e. carbohydrates, are stored in the plants thus sequestering the C. Organic components resistant to decay in sediments are a link between the C moving from the biosphere to geosphere for long term storage.

2.2.1 Carbon in the Marine Environment

In general inorganic C is less reactive in the natural environment; e.g. C stored in sedimentary rocks for millennia, whereas OC is more reactive due to its constant degradation and transformation, e.g. utilisation of OC compounds for respiration, and synthesis as in photosynthesis. Dissolved inorganic C in seawater represents the largest active reservoir of C on the earth's upper crust and is at least an order of magnitude larger than the other C reservoirs (Hedges and Keil, 1995). OM retained in sediments and soils are composed of a mixture of heavily degraded substances that are difficult to characterise in terms of the biochemical composition (Hedges and Keil, 1995). Rivers carry terrigenous organic substances to the ocean and are the main pathways towards the preservation of terrestrial OM in sediments (Hedges and Keil, 1995). It is estimated that only one third of the OC transported by rivers to the ocean is actually buried due to extensive re-mineralisation of OM in the water column (Hedges and Keil, 1995). Autochthonous OM in the marine environment is provided by phytoplankton that is grazed by protozoans and zooplankton (Hedges and Keil, 1995). Berner (1982) estimates the preservation rate for OM in marine sediments is 0.13×10^{15} g C yr^{-1}, and postulates that > 80% of total OC preservation happens close to river mouths. The organic materials (e.g. HSs, HUs, kerogen) that are preserved over long time scales must be highly unusual in structure, in order to survive degradation in the marine environment. Hedges and Keil (1995) estimated that about 90% of the preserved OM is composed of amorphous, highly insoluble kerogen macromolecules. These organic materials are difficult to study as they cannot be physically separated from its mineral matrix. Kerogen is defined as the fraction of OM in sedimentary rocks that is insoluble in organic solvents (Forsman and Hunt 1958; Dow, 1977; Durand, 1980), and in non-oxidising acids, and in bases (Aiken et al., 1985; Feller et al., 2010). OC preserved over long time scales provides a molecular record of life, as well as sequestering C and providing economically valuable fuel resources such as coal and petroleum (Hedges and Keil, 1995).

2.2.2 Oceans as Carbon Sinks

Biological productivity in the ocean plays a fundamental role in the sequestration of CO_2 from the atmosphere (Sigman and Haug, 2003). The size of the oceanic C sink is dependent on several competing effects on the CO_2 partial pressure (i.e. CO_2 vapour pressure) difference at the air–sea interface (Fung et al., 2005). If the partial pressure of a gas is increased in the atmosphere over a body of water, the gas will diffuse into the water until equilibrium is reached (PMEL, 2013). Partial pressure of CO_2 in the seawater is dependent on the temperature, the total amount of CO_2 dissolved in seawater, and the pH of the seawater (Takahashi et al., 2009). When the partial pressure of CO_2 in the seawater is less than that for the atmosphere the seawater will absorb CO_2 from the air; however, when the partial pressure of CO_2 in the seawater is greater than that of the atmosphere, CO_2 is emitted to the air (Takahashi et al., 2009).

CO_2 is exchanged between the atmosphere and the ocean by gas transfer, and between biota and other reservoirs by photosynthesis and respiration (Siegenthaler and Sarmiento, 1993). As the planet warms the ocean will become less efficient at storing C from the atmosphere. The ocean will become saturated with CO_2, lowering the pH of the ocean and decreasing the carbonate ion (CO_3^{2-}) concentration (Ridgwell and Edwards, 2007). The decrease in CO_3^{2-} will affect biological organisms which use carbonate ions to make their skeletons and shells. This will have a negative effect on biological diversity and coral reefs ecosystems. The ocean could switch from a sink to a source through the emission of large concentrations of CO_2 as the planet warms. For example, the equatorial Pacific Ocean region releases large amounts of CO_2 to the atmosphere due to oversaturation (Siegenthaler and Sarmiento, 1993), and when the partial pressure of CO_2 in the ocean exceeds that of the atmosphere, the ocean will release CO_2.

Transformed and recalcitrant OM can be retained in the sediments for thousands of years. Longer retention of C occurs in the deep ocean and is facilitated by the ‘biological pump’ which facilitates the transport of both OC and inorganic C ($CaCO_3$) from the ocean surface and releases these at depth (Sarmiento et al., 1998). Sediments provide a long term C sink for the remains of aquatic and terrestrial plants and animals when degraded and transformed. Following transformation, the OC is pumped to the deep ocean where it can be retained for millennia (Ducklow et al.,

2001). OM produced as a result of biological photosynthetic activity represents a net sink for CO_2. Primary productivity by marine phytoplankton is estimated to be ca. 54 Gt C yr^{-1} (Houghton et al., 2001) and approximately 11 Gt C yr^{-1} escapes consumption by zooplankton grazers and bacteria at the surface, and sinks to the ocean interior (Ridgwell and Edwards, 2007). Only about 0.05 Gt C yr^{-1} of this C is buried in marine sediments, with the remainder being re-mineralised in the water column, or in surface sediments (Ridgwell and Edwards, 2007).

2.2.3 Organic Matter Retention in Sediments

Sediments and soils are the largest sinks of OM (Simpson, 2002). Rivers transport both organic and inorganic C from the land to the ocean; part of this C will be retained by the sediments, and the remainder of the organic input is re-mineralised (Siegenthaler and Sarmiento, 1993). Marine biota have an important role in the sequestration of C as they act as a C pump through the production of particulate and dissolved OM that can be exported to deeper layers in the ocean for long term preservation (discussed in 2.2.2; Siegenthaler and Sarmiento, 1993). Autochthonous OM in marine environments is highly labile; however, the more resistant biochemicals are selectively concentrated into molecularly uncharacterised components (Hedges et al., 2000). There are various mechanisms that protect (i.e. delay the oxidation of OM) labile fractions against rapid re-mineralisation (Mayer, 2004).

An important development has been the identification of a variety of biomacromolecules from higher plants and algae that are resistant to biodegradation (e.g. lipids, proteins, polysaccharides and cutins, Largeau et al., 1986; Hedges et al., 2000). Fossilised biomolecules have been identified in kerogens isolated from ancient shales, and these can retain the characteristic structure of their biological sources (Largeau et al., 1986; Goth et al., 1988; Derenne et al., 1991; Hedges et al., 2000). Less than 1% of autochthonous OM is preserved in soils and sediments; therefore, even trace amounts of resistant biomacromolecules can be concentrated and represent major components of recalcitrant OM (Hedges et al., 2000). Factors controlling the burial (preservation at depth over time) of OM are poorly understood, and the prevailing suggestions are: (1), chemical condensations that make OM inedible; (2), the presence of minor

amounts of extremely recalcitrant biomolecules; (3), the inhibition of metabolism by anaerobic conditions; and (4), the adsorption to mineral surfaces (Mayer, 1995a). Biomolecules in the detrital pool presumably escape re-mineralisation due to their chemical structures and compositions (Mayer, 2004), as well as their interactions with inorganic materials. OM is inherently recalcitrant as it is difficult to break organic-organic linkages, and the degree of recalcitrance of a biomolecule can vary depending on their scale of its organisation, i.e. monomers *versus* supramolecular organisation (Mayer, 2004). Microorganisms are unable to transport large molecules (>600 Da) through their cell walls and larger substrates must first be broken down outside the cell by exo-enzymes, or by abiotic scission (Hedges et al., 2000). Therefore, highly structured polymers e.g. highly-ordered cellulose, are difficult for organisms to degrade whereas simple sugars and amino acids will be quickly re-mineralised in metabolism by organisms in the water column. Hydrophobic interactions are responsible for the maintaining the structures of macromolecules (Huque, 1989). In addition to cross-linked organic-organic associations and highly ordered structure, encapsulation of labile molecules in hydrophobic domains of more recalcitrant biomolecules also provides organic molecules with protection from re-mineralisation (Mayer, 2004). For example, proteins may form an interaction with algaenan (a refractory aliphatic biopolymer) resulting in their encapsulation and protection from degradation (Knicker and Hatcher, 1997). An example of how encapsulation can protect labile materials from degradation in the environment is the preservation of insects encapsulated in amber (fossilised tree resin); the amber protects the insect from degradation including even their most labile substances e.g. DNA (Clarke, 1974; Knicker and Hatcher, 1997). Aggregation of OM in hydrophobic species prevents degradations because most enzymes and ions require water to function. Therefore, when water is excluded from the immediate surroundings of macromolecules their degradation will decelerate (Hedges et al., 2000). In aqueous environments, folding of large molecules, and aggregation of small molecules creates internal hydrophobic microenvironments (Hedges et al., 2000).

Biotic exclusion relates to the enclosure of OM in clay minerals that prevents the access of organisms or their digestive enzymes (Mayer, 2004). Clay can sorb OM onto its mineral surfaces through van der Waals interactions, ligand

exchange, divalent cation bridging, electrostatic or hydrophobic bonding (Feng et al., 2005; Mikutta et al., 2007; Clemente and Simpson, 2013) and these interactions have a stabilisation effect on the OM. The interactions of OM with clay minerals has received the most attention and research has shown that OM and clay minerals form strong associations in both coastal and marine sediments (Weiler and Mills, 1965; Tanoue and Handa, 1979; Mayer, 1994; Keil et al., 1994a-c; Hedges and Keil, 1995), in fine grained sediments along continental shelves (Bordovskiy, 1965; Premuzic et al., 1982; Ergin et al., 1993; Hedges and Keil, 1995), and in soils (Qualls, 2004). Bock and Mayer (2000) found that the majority of OM from a near-shore sediment core was present as organo-clay aggregates that had definite associations between the OM and the very fine phyllosilicates. They suggest that the clay and the OM form aggregates rather than adsorption of the OM on the clay mineral surface. The OM can be preserved through physical entrapment in pores (of clays/sediment grains), or through strong associations with the mineral surfaces. The OM can be protected in the mesopores (<10 nm in length) of the sediment grain and these pores are inaccessible to the enzymes responsible for the initial stages of OM decay (Hedges and Keil, 1995; Zimmerman et al., 2004). However, some hydrolytic enzymes are 3-4 nm in size (Diaz and Balkus, 1996), and therefore OM present in pores < 3 nm can include the fractions that will be protected indefinitely (Mayer et al., 2004). Mayer et al. (2004) suggest that this physical protection only accounts for minor fractions of OM. Aggregation of the OM in either hydrophobic species or clay minerals are a major form of OM protection in the environment. Wiseman and Püttmann (2006) found that mineral interactions play a significant role in OC stabilisation through the formation of complexes or aggregates that help protect organic material from mineralisation, and this aggregation can be greatly influenced by Fe- and Al oxides.

2.2.4 Organic Inputs to the Sediments

The OM in estuarine sediments represents a range of precursor organic materials from marine and terrestrial environments. OM in estuarine sediments is composed of a broad spectrum of organic constituents from a variety of sources (phytoplankton or

benthic microalgae, terrestrial inputs, seagrasses, macro-algal beds, mangroves and salt marshes; Bouillon and Boschker, 2006).

However, our current understanding of the fate of OM in estuaries is limited (Hedges et al., 1997; Louchouarn et al., 1999; Raymond and Bauer, 2001; McCallister et al., 2006; Tremblay and Gagne, 2007). The sedimentary pool of OM is an assortment of materials due to the intense mixing by currents. Mineralisation is dependent upon the source material present and its degree of recalcitrance (Bouillon and Boschker, 2006). Labile organic compounds, e.g. phytoplankton, provide an energy source to benthic organisms (Tremblay and Gagne, 2007). Terrestrial OM inputs are significant in coastal regions and this OM is often retained in the sediments because it can already be significantly transformed by the time it reaches the ocean (Bouillon and Boschker, 2006). A study by Tremblay and Gagne (2006), found that HSs represented the dominant component of retained sedimentary OM (52-100%) with HU being the largest fraction (38-88%), followed by HAs (4-28%) and FAs (2-14%). The $\delta^{13}C$ signatures and the distribution of lignin (terrestrial biomarker) can give indications of the origins of the organic material (Fox, 1991). Differentiation is aided by the fact that the enrichments in ^{13}C in marine sources are different from those of terrestrial plants (Nissenbaum and Kaplan, 1972; Meyers 1994; 2003). Therefore, based on that variation of ^{13}C depletion, it is possible to identify whether the OM has originated from marine or from terrestrial environments (see Table 2.2). Nissenbaum and Kaplan (1972) studied marine HAs from a number of different locations and concluded that the HAs had a $\delta\ ^{13}C$ signature ranging between -22 and -23‰ whereas soil HAs range between -25 and -26‰. Identification of lignin using pyrolysis gas chromatography mass spectrometry (pyGC/MS) and ^{13}C and ^{1}H NMR spectroscopy assist in the identification of terrestrial OM. However, with time (and pressure) the OM can become significantly altered and it is then difficult to identify its origins.

Table 2.2. ^{13}C isotopes signatures of organic matter from marine and terrestrial environments.

Plant	$\delta^{13}C$ signature	Reference
Terrestrial C_4 plants	-12 to -16 ‰	O'Leary, 1988.
Terrestrial C_3 plants	-27 to -30‰	Hedges et al., 1986.
Seagrass	-15 to -3‰	Zieman et al., 1984; Wada et Yamamuro, 1999, Kuram Minagawa, 2001.
Phytoplankton (river flood plains)	-34.0 to -37.2‰	Hamilton and Lewis, 1992.
Algae (*Ascophyllum nodosum*)	-15.4 to -21.9‰	Golléty et al., 2010.
Seaweed	-15.6‰	Chikaraishi and Naraoka 2003.
Marine organisms	-12.3 to -19.6‰	Golléty et al., 2010.
Marine humates	-20 to -22‰	Nissenbaum and Kaplan, 1972.
Soil HAs and FAs (C_3 plants dominate)	-25 to -26‰	Nissenbaum and Kaplan, 1972.

2.2.5 Estuaries – a Complex Transitional Zone between Terrestrial and Marine Environments

Estuaries are important zones between the terrestrial and the marine environment. Pritchard, (1967) defines an estuary as *"...a semi-enclosed coastal body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage*". The environment is complex due to the mixing of freshwater and saline marine water, and saline concentrations will depend on river discharge and the tidal range. Due to the influence of the two environments, OM in estuaries is a mix of terrestrial plants, plankton, as well as smaller contributions from micro- and macro-heterotrophs and macro-benthic algae (natural sources of OM), and wastewater discharges (anthropogenic sources of OM) (Thoumelin et al., 1997). Removal of OM in an estuary is a result of decomposition by living organisms, and transport of OM towards the sea (Williams, 1975; van Heemst et al., 2000). Terrestrial OM and OM from riverine and estuarine primary and secondary productivity are major sources of OM to the ocean (Meybeck 1982; Hedges, 1992; Minor et al., 2001). Dissolved organic carbon (DOC) is exported outside the estuary by the current whereas

particulate organic carbon (POC) can become trapped in the sediments. Processes such as sediment resuspension, diffusion from sediments, biodegradation, and flocculation at freshwater – marine water interfaces can influence the fate of the OM (Thoumelin et al., 1997). Variations in river discharge and in tidal conditions, and weather events such as storms, can control the residence time of OM, and affect important reactions such as flocculation, solubilisation, respiration and burial of C (Goni et al., 2005).

2.3 Humic Substances

Humic substances (HSs) from soils and sediments have a number of compositional differences due to the differences in the organic inputs to the humification process. However, their functions and chemistries are similar, and extraction methods developed for soils can be applied to sediments. Therefore, reference is made to soils throughout the review of the literature and in the interpretation of the data in later chapters. Both terrestrial and marine OM are discussed as the organic components in the estuary are a mixture of terrestrial and marine detritus.

2.3.1 Definitions and Descriptions of Humic Substances

A large proportion of recalcitrant OM in waters and sediments is composed of humic substances (HSs) that are formed from the breakdown of plant, animal, and microbial tissues (Coates et al., 2002; Giani et al., 2010). HSs, one of the most widely distributed classes of natural products, are ubiquitous in water, soil and sediment (Ikan et al., 1990). The breakdown and transformation of OM are not genetically controlled (Clapp et al., 2005) and their random formation produces an immense variety of humic structures. The operational definition of HSs state that they are formed from the biological and chemical transformations of plant and animal debris and show no morphological resemblances to the organic materials from which they were formed (Kononova, 1966; Hayes and Swift, 1978). There has been extensive research on HSs since their extraction from peat by Achard (1786); however, their structures and aspects of their chemical compositions remain unresolved despite many advances in analytical approaches. Traditionally HSs were defined as "…*a series of high-MW, brown to black, colored substances, formed by secondary synthesis reactions"* (Stevenson, 1982). Aiken et al. (1985) expanded on this

definition to describe these as "…*a category of naturally occurring, biogenic, heterogeneous organic substances that can be characterized as being black-to-yellow in color, of high molecular weight (MW), and refractory"*. These descriptions are vague and show the uncertainty about the exact nature of HSs composition and structure; but it is accepted that the compositions of HSs include aromatic rings, and aliphatic species containing O-, N- and S- functional groups (Stevenson, 1994; Romarís-Hortas et al., 2007). Piccolo (2001) and colleagues have challenged the early definitions and described the conformation of HSs as a "*...self-assembly of different small molecules into supra-molecular structures of different sizes"*. Based on the work by Piccolo (2001) a new concept has been put forward by Sutton and Sposito (2005), that describe HS as "*... supramolecular associations, in which many relatively small and chemically diverse organic molecules form clusters linked by hydrogen bonds (H- bonds) and hydrophobic interactions"*. All these experiments on the size and associations of HSs were carried out after preliminary work by Piccolo (1996) and Kenworthy and Hayes (1997) who placed doubt on the belief that HSs may be polymeric structures. HSs are believed to be composed of three fractions, namely:

Humic Acids (HAs): extractable in base and are insoluble in aqueous media below pH 2.

Fulvic Acids (FAs): extractable in base and remain soluble at all pH values in aqueous media.

Humin (HU): not soluble in aqueous media at any pH value

HAs and FAs differ in their colour, functional groups, molecular weight, degree of polymerisation, and degree of hydrophobicity (Moros et al., 2008; Uyguner-Demirel and Bekbolet, 2011). In general, HSs increase in molecular size and are darker in colour in the order of FAs, HAs and HU (Stevenson, 1994; Tan, 2003; Prentice and Webb, 2010). When bonding forces are broken or weakened, components of the HSs associations are dissolved in aqueous media. HSs are defined operationally (first introduced by Sprengel, 1826) in terms of their solubility in an aqueous medium as a function of pH (MacCarthy, 2001). These humic fractions do not represent pure compounds; each fraction is composed of a complex and heterogeneous mixture of organic substances (Hayes et al., 1989). These humic fractions also contain components with definite chemical classes (i.e. non-humic substances) such as

proteins, sugars, and lipids. These biomolecules may be entrapped in the humic matrix by hydrophobic interactions or by non-covalent bonds (Lichtfouse et al., 1998a). Entrapment in hydrophobic domains eliminates water and these biomolecules are consequently protected from microbial activity (Piccolo and Conte, 2000). The fate of HSs is to eventually become re-mineralised, or to become altered into fossilised C.

2.3.2 Non-Humic Substances

Non-humic substances (NHSs) are the main food source for micro-organisms and fungi. NHSs include the following biomolecules: carbohydrates (polysaccharides, oligosaccharides and monosaccharides); proteins (peptides and amino acids); lipid molecules (fatty acids, sterols, terpenes, chlorophyll, fats, waxes and resins); and low molecular organic acids (Stevenson, 1982) that have not been transformed. Such molecules can be regarded as components of HSs only when these are covalently linked to the humic core and satisfy the solubility criteria for HSs (Hayes and Swift, 1978; Aiken et al., 1985; Stevenson, 1994). NHSs are less complex and less resistant to microbial attack relative to HSs.

2.3.3 Humic Acids

HAs are the fraction of the HSs that in laboratory extractions are soluble at high pH values but coagulate, as a dark brown to black, sludge-like material, in aqueous media at low pH (pH < 2) (Moros et al., 2008). Clapp et al. (1996) state that HAs are composed of a wide variety of compounds with variable MW and physico-chemical properties. These complex substances challenge the design of experiments and interpretations of the analytical data generated (Clapp et al., 1996). HAs are associations of mainly hydrophobic species that form hydrophobic associations that are stabilised at neutral pH by hydrophobic dispersive forces (van der Waals, π-π, and CH-π bondings; Piccolo, 2002). These associations grow progressively in size, through the formation of intermolecular hydrogen bondings at lower pH values, forming a precipitate (Piccolo, 2002). There is a vast amount of research on the HA fraction as these are the most extractable component of HSs. NHSs are generally co-extracted with HAs as these components are generally soluble in aqueous alkaline solutions and some can precipitate in acid (Hayes and Clapp, 2001). This problem

can be overcome by fractionating the extracts on XAD-8 ((poly)methylmethacrylate) resin to remove NHSs.

2.3.4 Fulvic Acids

Following the precipitation of HAs (after alkaline extraction) by acidification, the FAs remain in solution. In the older literature fulvic acids (FAs) were regarded as the components that remained in solution when the alkaline medium was acidified. However, based on the definitions of the International Humic Substances Society (IHSS; Swift, 1996), the materials in solution in the acidified extract are now considered to be the FAs fraction because these contain carbohydrates, peptides, and other biological molecules that are not part of the humic materials. Similar to the HAs, FAs must be fractionated on XAD-8 resin to remove NHSs. The true FAs (hydrophobic components) are adsorbed on XAD-8 resins, and the polar contaminants are washed through and removed (Aiken, 1985; Swift, 1996; Hayes et al., 2008). FAs have lower MWs than the HAs and contain more acidic functional groups, especially COOH groups (Stevenson 1982; Aiken et al., 1982). Piccolo (2002) suggests that FAs remain in solution at all pH values due to the presence of sufficient acidic functional groups to keep the fulvic clusters dispersed. Simpson et al., (2002) has suggested 1000 Da as the average MW for the FAs, based on NMR studies.

2.3.5 Humin

Humin (HU) represents the most important organic C fraction as it can remain stored in the geosphere for thousands of years forming an integral part of the global C cycle. HU is operationally defined as the fraction of HSs that is insoluble in aqueous media at any pH value (Hayes, 2006), i.e. non-extractable natural OM (Simpson et al., 2011). Compositional information obtained by Song et al. (2011) indicates that HU is composed of components from plants and from microorganisms whose structures are known, and there is not sufficient evidence to indicate that these components have been microbiologically or chemically transformed. Therefore, HU should not be regarded as fitting the classical definitions of HSs (i.e. a substance that has been significantly altered; Clapp et al., 2005; Song et al., 2011; Hayes et al., 2012) and should be regarded as a separate organic entity rather than part of the HSs.

HU is highly refractory and accounts for the largest fraction of the 'extractable' (sparingly soluble in organic solvents) organic components retained in soils and sediments. It is estimated to account for >50% of OC in soils and sediments (Kononova, 1966; Stevenson, 1982; Simpson et al., 2011) and ca. 70% of the OC in unlithified sediments (Durand and Nicaise, 1980; Peters et al., 1981; Hatcher et al., 1985; Ishiwatari, 1985; Vandenbrouke et al., 1985; Mayer, 1994; 1995b; Keil and Hedges, 1995; Rice 2001). HU is the least studied of the humic fractions (Rice, 2001; Simpson et al., 2011) because these recalcitrant organic materials are difficult to separate from the mineral matrix (Tremblay and Gagné, 2007), and it is difficult to characterise using analytical methods that require the analyte to be soluble. The HU fraction is much more strongly associated with the mineral matrix than the HAs or FAs, and the HU often forms strong complexes with inorganic colloids (Lichtfouse et al., 1998a; Rice, 2001; Prentice and Webb, 2010). The mineral/inorganic composition of the clay-sized fraction of soil and sediments (as discussed in Section 2.2.3) will influence the extraction of HU in organic solvents. HU from an andisol (Chang et al., 2014) and from a ferrisol (Song et al., 2011) were sparingly soluble in DMSO-H_2SO_4, with only aliphatic hydrocarbons solubilised in the case of the andisol. The limited solubility is attributed to the presence of aluminium and of iron hydroxides in these soil types. These species form aggregates with both the OM and the clay minerals (Wiseman and Püttmann, 2006), thereby preventing the solvent coming into contact with the OM. In comparison, HU was more easily isolated from soils predominantly composed of aluminosilicates clays (for example a mollisol and a gravelly brown earth) and these isolates were rich in aliphatic hydrocarbons, as well as having peptide and carbohydrate components (Song et al., 2011). Hydrofluoric (HF) acid can be used to isolate HU when it is strongly bound (as in the case of the ferrisol and andisol) to inorganic species. However, HF acid digestion can result in losses of the OM, especially of the carbohydrate components and can give rise to alterations to the isolates, depending on the concentration of the solution and the length of the extraction time. Schmidt et al. (1997) and Gélinas et al. (2001) both found that there were no evident changes in bulk chemical composition of the OM following HF digestion of the inorganic components, except for the loss of some carbohydrates.

DMSO-H_2SO_4 has been shown to be a suitable solvent for the extraction of HU (Hayes 1985; 2006; Song et al., 2011; Chang et al., 2014). Following extraction of the soluble HU, (DMSO-) insoluble HU can be isolated by dissolving the mineral matrix using HF. The residue that remains following HF digestion represents the most stable/highly resistant materials in the C sink. Components in the HU fraction are likely to be in the form of macromolecules and/or very stable aggregates. ^{13}C NMR studies have shown that HU tends to be enriched in aliphatic and substituted aliphatic C and has less aromatic C in comparison to HAs (Almendros et al., 1991; 1996; Nierop et al., 1999; Salloum et al., 2001; Kang et al., 2003; Guignard et al., 2005; Wang and Xing, 2005; Keeler et al., 2006; Simpson and Johnson, 2006; Bonin and Simpson, 2007; Simpson et al., 2011). HU has the longest turnover time of all the fractions regarded as humic because of the resistance of its components to chemical, physical, and biodegradation (Prentice and Webb, 2010). Solid-state ^{13}C NMR studies by Song et al. (2011) showed that DMSO-acid extracted HU is composed primarily of aliphatic C species and has low amounts of carbohydrate, peptide, and lignin-derived components. The recalcitrant nature of HU arises from the fact that non-hydrolysable aliphatic biomolecules (e.g. from plant cuticles) resist degradation due to the arrangement of their molecular structures (Song et al., 2011). Such aliphatic biopolymers are likely to be concentrated in HU due to hydrophobic associations and these aliphatic components bind preferentially to clays (Hayes et al., 2009; Simpson et al., 2011). Their polarity and their interactions with clay minerals minimises their solubility in aqueous solvents. The main compositions of HU (aliphatic species, peptides, carbohydrates, peptidoglycan and lignins) are similar to those in soluble soil extracts but the HU has a greater abundance of macromolecular components and contributions from microbial cell walls (peptidoglycan) (Simpson et al., 2011). The peptide and saccharide components of HU can resist degradation because of protection arising from hydrogen bonding, and from encapsulation (Knicker et al., 2001; Lichtfouse et al., 1998b), as well as through intimate associations with and sorption by the inorganic soil colloid surfaces (Song et al., 2011).

Traditionally, HU research was largely in the domain of geochemistry because it was considered to be a part of the coalification and petroleum genesis processes. However, research is now focused on the HU fraction due to its long term

retention times, and because of its dominant role in the binding of organic contaminants. As the knowledge of HU retention is expanded, a deeper insight will be gained into C sequestration and a further understanding of the global C cycle will be attained.

2.3.6 Humic Substances in Aquatic Sedimentary Environments

Humic substances are a large sink for OC as they are the main component of dissolved OM in waters and of the OM in soil and peat (Moreda-Pineiro et al., 2006). They impact the solubility and facilitate the mobility, transport, concentration, and accumulation of trace elements in marine environments (Hirata, 1981). These organic materials contain a variety of oxygen containing functional groups (carboxylic, carbonyl and phenolic) that provide these with their great complexing abilities (Lund, 1990). Originally HS studies were focused primarily on soils and peat. More recently there has been a greater focus on sedimentary OM but the emphasis is often focused on the origin of petroleum (Hedges and Oades, 1997). Extraction of HSs from sediments is fraught with difficulties due to low extraction yields and limited published methodologies, as well as the difficulties associated with obtaining sediment cores (geographical location, technical equipment required, and financial cost). However, organic materials retained in the sediments are a key part of the global cycling of C, and these provide a record of changes that have occurred in the environment over long time scales.

Early studies by Degens et al. (1964) found that HAs represented 30-60% of OM in sediments from the basins of Southern California, and Bordovskiy (1965) found that HAs composed 40% of the OM in Bering Sea sediments (Nissenbaum and Kaplan, 1972). Marine HSs have been investigated using methodologies that were developed for soils (Hedges and Oades, 1997). OM isolated from marine environments (that have no terrestrial influences) is mainly derived from plankton and contains no contributions from lignin (Vandenbrouke et al., 1985) whereas terrestrial OM has strong contributions from lignin. Lignin derived materials can be transported by rivers and form part of the sedimentary C pool in coastal regions (Rullkötter, 2006). Aliphatic structures are the major compositions in marine HSs while terrestrial HSs are generally composed of more aromatic structures that are derived from higher plants (Hatcher et al., 1981). Marine organisms that contribute

to the OM pool have high protein contents; algal cell walls are composed of highly structured glycoproteins (Roberts, 1974; Table 2.3). However, aliphatic hydrocarbons account for the main compositions of the preserved OM in the aquatic environment as these molecular species are refractory, whereas components derived from proteins and carbohydrates are easily degraded (chemically and biologically; Gelin et al., 1999). Algaenans, (nonhydrolysable, aliphatic bio-macromolecules biosynthesised by marine algae), are major contributors of OM to the long term C sink as these biomolecules are extremely difficult to degrade (Gelin et al., 1999).

Table 2.3. Biochemical composition of marine organisms (Romankevitch 1984; Rullkötter, 2006).

Organism	Protein (%)	Carbohydrate (%)	Lipids (%)	Ash (%)
Phytoplankton	30	20	5	45
Phytobenthos	15	60	0.5	25
Zooplankton	60	15	15	10
Zoobenthos	27	8	3	62

2.4 Formation of Humic Substances

A number of theories (or pathways) have been proposed to explain the formation of HSs (usually relating to the terrestrial environment) (Stevenson, 1994; Figure 2.1), but many of these have been disproven as the major pathway for the formation of HSs. Pathway 1 refers to the sugar-amine theory (Maillard reaction), pathways 2 and 3 refer to the formation of HSs via quinone formation, and pathway 4 refers to the ligno-protein theory.

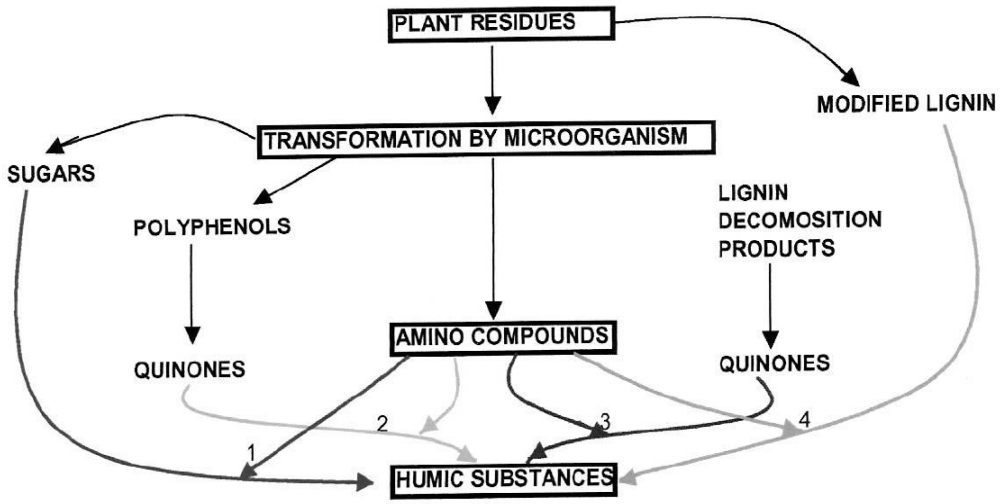

Figure 2.1. Proposed mechanisms for the formation of humic substances from the decay of plants (Stevenson, 1994, p. 189).

Burdon (2001) discussed these formation pathways in detail and he dismissed some of these pathways of formation as the main route of HSs formation for a number of reasons; for example, the NMR spectra of laboratory synthesised products are not sufficiently similar to the spectra of HSs isolated from the environment; the absence of lignin in HSs isolated in marine environments; and the lack of sufficient free monosaccharides and amino compounds required for the Maillard reaction to proceed to any considerable extent. HSs can accumulate in soils and sediments that do not receive OM derived from lignin. Therefore, the lignin and polyphenol theory fails to explain how HSs can arise from such precursors (Stevenson, 1994). Aromaticity can be attributed to non-plant sources, for example, microorganisms can produce numerous phenolic and hydroxyl aromatic acids from non-aromatic C sources (Stevenson, 1994). Burdon (2001) dismisses the concept that HSs are created from biotic reactions because it is inconceivable that microorganisms would synthesise a substrate (i.e. HSs) that they cannot use, expending considerable energy in the process. It is plausible that each formation pathway can contribute to HSs formation to an extent.

2.5 Organic Matter Transformations in the Marine Environment

HSs are formed from the degradation products of plant material (Cuthbert and Giorgio, 1992; Wu et al., 2002), and of necrotic microorganisms (Buffle, 1990; McDonald et al., 2004). This degradation and transformation process is called humification which stabilises organic components resulting in their preservation in the environment (Kögel-Knabner, 2002). Soil HAs generally tend to have a high aromatic content arising from the breakdown of vascular plants and retention of refractory biopolymers such as lignin (Wershaw et al., 1990; Rasyid et al., 1992), whereas marine HSs have less aromatic compounds and higher carbohydrate and protein compositions (Ertel and Hedges, 1983; Moreda-Pineiro et al., 2006). Burial of OM in marine sediments results in lower oxygen contents, loss of sugar compounds, and changes in the forms of organic nitrogen (due to ammonification and nitrification processes; Feller et al., 2010). Formation of HSs and HU occur in the early stages of diagenesis (later stages involve the transformations to fossil fuels) where microorganisms are actively involved in the degradation and transformation of fresh OM which is subsequently buried in the sediments (Feller et al., 2010). As degradation of the organic materials proceeds towards the genesis of fossil C, functional groups and nitrogen are removed, and organic sulfur is incorporated in marine OM (a feature that differentiates marine from lacustrine or continental immature kerogens; Huc, 1990; Feller et al., 2010). Compositions and structure of preserved OM are still the focus of many investigations in order to understand C cycles, C sequestration, and fossil fuel formation, as well as understanding chemical process mediated by HSs and HU.

2.6 Structures of Humic Substances

The exact structures of HSs remain unknown. However, a number of advances were made in the late 1990's and early 2000's in relation to the size and conformations of HSs. Traditionally it was believed that HSs were macromolecular (with some exceeding 10^6 Daltons (Da)), based on studies of HSs in aqueous solutions using the following techniques: gel permeation chromatography; low-angle x-ray scattering; ultracentrifugation; viscometry; and light reflectance spectroscopy (Hayes et al., 1989). However, recent research suggests that HSs are a collection of small molecules in supramolecular structures due to aggregation (Piccolo, 2001). The size

and conformation of the HSs is dependent on the properties of the media used e.g. pH or ionic strength. Simpson et al. (2011) state that in order to solve the structure of HSs a multidisciplinary approach is required that uses the most modern advances in science.

2.6.1 Random Coil Model

The reversible coiling model (proposed by Cameron et al., 1972) was extensively used to describe the structure of humic materials, and the model became popular following experiments by Ghosh and Schnitzer (1980). The model proposed that HSs behave like polymers. The macromolecular confirmation of these are not unique but can differ depending on the pH and the ionic strength of the extraction medium. Ghosh and Schnitzer (1980) proposed that "*…HSs are rigid spherocolloids at high sample concentration, at high ionic strength, and at low pH,* whereas *at a high pH values, low sample concentration, and ionic strength, they behave as flexible linear polymers"*. This theory is dismissed by Piccolo (2001) based on the fact that the experiments were conducted using whole humic extracts with full polydispersity.

2.6.2 Micelle Theory/Supramoleular Confirmations

Wershaw (1986, 1993) was the first to present a real alternative to the random coil/polymer model. Piccolo (2001) considered his explanation to be viable as it relied on the aggregation of different HSs particle sizes in contrast to an alternative belief that HSs were polydisperse linear polymers. Early reports of HSs having a large MW were based on the MW of biopolymers such as cellulose, but the figures were too high for transformation products from partly degraded plant materials and partly formed microbial substances (Burdon, 2001). Piccolo and Conte (2000) and Piccolo (2002) have shown HSs to have a considerably smaller MW (ca. 1000 Da), and large MW figures are attributed to the aggregation of these smaller molecular species into in a supramolecular association. Several studies using low and high pressure size exclusion chromatography have shown that HSs tend to reflect the behaviour of molecular associations rather than polymers (Piccolo, 2001). Based upon experimental work (Piccolo, 2001) Piccolo and colleagues expanded on the supramolecular concept - a new view of the structures of HSs. Piccolo's earlier studies were put in context by the observations of Kenworthy and Hayes (1997) and

from subsequent work by the Piccolo group. Piccolo (2001) describes HSs as "*...superstructures of only an apparent large size and self-assembled by relatively small heterogeneous molecules held together by mainly hydrophobic dispersive (van der Waals, π-π, CH-π) forces...*". Following the research by Piccolo and colleagues, Sutton and Sposito (2005) proposed that HSs are "*...collections of diverse, relatively low molecular mass components forming dynamic associations which are stabilized by hydrophobic interactions and hydrogen bonds*". They expand on the arrangement of HSs to describe the aggregation as micellar which allows for protection of the hydrophobic core which is provided by the hydrophilic exterior regions in an aqueous environment (von Wandruszka et al., 1999; Sutton and Sposito, 2005). Simpson (2002) used solution-state multidimensional (DOSY, Diffusion Ordered Spectroscopy) NMR spectroscopy to study aggregation and disaggregation of the HSs. At high concentrations HSs have MW values as high as 66 kDa (similar to the weight of proteins) due to aggregation of the small molecules. However, when the HSs are disaggregated (using organic acids), MW values range between 200-3000 Da (Simpson, 2002). Sutton and Sposito (2005) found similar MWs for humic molecules using electrospray ionisation (ESI) and laser desorption ionisation (LDI) techniques coupled with mass spectrometry (MS), thus supporting the view that HSs are a diverse collection of low MW organic molecules. This supramolecular theory agrees with research published by Piccolo (1996); Chien et al. (1997); Tombácz (1999); Wershaw (1999); Martin-Neto et al. (2001); Simpson et al. (2002); Nanny and Kontas (2002); Piccolo (2001; 2002); Sutton and Sposito (2005).

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Chapter 3 Major Components of Organic Matter

A prerequisite to the study of HSs and recalcitrant OM is an understanding of the compositions of natural OM in terrestrial and aquatic environments. The origins and chemistry of the most important biomolecules are outlined in this chapter.

3.1 Carbohydrates

Carbohydrates are necessary to sustain most non-autotrophic organisms. They are produced by plants and some algae through the photosynthesis process in which carbon dioxide (CO_2) and sunlight are utilised to produce complex organic carbon (OC) molecules. Necrotic plants and leaf litter are degraded in the terrestrial environment to provide a continuous source of carbohydrates to the soil. Carbohydrates account for the largest identified fraction of dissolved OC in the sea (Myklestad et al., 1997). Phytoplankton provides the primary source of these carbohydrates, and of the OM in the marine environment (Romankevich, 1984) with the majority of the organic materials being directly released as dissolved OC (Biersmith and Benner, 1998). Carbohydrates are composed of saccharide units. Sugars are composed of monosaccharides (one unit) e.g. glucose, or disaccharides (two units) e.g. lactose. When many sugar units join together a polysaccharide is formed e.g. cellulose. Simple sugars (monosaccharides) will be degraded rapidly in the environment whereas polysaccharides are more persistent as these are more difficult to decompose, especially if their structures are highly ordered.

3.1.1 Sugars

HSs can contain sugars from either biomass (i.e. plants, algae) or from microorganisms. The major sugars in plant cell walls include pentoses (five C atoms in the molecule; arabinose and xylose) and hexoses (six C atoms in the molecule; glucose, galactose) (Pettipher and Latham, 1979) (Fig 3.1). Microbial sugars are composed of hexoses (mannose and galactose) and deoxyhexoses (fucose and rhammnose; Sauheitl et al. 2005). Microbial biomass (living organisms 5-10 μm^3) is considered an important part of the total OM present in soils, accounting for 1-5% (Alef and Nannipieri, 1995; Glaser et al., 2004). Marine microorganisms and algae produce extracellular secretions in order to create a micro-environment in which their cells are protected from toxins, grazing species, and rapidly changing

environmental conditions (Biersmith and Benner, 1998). These secretions are composed of complex heteropolysaccharides that contain a variety of aldoses (monosaccharides that contain one aldehyde group per molecule; Biersmith and Benner, 1998). Despite their extremely labile nature sugars are an integral part of the OC pool, and thus must be afforded a degree of protection either by physical preservation, encapulsation, or by covalently bonding with humic substances (HSs) (Derrin et al., 2007) and humin (HU).

Aminosugars, carbohydrates that contain amine-derived functional groups, are considered to be abundant components of marine OM (Benner and Kaiser, 2003). These sugars are an integral component of bacterial cell walls; for example, muramic acid which is found in the repeating disaccharide backbone in a peptidoglycan (Ogawa et al., 2001). They are also components of biopolymers such as chitin (a homopolymer of N-acetyl-D-glucosamine from phyto- and zooplankton), polysaccharides, glycoproteins and glycolipids, all of which are common to many organisms. Therefore, these are important structural components of prokaryotic cell walls and are an integral component of fresh OM (Carstens et al., 2012).

Uronic acids are found in the cell walls of grasses (Kim and Carpita, 1992). It is proposed that these are an integral part of the cell wall as they form ester linkages between polysaccharide chains; these form between the carboxyl groups of uronic acid residues in one chain and the hydroxyls on a another polysaccharide chain (Iiyama et al., 1994).

Glyceraldehyde ($C_3H_6O_3$) is an intermediate compound in carbohydrate metabolism. It is utilised by algae for energy and growth (Saunders, 1957). D-glyceraldehyde-3-phosphate is the central molecule in glycolysis (Toxvaerd, 2005).

Sugars can be released from organic samples by acid hydrolysis, and identified using ion chromatography. Ratios of the sugars present can give indications on the origins of the organic materials. The ratio (MAN+GAL)/(XYL+ARA) is used to assign the origin of the polysaccharides; values close to 0.5 suggest plant origins whereas values close to 2 suggest microbial origins (Oades, 1984).

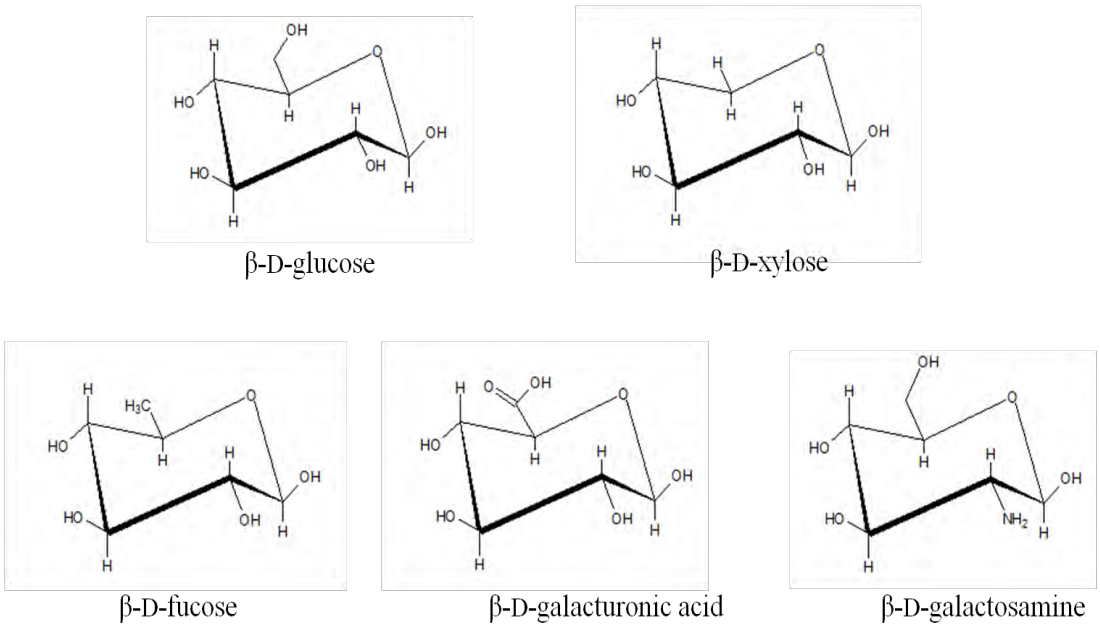


Figure 3.1. Basic structures of major sugar monomers: hexoses (glucose); pentoses (xylose); deoxyhexoses (fucose); uronic acids (galacturonic acid); and aminosugars (galactosamine).

3.1.2 Cellulose

Cellulose is a fibrous, tough, water-insoluble biomolecule that is located in cell walls (O'Sullivan, 1997) of plants, algae, and fungi (Peberdy, 1990; De Leeuw and Largeau, 1993; Kögel-Knabner, 2002). Cellulose is abundant in vascular plants and can be absent, or present at lower concentrations in algae. Cellulose contents in marine algae are smaller and tend to be more variable compared to higher plants; there are low cellulose contents in both red and brown algae (Frei and Preston, 1964), whereas most green algae have a cellulosic wall (Baldan et al., 2001). Plants have a high C/N values (>20) due to the high concentration of cellulose, whereas algae have a lower C/N value (4-10) attributable to their higher protein compositions (Meyers, 1994).

Cellulose is reported to be formed at, or outside the plasma membrane in higher plants (Lamport, 1970; Brett and Waldron, 1990; O'Sullivan 1997). It is composed of aliphatic monomeric units that form a predominantly crystalline (linear) polymer (~15% amorphous) due to its repeating glucose units (β-(1→4) glycosidic bonds, Figure 3.2) and H-bridges (between hydroxyl groups; Kögel-Knabner, 2002) to form linear polymeric chains of over 10,000 glucose residues (Teeri, 1997). The cellulose chains attach to each other along their lengths and are held in this conformation by H bonds and van der Waals forces (Teeri, 1997), and the these

chains tend to aggregate thereby forming highly ordered structural entities (Ibrahim et al., 2010). A single cellulose crystal contains tens of glucan chains that are in parallel orientation (Teeri, 1997). Despite its highly ordered structure, cellulose is not uniform (i.e. has amorphous domains) because it is a natural polymer and is therefore subject to imperfections in its synthesis (Teeri, 1997). Cellulose crystals in the fibres of higher plants associate with other cell-wall components, such as lignin and hemicelluloses, and results in even more complex structures. Non-crystalline cellulose is more easily degraded by enzymes (Béguin and Aubert, 1994; Pérez et al., 2002). The associations formed between cellulose with hemicelluloses and lignin can affect its biodegradation (Pérez et al., 2002).

Algal cellulose is rarely composed of pure β-(1→4) glucan because it more often contains sugars other than glucose, usually xylose (Mackie and Preston, 1974, Baldan et al., 2001). Xylans (mainly β-(1→3) linked) and mannans (mainly β-(1→4) linked), are less rigid biopolymers that can replace cellulose in the algal cell walls to provide structure (McCandless, 1981; Baldan et al., 2001). These form microfibrils that are embedded in an amorphous matrix in the algal cell wall (Baldan et al., 2001).

Cellulose can be chemically or enzymatically hydrolysed to soluble sugars (Teeri, 1997). Meyers (1994) found that cellulose-rich coastal (continental shelf) OM is typically less degradable than algal OM. Eubacteria and fungi are the main microorganisms involved in the degradation of cellulose, however, some anaerobic protozoa and slime moulds are capable of degrading cellulose (Pérez et al., 2002). Cellulose degrading microorganisms, along with non-cellulose degrading organisms, produce a suite of enzymes with different specificities that work together to completely degrade cellulose, releasing CO_2 + H_2O under aerobic conditions, and CO_2, CH_4 and H_2O under anaerobic conditions (Béguin and Aubert, 1994; Leschine, 1995; Pérez et al., 2002). Cellulases (endoglucanases and cellobiohydrolases) hydrolyse the β-(1→4) linkages, of cellulose to release cellobiose molecules (Pérez et al., 2002). Endoglucanases (endo-(1→4)-β-glucanases, EGs) hydrolyse internal bonds (usually in non-crystalline regions) releasing new terminal ends whereas cellobiohydrolases (exo-(1→4)-β-glucanases, CBHs) act on existing or endoglucanase-generated chain ends (Pérez et al., 2002). Finally, cellobiose is hydrolysed by β–glucosidase releasing two glucose molecules (Pérez et al., 2002). Products of cellulose hydrolysis are available as carbon and energy sources for

cellulose degrading microorganisms or other organisms living in the environment where cellulose is being degraded (Pérez et al., 2002).

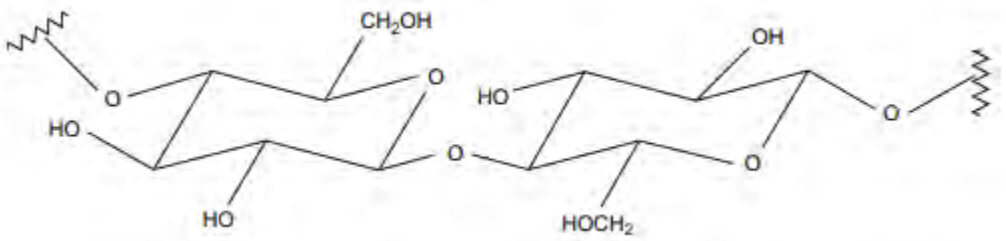


Figure 3.2. Repeating unit of cellulose chain (cellobiose unit) (O'Sullivan, 1997, p. 175).

3.1.3 Hemicelluloses

Hemicellulose is the second most abundant type of polysaccharide in nature, with xylan being the most abundant sugar in this heterogeneous biopolymer (Saha, 2003). Hemicelluloses are low molecular-weight polysaccharides that can be solubilised in an aqueous alkali solution (Timell and Syracuse, 1967). Hemicelluloses do not have a structural role in the cell walls of higher plants rather their role is to fill the voids around cellulose fibrils and provide couplings to the lignins (Figure 3.4), but may influence the aggregation of cellulose during the formation of the cell wall (Atalla et al., 1993). Hemicellulose is composed of a variety of sugar units that have a range of structural linkages i.e. α- or β-(1→2; 1→3; 1→4; 1→6) (Figure 3.3, Pettersen, 1984). Unlike the relatively homogeneous cellulose biopolymer (which is exclusively composed of glucose), hemicelluloses are composed of pentoses (xylose and arabinose), hexoses (mannose, glucose, and galactose) and sugar acids (Saha, 2003). Hemicellulose compositions differ depending on origins; hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans (McMillan, 1993; Saha 2003). Xylans are heteropolysaccharides that have a homopolymeric backbone composed of 1→4-linked β-D-xylopyranose units (Saha 2003). In addition to xylose, xylans may also be composed of arabinose, glucuronic acid, and acetic, ferulic, and *p*-coumaric acids (Saha, 2003).

Unlike the linear cellulose polymers, hemicelluloses have branches with short side chains composed of different sugars that do not form aggregates, even when they are co-crystallised with cellulose chains (Pérez et al., 2002). These short chain polymers are easy to degrade into monomeric sugars and ethanoic acid (Pérez et al.,

2002) but it requires the co-operative action of a variety of hydrolytic enzymes e.g. endo-(1→4)-*β*-xylanase generates oligosaccharides from the cleavage of xylan; and xylan (1→4)-*β*-xylosidase converts xylan oligosaccharides to xylose (Jeffries, 1994) in association with xylan esterases, ferulic and *p*-coumaric esterases, α-L-arabinofuranosidases, and α-4-*O*-methyl glucuronosidases (Pérez et al., 2002).

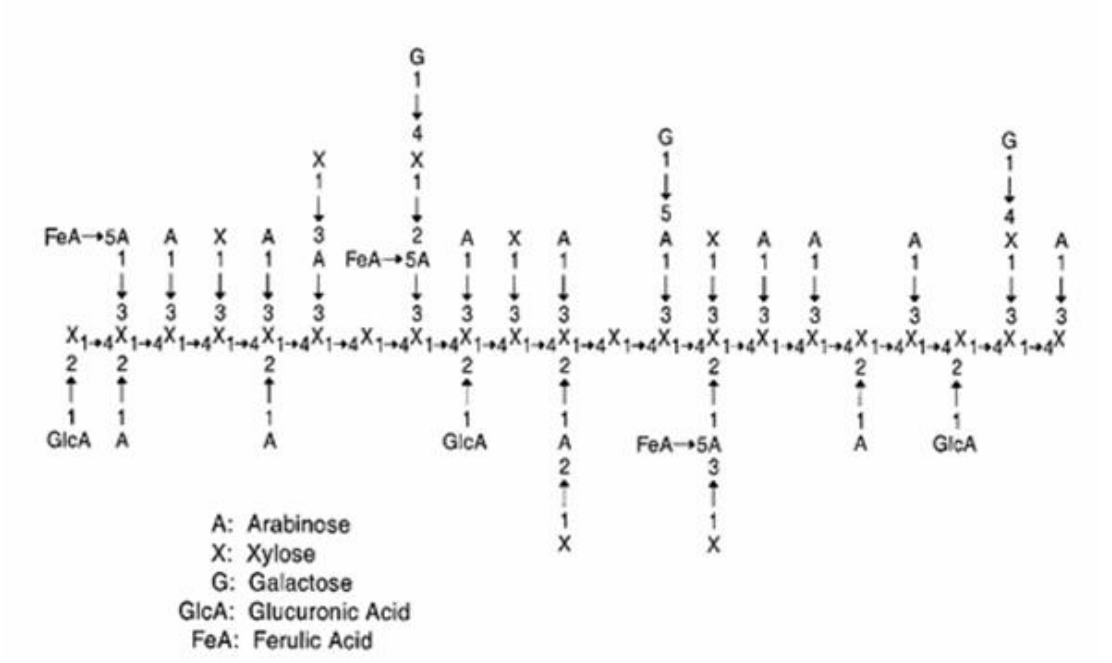


Figure 3.3. Schematic structure of corn fiber heteroxylan (adapted from Saulnier et al., 1995; Saha, 2003 p. 280).

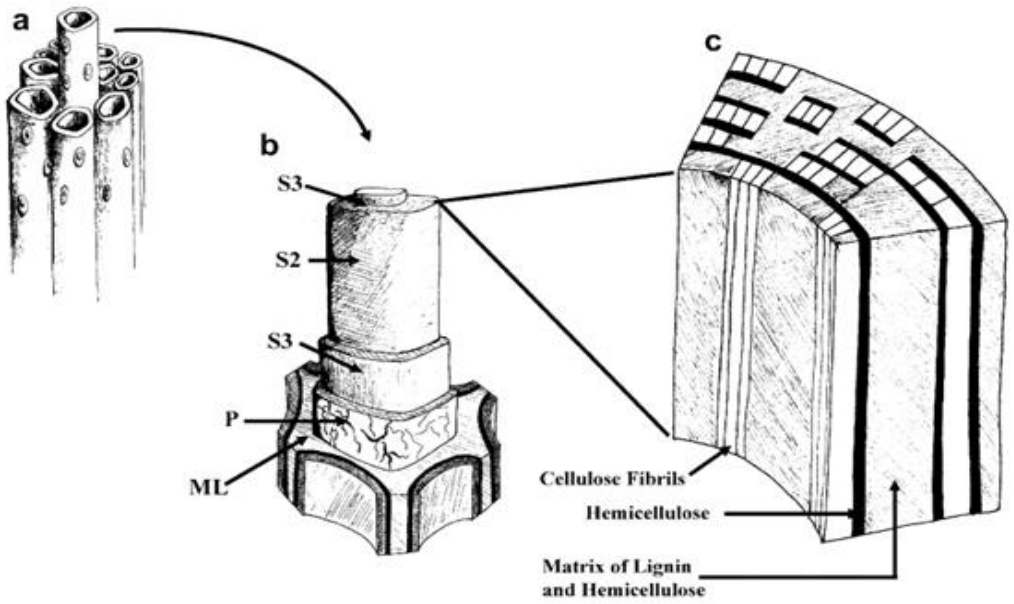


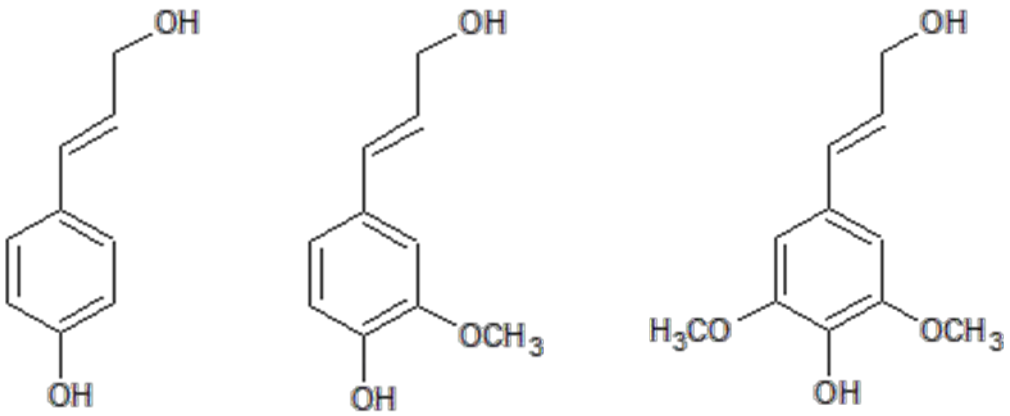
Figure 3.4. Configuration of wood tissues. a adjacent cells; b cell wall layers, S1, S2, S3 secondary cell wall layers, P primary wall, ML middle lamella; c Distribution of lignin, hemicelluloses and cellulose in the secondary wall (adapted from Kirk and Cullen 1998; Pérez et al., 2002, p.54).

3.2 Lignin

Lignin is an aromatic polymer, and along with cellulose, is the most abundant polymer in nature (Pérez et al., 2002). Lignin, and other minor phenolic compounds, account for up to 30% of some secondary cell walls (Scheller and Ulvskov, 2010, Figure 3.4). It has many functions including; structural support, and provision of resistance against microbial attack and against oxidative stress. The hydrophobic nature of lignin shields the polysaccharides from degradation in the cell wall by restricting the access of both enzymes and chemicals (Mansfield, 2009). Lignin, an aqueous insoluble, amorphous heteropolymer, is composed of phenylpropane units joined together by different types of linkages (Figure 3.6, Pérez et al., 2002). The polymer is synthesised by the generation of free radicals, that are released in the peroxide-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol (*p*-hydroxyphenyl propanol), and sinapyl alcohol (syringyl propanol) (Figure 3.5, Sánchez, 2009). Softwood lignins are predominantly composed of coniferyl alcohol, whereas coniferyl and sinapyl alcohols are the major components of hardwood lignins (Pérez et al., 2002). The

units in lignin polymers are linked by C-C and aryl-ether linkages (Pérez et al., 2002, Sánchez, 2009).

The degradation of lignin is very challenging because of its structural complexity, its high molecular-weight, and its insolubility. The degradation of lignin is outlined in detail by Pérez et al. (2002). Enzymes (extra-cellular, oxidative, and unspecific) can liberate highly unstable products (radicals) that undergo many additional oxidative reactions to catalyze the initial steps of lignin depolymerisation (Pérez et al., 2002). White rot fungi are the microorganisms that most efficiently degrade lignin from wood (Pérez et al., 2002). However, bacterially mediated lignin degradation and the presence of lignin-degrading enzymes has been reported for actinobacteria from the *Streptomyces* genus (Berrocal et al., 1997; Pérez et al., 2002). Peroxidases (lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs)) and laccases (blue copper phenoloxidases) are involved in lignin degradation mediated by white-rot fungi (Pérez et al., 2002). MnPs oxidise phenolic compounds and LiPs and laccases can oxidise phenolics and non phenolics (Kirk and Cullen, 1998; Gianfreda et al., 1999; Pérez et al., 2002). Enzymes including cellobiose oxidising enzymes, aryl alcohol oxidase, and aryl alcohol dehydrogenases also have major roles in the degradation of lignin (Cullen, 1997; Pérez et al., 2002).



***p*-coumaryl alcohol** **coniferyl alcohol** **sinapyl alcohol**

Figure 3.5. Structure of the phenyl propionic alcohols involved in lignin biogenesis.

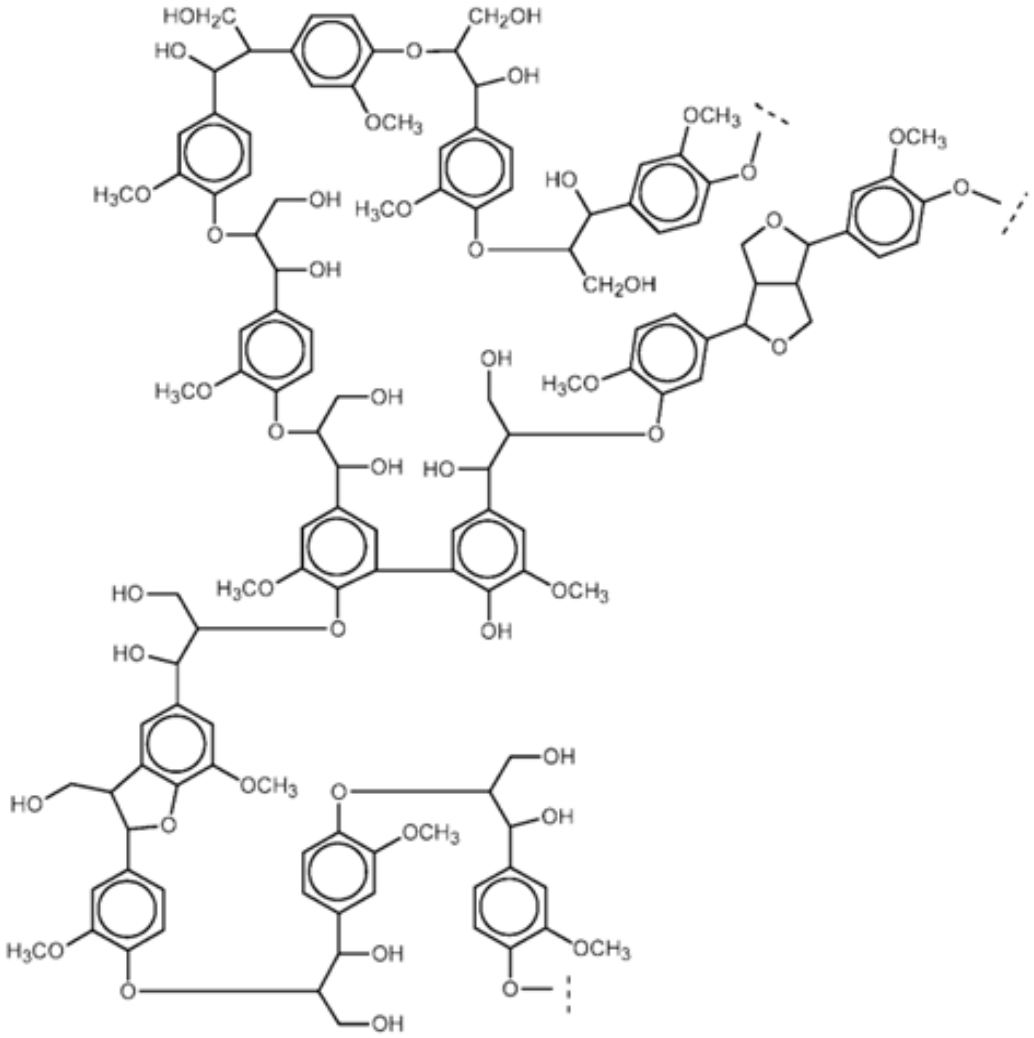


Figure 3.6. Lignin from gymnosperms showing the different linkages between the phenyl-propane units (Pérez et al., 2002, p. 55).

3.3 Proteins and Amino Acids

3.3.1 Amino Acids

There are 20 (standard) amino acids that have endless possible combinations to form a substantial variety of peptides/proteins. The basic structure of all amino acids is the same (Figure 3.7); however, the R′ group varies. The amino acid chains vary from being highly hydrophobic to highly hydrophilic, and with intermediate polarities. The relative hydrophobicity or hydrophilicity of an amino acid is called hydropathy. This is an important determinant of protein-chain folding because hydrophobic side chains tend to be clustered in the interior of a protein whereas

hydrophilic residues are usually found on the surface (Horton et al., 2006). Amino acids in OM can be released by acid hydrolysis followed by separation and identification using HPLC or gas chromatography (Stevenson and Cheng, 1970; Kögel-Knabner, 1995: 2002) or by NMR spectroscopy.

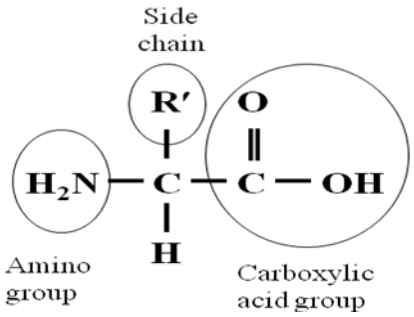


Figure 3.7. General structure of amino acids.

3.3.2 Protein/Peptide

Proteins represent the most versatile biomolecules and they are essential for the function of virtually all biological systems as they are responsible for numerous functions: catalysis; transport and storage of other molecules; mechanical support; immune protection; cellular signalling; transmission of nerve impulses; and the control of growth and differentiation (Berg et al., 2002). Protein polymers are composed of a linear sequence of monomer units (amino acids) that are linked together by a peptide bond (covalent bond between the α-amino group of one amino acid and the α-carboxyl group of another amino acid). Each polymer has a unique monomer sequence that is genetically coded (Hames and Hooper, 2011) and folds into a 3D structure to become biologically active. Proteins posses a wide range of functional groups that include: alcohols; thiols; thioethers; carboxylic acids; carboxamides; and a variety of basic groups (Berg et al., 2002).

Proteins can be studied using X-ray crystallography, ^{1}H, ^{13}C and ^{15}N NMR spectroscopy, and mass spectrometry.

3.3.3 Protein Compositions and Sources in Organic Matter

Nitrogen (N) has a significant influence on the cycling of OM in the biosphere (Knicker and Hatcher, 1997). When N is incorporated into OM, it is moved from the active pool and is no longer available for biological productivity (Knicker and

Hatcher, 1997). It is reasonable to assume that protein in a sedimentary environment would be rapidly re-mineralised by microorganisms; however, it can persist in HSs and HU which suggests it contains recalcitrant species or that it has employed methods of protection from degradation. It was traditionally believed that refractory N was predominantly composed of heterocyclic N- compounds (Flaig et al., 1975; Schnitzer, 1985; Knicker and Hatcher, 1997). Aromatic N-heterocycles are typically found in ancient sediments (Patience et al., 1992; Derenne et al., 1998) and in advanced abiotic condensation products from the reaction of amino acids with carbohydrates (melanoidins; Benzing-Purdie et al., 1983; Hedges et al., 2000). Almendros et al. (1991) demonstrated, using ^{15}N NMR spectroscopy, that a significant amount of N is present in amide form, possibly from protein as amide linkages are difficult to form abiotically (Hedges et al., 2000). Knicker and Hatcher (1997), using both ^{15}N NMR spectroscopy and TMAH(tetramethylammonium hydroxide)-pyGC/MS, reported that refractory N (from an organic rich sediment) is composed primarily of amide-linked nitrogenous substances from protein. They proposed that this protein is protected from degradation by encapsulation within the macromolecular matrix. Derenne et al. (1993) characterised a refractory biopolymer from hydrolysed residues of algae and proposed that it was composed of long-chain alkyl amides. Such a polymer would account for the recalcitrance of protein in the environment. Encapsulation of labile protein into refractory OM can help to explain the fate of labile N in various biogeochemical deposits (Knicker and Hatcher, 1997).

Microorganisms have an important role in the recycling of immense quantities of OM and their cells may be important direct sources of OM (McCarthy et al., 1998, Hedges et al., 2000). It has been suggested by Simpson et al. (2007a) that microbial biomass (predominantly composed of protein, on a dry basis) inputs to OM are underestimated. It was accepted that microbial biomass accounted for ca. 5% of the organic N in soil OM; however research by Simpson et al. (2007a) show that bacterial biomass accounted for ca. 85% of the organic N in the (black, gray and brown) soils studied. Studies show that soil OM contains large contributions from peptidoglycan, lipoprotein, and cellular proteins and peptides (Kelleher and Simpson 2006; Simpson et al., 2007b), all of which suggest microbial origin (Simpson et al., 2007b), in agreement with the amide-N identified

in ^{15}N NMR by numerous researchers. Amide-rich N- constituents of ultrafiltered oceanic DOM yield high ratios of D-alanine, D-serine, D-glutamic acid and D-aspartic acid versus the common L-counterparts (McCarthy et al., 1998), suggesting microbial sources as these D-amino acids are characteristic of peptidoglycan-rich cell walls of eubacteria (Hedges et al., 2000). Algae, and to a lesser extent terrestrial plants are other important sources of protein to the OM compositions. Aquatic plants are a considerable source of protein: phytoplankton contains 24–50% protein (Knicker, 2011); and algae can contain 6-71% protein, depending on species (Becker, 2007).

3.4 Lipids

Lipids are a heterogeneous group of biomolecules that occur both in lower and higher plants and in microorganisms (Kögel-Knabner, 2002); eukaryotic lipids play only a minor role in the compositions of recalcitrant OM. Lipids (fats, oils, resins, waxes) are water insoluble molecules that have a diverse range of functions: acting as an energy store; they are components of membranes; and they can serve as hormones and intracellular second messengers (Hames and Hooper, 2011). Fatty acids are the simplest lipids (CH_3-$(CH_2)_n$-COOH), and these are components of many more complex lipids e.g. waxes, triacylglycerols, glycerophospholipids and sphingolipids (Horton et al., 2006). The relative abundance of particular fatty acids varies with the type of organism; for example branched fatty acids are common components of bacterial membranes (Horton et al., 2006).

Waxes are non-polar polyesters of long chain fatty acids and long chain monohydroxylic alcohols that provide protection in plant cell walls (Horton et al., 2006). Wax esters appear to be minor components of bacteria (Dinel et al., 1990). The main function of waxes is to provide a protective waterproof coatings for plants (some leaves and fruits) and animals (skin, fur and feathers) (Horton et al., 2006).

Lipoproteins are globular particles consisting of a hydrophobic core of triacylglycerols and cholesterol esters surrounded by a coat of protein, phospholipid and cholesterol (in eukaryotes) (Hames and Hooper, 2011). Bacterial lipoproteins are responsible for various important cellular functions, such as biogenesis and maintenance of cell surface structures, and the transport of

substrates (Okuda and Tokuda, 2011). Other lipids include steroids (mainly in eukaryotes and rarely in prokaryotes), vitamins and terpenes, all of which are classified as isoprenoids (Horton et al., 2006).

Lipases are the enzymes involved in the hydrolysis of triacylglycerols to fatty acids (Hames and Hooper, 2011). Availability of oxygen, microbial populations, and the pH of the local environment will affect the decomposition of lipids; for example, in acid soils filamentous fungi and acetomycetes are favoured, whereas in alkaline soils decomposition of lipids is promoted by soil microorganisms that produce lipases, which hydrolyse complex lipid molecules to forms more readily utilised by microorganisms (Dinel et al., 1990).

3.4.1 Sources of Lipids associated with Organic Matter

The main roles of lipids are in the protection and organisations of the cells. Plants have the largest range of lipids in comparison to bacteria and algae (Dinel et al., 1990). Algae are a source of hydrocarbons, wax esters, and primary alcohols (Dinel et al., 1990). The hydrocarbons in algae are a homologous series of medium-chain alkanes ranging from C_{15} to C_{18}, and the distribution of alkanes is affected by abiotic factors, the algal species, and the age of the organism (Dinel et al., 1990). Bacteria produce three main categories of compounds: wax esters, aliphatic hydrocarbons, and extremely complex, high molecular weight, poly-functional compounds (Dinel et al., 1990). The hydrocarbon composition of bacterial lipids may vary in C number distribution and the degree of saturation (Dinel et al., 1990). The lipid components of plants include: hydrocarbons, ketones, primary alcohols, secondary alcohols, and wax esters (Dinel et al., 1990). Leaf waxes are composed of long chain fatty acids (C_{22} to C_{36}) (Huang et al., 1998). Grasset et al. (2009) found (using thermochemolysis) that lipids in clay associated OM were mainly short-chain fatty acids and hydrocarbons that had a microbial origin. Insects are also a source of lipids in the soil environment as their bodies are equipped with cuticular layers of hydrophobic materials to prevent excessive desiccation, and hydrophobic materials constitute over 90% of the cuticular lipids of cockroaches (Dinel et al., 1990).

The large compositions of aliphatic hydrocarbons in HSs and HU play an important role in their long term stabilisation in the environment. The presence of lipids does not conform to the classification of HSs, and their removal should be

regarded as a prerequisite for studies of humic compositions. Some of the lipid components are removable in non-polar solvents; however, it is challenging to remove all of the lipid materials because these can form strong associations with other components in the humic fractions, especially the HAs and HU components (Clapp et al., 2005). Lipids show resistance to decomposition and are preserved for long periods (Oro et al., 1965), as demonstrated by Eglinton et al. (1968) when they isolated hydroxy fatty acids (10,16-dihydroxyhexadecanoic acid and ω-hydroxy acids in the C_{16} to C_{24} range) from a 5,000 year old lacustrine sediment (Dinel et al., 1990). There is clear evidence that lipids have selective resistance to microbial degradation (Dinel et al., 1990). Accumulation of aliphatic compounds in soil OM is enhanced when there is a high input of long chain aliphatic plant biomass, microbial activity, and a low soil pH (Bull et al., 2000; de Assis et al., 2011). Long-chain (> C_{20}) lipids, may be important with regard to C stabilisation and humification processes (de Assis et al., 2011). Resistant long chain aliphatic lipids/waxes will be further discussed in 3.5, 3.6 and 3.7).

Lipids can be analysed using mass spectrometry methods (LC/MS, GC/MS, pyGC/MS, ESI-FTMS) and ^{1}H and ^{13}C NMR spectroscopy. Some methods may require lipid extraction in organic solvents prior to characterisation. Applications of pyGC/MS have significantly advanced our awareness of long chain hydrocarbons, esters, acids, and alcohols from the soil environment. Alkanes, alkenes, fatty acid *n*-alkyl esters, and alkyl aromatics were among the products identified by Schnitzer and Schulten (1995) from soils using pyGC/MS (Clapp et al., 2005).

3.5 Algaenan

There have been numerous studies on algaenan because it is of great geochemical importance (Gelin et al., 1999; Largeau et al., 1984; 1986) because of its long term persistence in the environment thus representing an important biomolecule involved in the sequestration of C. The algaenans are protective tissues in algae, but their exact physiological function has not been elucidated (Blokker et al., 2006). These resistant biomolecules are ubiquitous in freshwater green algae (de Leeuw and Largeau, 1993; Ogawa and Tanoue, 2003), and they can also be synthesised by cyanobacteria and by marine algae (Largeau, 1995; Gelin et al., 1996, 1999; Ogawa and Tanoue, 2003). Algaenans present in sediments originate

not only from dead cell material; when algae reproduce the parental cell wall is released as a waste product that is predominantly composed of the resistant biopolymer (Blokker et al., 1998b). Algaenan has been described as an aliphatic, insoluble, and chemically resistant biopolymer (Tegelaar et al., 1989a) that is a major component of the outer cell wall in *Botryococcus braunii* (*B. braunii*; Berkaloff et al., 1983; Blokker et al., 1998a, 2000; Derenne et al., 1989; Gatellier et al., 1993; Gelin et al., 1994; Kadouri et al., 1988; Simpson et al., 2003). Algaenan, despite comprising of only a few percent of the total biomass of the algae, appears to be selectively preserved upon degradation, and becomes a major constituent in kerogen (Largeau et al., 1984, 1986; Goth et al., 1988; Derenne et al., 1991; Gillaizeau et al., 1996). Studies by Simpson et al. (2003) found that the hexane insoluble botryals (α-branched aldehydes originating from aldol condensation) isolated from *B. braunii* are composed of a mixture of low molecular weight unsaturated aliphatic aldehydes and unsaturated hydrocarbons with an average chain length of about 40 carbons. There is clear evidence for the selective preservation of algaenan, for example, studies by Nguyen et al. (2003) showed that algaenan (from *B. braunii*) is non-hydrolysable and insoluble, which accounts for the slower rate of OM degradation compared to diatoms (predominantly silica based cell walls; Kröger et al., 1994), dinoflagellates (principally composed of cellulose; Brock and Madigan, 1984) and cyanobacterium (predominantly composed of peptidoglycan and lipopolysccharides; Brock and Madigan, 1984). Zang et al. (2001) used 2D ^{13}C-^{15}N NMR spectroscopy to study degraded *B. braunii* and the spectra indicated that algaenans may be involved in the protection of peptide bonds from enzymatic attack (Nguyen et al., 2003).

Further research on algaenans is of great importance as the elucidation of the chemical structure of algaenans will provide a greater understanding of C sequestered in sediments. Spectroscopic methods like FTIR and solid-state ^{13}C NMR provide information concerning the nature and functional groups of the algaenan biopolymer, but the finer details of the chemical structure of the building blocks cannot be elucidated (Blokker et al., 1998b). The use of chemical degradation methods to release the monomeric units of algaenan is very difficult because of the chemical stability of the ether bonds (Blokker et al., 1998b).

pyGC/MS has proved to be a more successful method to provide such information; however, thermal rearrangement reactions can result in the loss of functional group information (Blokker et al., 1998b). Chemical reagents such as hydrogen iodide (HI) and ruthenium tetroxide (RuO_4) are used to cleave ether-linkages (Panganamala et al., 1971; Sharpless et al., 1981; Amblés et al., 1996) and have been successful in revealing the chemical structure of such biopolymers (Gelin et al., 1997; Schouten et al., 1998; Blokker et al., 1998b). Ruthenium tetroxide oxidation coupled with GC/MS has shown that the building blocks of algaenans (from both *B. braunii* and *T. minimum*) are α,ω-dicarboxylic acids with either ester or ether bonds that link the these hydroxyacids to each other; double bonds may be biochemically oxidised to form ether bonds which in turn will cross-link the polymer (Figure 3.8) (Schouten et al., 1998).

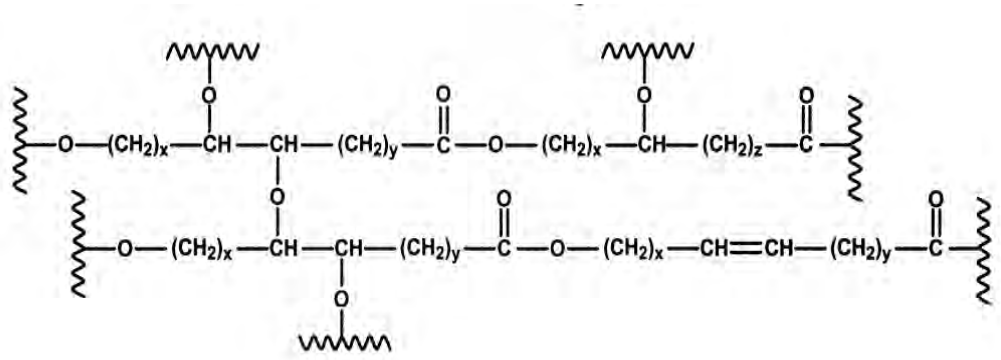


Figure 3.8. Proposed simplified structure of algaenans (based on analyses of *T. minimum, S. communis* and *P. boryanum*) consisting of linear polyester chains cross-lined via ether bonds (Blokker et al., 1998b, p. 1465).

3.6 Cutin/Cutan

Plant cuticles are synthesised and secreted by the epidermis during plant development (Kunst et al., 2005). Cutin and cutan are the most notable polymeric lipid components found in plant cuticles (Deshmukh et al., 2003). Cutin is described as the component that can be solubilised upon saponification treatment, whereas cutan is a non-saponifiable and a non-extractable component; their function is to provide the plant with a protective barrier from the external environment, as well as separating different organs of the plant (Kolattukudy, 1980; Deshmukh et al., 2003).

Cutin can account for 40% to 80% of cuticular weight in plant organs (Holloway, 1982; Kunst et al., 2005). Cutin is composed of oxygenated fatty acids; 16 or 18 carbon ω-hydroxyacids, with hydroxyl or epoxy groups in the mid chain positions (Kunst et al., 2005). Embedded in the cutin matrix are cuticular waxes (very-long-chain fatty acids derivatives) that are readily removed using non-polar solvents (Kunst et al., 2005). These waxes are arranged into an intracuticular layer in close association with the cutin matrix, and there is an epicuticular film exterior to this (Kunst et al., 2005). Cutin forms a three-dimensional network, formed by extensive ester cross-linking of the monomeric species (Kolattukudy, 1984), that is associated with polysaccharides and intracellular wax, as well as small amounts of phenolic compounds, such as *p*-coumarate and ferulate (Holloway, 1982; Kunst et al., 2005). In addition to ester functionalities, Deshmukh et al. (2005) observed epoxy groups, free primary alcohols and carboxylic acid groups, as well as evidence for α-branched fatty acids/esters in cutin. The α-branched carboxylic acids offer opportunities for cross-linking, and explain the presence of amorphous chains in the cutin/cutan mixture (Deshmukh et al., 2005). Dihydroxypalmitic acids are the most abundant monomers of the C_{16} fatty acids (one hydroxyl group at the ω-carbon and the other hydroxyl positioned at a mid chain carbon), whereas C_{18} cutin monomers are oleic acid, ω-hydroxyoleic acid, 9,10,18-trihydroxystearic acid and 9,10-epoxy-hydroxystearic acid, as identified by GC/MS following ester breaking reactions (Walton and Kolattukudy, 1972; Kolattukudy, 1980; Kunst et al., 2005). Early models depict cutin monomers linked head to tail via their primary functional groups in a linear manner, and partially cross linked through their secondary hydroxyl groups (Kunst et al., 2005). More recently glycerol has been reported to be a major constituents of cutin accounting for up to 14% of the total monomers (Moire et al., 1999; Graça et al., 2002; Kunst et al., 2005). Degradative studies of plant cuticles are invasive and, only allow assumptions to be made about cross-linking and other connectivities between monomers (Deshmukh et al. 2003). Solution-state NMR experiments are challenging because of the insolubility of the cuticular biopolymers; but solid-state NMR has been successful in characterising intact cutin from lime (Zlotnik-Mazori and Stark, 1988; Pacchiano et al., 1993; Fang et al., 2001) which confirms that aliphatic, alkene, aromatic, keto, and ester functionalities contribute to the compositions of

cutin (Zlotnik- Mazori and Stark, 1988; Deshmukh et al., 2003). To provide evidence for presence of cross-linking, Deshmukh et al. (2003) used 1- and 2-D HR-MAS NMR coupled with a 1-D CPMAS and Bloch decay NMR to characterise the molecular architecture of cutin isolated from tomato fruit cuticle.

Cutan, the resistant residue remaining after depolymerisation of cutin, is only partially understood (Deshmukh et al., 2005). Deshmukh et al. (2005) used both ^{1}H and ^{13}C NMR spectroscopy (chemical shift assignments are presented in Table 3.1), to provide insights into the structure and composition of cutan. They describe cutan (from *Agave americana* leaf) as being mainly composed of free primary hydroxyls and long-chain carboxylic acids that are proposed to form ester linkages with trihydroxylated benzene units. They also identified benzenecarboxylic acids that form ester links with fatty alcohols in cutan. The long-chain polymethylenic groups in cutan are shown to express a high degree of crystallinity similar to that observed in OM from soils, sediments, and fossil materials (Nip et al., 1986a,b; Tegelaar et al., 1991, 1995; Collinson et al., 1994; van Bergen et al., 1994; Deshmukh et al., 2005). pyGC/MS studies of *A. americana* cutan identified a homologous series of *n*-alkanes, *n*-alk-1-enes, and α,ω-alkadienes (Nip et al., 1986a,b; Tegelaar et al., 1989b), attributed to the thermal degradation of the polymethylenic chains (Deshmukh et al., 2005). Thermochemolysis (using TMAH) of *A. americana* cutan yields fatty acid methyl esters (FAMEs, C_{15}–C_{31}, with a high concentration of C_{27}–C_{31}), as well as a number of 1,3,5-trimethoxylated benzene derivatives and benzene carboxylic acid derivatives (McKinney et al., 1996; Deshmukh et al., 2005). The ^{13}C CPMAS NMR data and the pyGC/MS data have been combined to propose a structure for cutan; a backbone of 1,3,5-trihydroxylated aromatic rings (Deshmukh et al., 2005) with the hydroxyl groups forming ester linkages with fatty acids (McKinney et al., 1996; Deshmukh et al., 2005). The structure of cutan (Figure 3.9) proposed by Deshmukh et al. (2005) has an aromatic backbone with carboxylic acid groups, and aryl-OH groups. These groups can form ester linkages with long-chain alcohols, and long-chain carboxylic acids. The proposed structure incorporates units that include α-branched carboxylic acids, esterified secondary alcohols, free primary alcohols, and olefinic linkages.

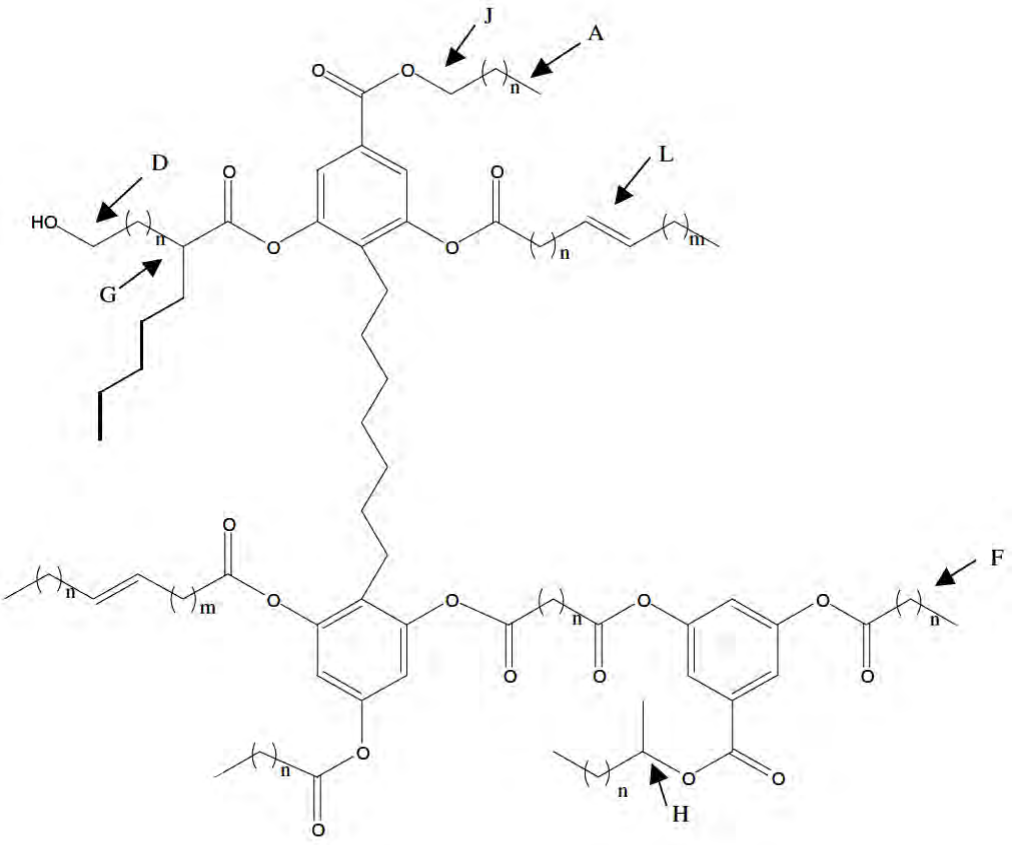
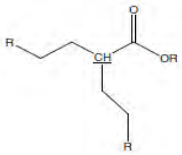
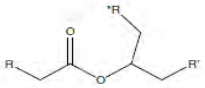
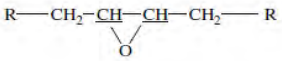


Figure 3.9. Structure proposed by Deshmukh et al. (2005) for *Agave americana* cutan, showing various types of functional units (A-L, listed in Table 3.1). *n* varies from 25-32 based on the work by McKinney et al. (1996) and Schouten et al. (1998). Values for m are at least 6 to allow sufficient remoteness from the carbonyl group to display a chemical shift assignable to L structures. Values of m + n are less than 31 (Deshmukh et al., 2005, p.1083).

Table 3.1. Chemical shifts of resonances identified in *Agave americana* cutan and a cutin/cutan mixture (Deshmukh et al. 2005, p. 1075).

Symbol	Type	^{1}H shift (ppm)	^{13}C shift (ppm)
A_1	$\underline{CH_3}$-CH_2-CH_2-CH_2-R	0.8	14
A_2	CH_3-CH_2-$\underline{CH_2}$-CH_2-R	1.3	30
B_1	R-CH_2-CH_2-CH_2-CO-O-$\underline{CH_2}$-CH_2-CH_2-R	4.0	67
B_2	R-CH_2-CH_2-CH_2-CO-O-CH_2-$\underline{CH_2}$-CH_2-R	1.5	30
B_3	R-CH_2-CH_2-CH_2-CO-O-CH_2-CH_2-$\underline{CH_2}$-R	1.2	26
C_1	R-CH_2-CH_2-$\underline{CH_2}$-CO-O-CH_2-CH_2-CH_2-R	2.3	34
C_2	R-CH_2-$\underline{CH_2}$-CH_2-CO-O-CH_2-CH_2-CH_2-R	1.7	22
D_1	R-CH_2-CH_2-$\underline{CH_2}$-OH	3.3	63
D_2	R-CH_2-$\underline{CH_2}$-CH_2-OH	1.4	34
D_3	R-$\underline{CH_2}$-CH_2-CH_2-OH	1.3	26
F_1	R-CH_2-$\underline{CH_2}$-CO_2H	2.2	36
F_2	R-$\underline{CH_2}$-CH_2-CO_2H	1.6	25
G		2.4	45
H		4.8	74
J	Ar-CO-O-$\underline{CH_2}$—CH_2-R	4.2	67
K		2.7	56
L	R-CH_2-$\underline{CH}$=$\underline{CH}$-CH_2-R	5.25	130

3.7 Suberin/Suberan

Along with cutin, plants synthesise a second type of insoluble lipid polyester polymer derived from fatty acids, named suberin (Molina et al., 2006). The function of suberins is to control the movement of water and solutes and to provide strength to the cell wall (Nawrath, 2002; Molina et al., 2006). Desuberisation can result in the collapse of the cell walls (Rocha et al., 2001). Suberin, a major component of the outer bark cell walls, is organised in a characteristic lamellar structure (Gandini et al., 2006). Suberin does not have a distinct chemical structure as its compositions can vary (Gandini et al., 2006). The monomer unit composition of suberin has been determined for many plant species; but a detailed macromolecular structure, and how it associates with other cell wall biopolymers has not been elucidated to date (Gandini et al., 2006). Suberin is composed of both aliphatic and aromatic species (Holloway, 1984; Bernards et al., 1995; Kolattukudy, 2001; Bernards, 2002); the aliphatic components are mostly C_{16} to C_{28} ω-hydroxy fatty acids and C_{16} to C_{26} α,ω-dioic acids (these are diagnostic for suberin) with little mid-chain oxygen functionality, and the aromatic component is a "*hydroxycinnamate derived polymer,*

primarily comprised of ferulic acid, N-feruloyltyramine, cinnamic acid, p-coumaric acid or caffeic acid" (Bernards et al., 1995; Molina et al., 2006). Glycerol has been shown to be an important component of suberin (Bernards 1998; Graça and Pereira 1997, 1999, 2000a,b, 2002; Gandini et al., 2006) because it can account for ca. 20% (by weight) of suberin in oak, cotton and potato (Moire et al., 1999; Graça and Pereira, 2000b,c; Molina et al., 2006). The most recent model for the suberin macromolecule (based on suberised potato cell walls) was proposed by Bernards (2002) (Figure 3.10). Solid-state NMR studies have shown that there are two distinct aromatic domains in suberised cell walls; the first aromatic domain is located inside the aliphatic domains, and is mainly composed of hydroxycinnamates esterified with glycerol or ω-hydroxyfatty acids; and the second aromatic domain is a lignin-like polymer that is composed of cross-linked hydroxycinnamic acid type molecules that are covalently bonded to the aliphatic suberin (Sitte, 1962; Pascoal Neto et al., 1996; Lopes et al., 2000; Gandini et al., 2006). It is proposed that ether or ester bonds may form between suberin and polysaccharides (Sitte, 1962; Lopes et al., 2000; Yan and Stark 2000; Gandini et al., 2006). Studies of suberin by Rocha et al. (2001) identified both rigid and mobile aliphatic methylene species in suberin. Crystalline aliphatic suberin has been identified in *Q. suber* cork (Lopes et al., 2000; Bernards, 2002; Gandini et al., 2006).

Difficulties arise in estimating the suberin content because of its complex macromolecular structure, and because of the similarity of the aromatic domains in suberin and lignin (Kolattukudy, 1980; Kolattukudy and Espelie, 1989; Bernards, 1998; Kolattukudy, 2001; Bernards, 2002; Gandini et al., 2006). In order to study suberin there must be a solvent extraction step to isolate the low molecular weight components, followed by the chemical scission of the ester bonds in the macromolecule (Kolattukudy and Espelie, 1989; Gandini et al., 2006). Enzymes can be used to cleave bonds between polysaccharides and aliphatic suberin to isolate polymeric suberin (Rocha et al., 2001). The isolated suberin polymers/fragments can be qualitatively and quantitatively characterised using GC/MS, FTIR and NMR spectroscopy.

Suberan, a non-hydrolysable biopolymer, is ubiquitous in periderm tissue of higher plants (Ogawa and Tanoue, 2003). Unlike suberin, suberan is not saponifiable. Turner et al. (2013) describe suberan as a residue remaining after the

saponification of suberin. Suberans (and cutans) are suggested to be responsible for the enrichment of aliphatic compounds in soils (Tegelaar et al., 1989c, Augris et al., 1998). Therefore, the elucidation of the chemical nature of suberan is important in order to understanding diagenetic and humification processes, especially because below ground plant biomass and the bark layer of trees make significant contributions to both soil and estuarine/coastal sedimentary OM (Turner et al., 2013).

Tegelaar et al. (1995) using pyGC/MS showed suberan to be composed of a homologous series of *n*-alkanes, *n*-alk-1-enes and α,ω-alkadienes that were mostly C_{20} to C_{22} in chain length but ranged from C_6-C_{37}. Suberan retains ester functionality following saponification and this is attributed to the hydrophobic nature of the material (due to the polymethylene compositions) that provides some protection from extensive alkaline hydrolysis (McKinney et al., 1996; Schouten et al., 1998; Deshmukh et al., 2005; Turner et al., 2013). The data obtained by Turner et al. (2013) for suberan indicate that the chain length, the dominant ester functionality, and functional groups present are all similar features observed for suberin; however, suberan is structurally distinct. There is a possibility that suberan could be formed as as an artefact during the isolation of suberin; alternatively, it may be a distinct polymer that co-exists with suberin (Turner et al., 2013).

The crystallinity of suberan and its potential recalcitrance to further saponification are of importance in relation to the humification of OM. Soil OM has polymethylenic structures with highly ordered structures (Hu et al., 2000) and it is probable that the polymethylenic components derive partly from suberan-like units (Turner et al., 2013). The persistence of suberan structures in OM may be due to their resistance to hydrolytic processes (Turner et al., 2013).

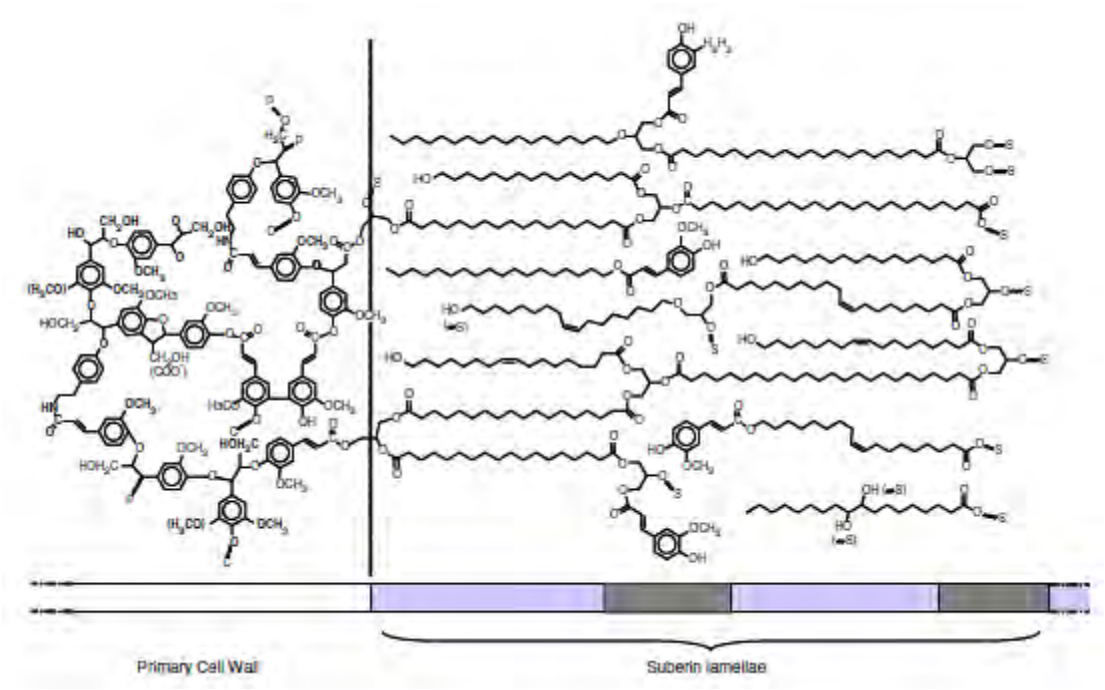


Figure 3.10. The suberin model proposed by Bernards (2002); C: carbohydrate, P: phenolics, S: suberin (Gandini et al., 2006, p. 881).

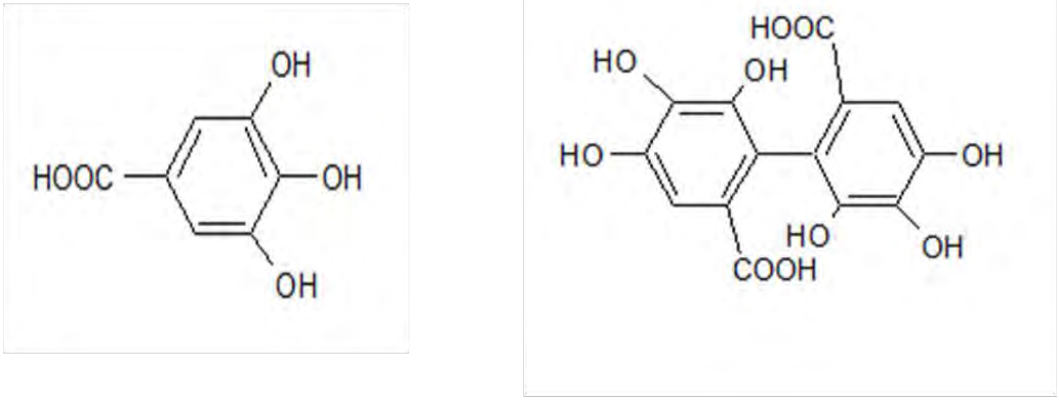
3.8 Tannins

Tannins are ubiquitous in the environment as they are components of many different families of higher plants. The chemistry of tannins can vary widely depending on the species they originate from, and nearly every part of the plant has high tannin concentrations (Khanbabaee and van Ree, 2001). An increased production of tannins can be associated with poor health; therefore, tannins must have a biological role in protection against infection, insects, or animal herbivory (Haslam, 1989; Porter, 1989). Tannins are polyphenolic secondary metabolites that are exclusive to higher plants (Khanbabaee and van Ree, 2001). These compounds are identifiable by their strange smell and astringent taste, and they appear as either a pale yellow/white amorphous powder, or as a shiny, uncoloured, loose mass (Falbe and Regitz, 1995; Khanbabaee and van Ree, 2001). The structure of a large number of tannins (>1,000) from natural products has been determined (Quideau and Feldman, 1996) and the nomenclature of these compounds can be misleading (Haslam, 1989; Khanbabaee and van Ree, 2001). There have been a number of definitions of tannins (e.g. Bate-Smith and Swain, 1962; Haslam, 1989; Griffiths, 1991) but these definitions are not sufficiently comprehensive for these diverse compounds. Khanbabaee and van Ree (2001) provide an updated definition of compounds that can be classified as tannins:

they "…*are polyphenolic secondary metabolites of higher plants, and are either galloyl esters and their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol-, catechin- and triterpenoid cores (gallotannins, ellagitannins and complex tannins), or they are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns (condensed tannins)"*. Hydrolysable tannins are mainly composed of sugar (mostly D-glucose) and phenolic acids (Kögel-Knabner, 2002). This group of tannins can be differentiated into: (a), gallotannins (composed of a central sugar unit esterified with gallic acid); and (b), ellagitannin (composed of a sugar unit esterified with ellagic acid) (Figure 3.11) (Kögel-Knabner, 2002). Condensed tannins (Figure 3.12) are mainly composed of polyhydroxy-flavan-3-ol with C-C bonds between C-4 and C-8 and between C-4 and C-6 (Kögel-Knabner, 2002). Condensed tannins are the most heterogeneous because of the variety of functional groups possible (Kögel-Knabner, 2002).

Hydrolysable tannins (as their name suggests) can be split hydrolytically (using hot water or tannases) into their components, whereas condensed tannins are much more difficult to split due to their structure (Khanbabaee and van Ree, 2001). It is difficult to quantify tannins due to the complexity and the diversity of their chemical compositions. In nature, it is reasonable to assume that hydrolysable tannins are decomposed more rapidly than condensed tannins (Kögel-Knabner, 2002). Lignin and tannin compounds are the most likely source of phenolic compounds in HSs and HU. Colourmetric methods are not reliable for tannin identification/quantification as many of these compounds may not be extractable or there may be slight structural modifications that will change their reactivity with the colour reagent (Kögel-Knabner, 2002). Tannins can be identified using ^{13}C NMR spectroscopy by their chemical shift at 150 ppm (O-aryl); however, this resonance is also indicative of lignin. Therefore, dipolar dephasing is required to identify a resonance ca. 105 ppm (quarternary carbons) that is indicative of tannins (Wilson 1987; Skjemstad et al. 2008). Almendros et al. (2000) studied the composting of forest and shrub biomass and concluded that the apparent stability of the aromatic domain during composting is compatible with selective preservation of tannins together with aliphatic structures. The ^{13}C NMR spectra from that study also

indicated that the selective preservation of tannins may control the decomposition of other plant macromolecules.



gallic acid **ellagic acid**

Figure 3.11. Structural units of hydrolysable tannins: gallic acid; and ellagic acid.

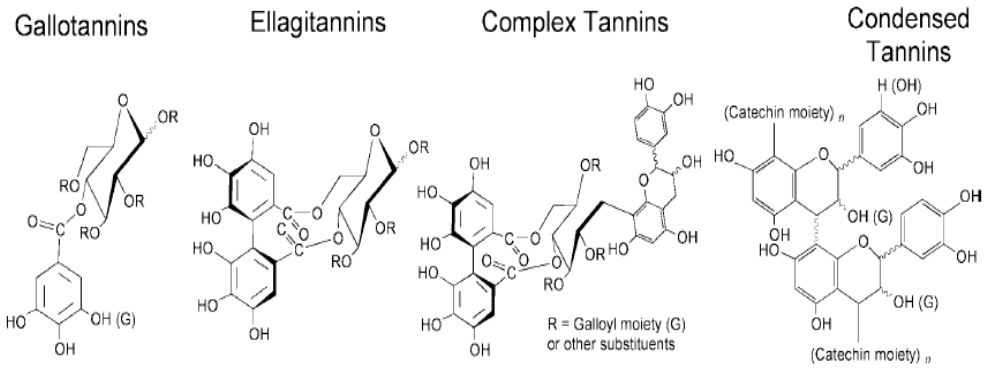


Figure 3.12. Classification of tannins (Khanbabaee and van Ree, 2001, p. 643)

3.9 Conclusions

It is evident that the structures and chemical compositions of OM molecules are important in relation to their long term persistence in the environment. OM from different organisms is characterised by different degrees of chemical reactivity; algal biomass is more degradable relative to terrestrial biomass, and some algal groups are selectively preserved relative to others in the fossil record (Goth et al., 1988; Arndt et al., 2013). The selective preservation of recalcitrant OM at the expense of labile components is perhaps one of the main reasons that OM becomes less degradable with burial depth (Arndt et al., 2013). Terrestrial OM is relatively more refractory than aquatic OM (Hedges et al., 2000), because the chemical composition of terrestrial OM is dominated by moderately resistant (e.g. cellulose, lignin and cutin) or highly resistant (e.g. cutan) biopolymers (de Leeuw and Largeau, 1993); however, biomolecules such as algaenan are also difficult to degrade in the environment. Alternatively, the refractory nature of terrestrial OM could reflect the extensive degradative modifications that have occurred during burial in soils and subsequent transport compared to the relatively fresher aquatic OM. Both mechanisms assume that terrestrial OM has a lower degradability fundamentally due to its chemical composition. However, protection mechanisms (from associations with minerals or with other OM) could also be involved in the lower degradability of the terrestrial OM (Arndt et al., 2013).

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Chapter 4 Extraction and Fractionation of Humic Substances associated with Sediments

There is no standard method for the extraction of humic substances (HSs) and humin (HU) from soils or sediments. Many solvents are capable of solubilising HSs, but the choice of solvent will have an impact on the components isolated and the quantity of HSs recovered. The method used by the International Humic Substances Society (IHSS, Swift, 1996) (http://www.humicsubstances.org/) is well documented and scientists have utilised this method as a standard approach. However, this method recovers only HAs and FAs, and it does not completely remove the organic components which are soluble in aqueous solvents. The use of chemical procedures to isolate OM is controversial as some scientists consider that the chemicals alter the organic compositions resulting in the formation of artefacts. However, it is very difficult to get an awareness of the components of the OM without chemical extraction because of the heterogeneity of OM and the variances in compositions found in different geographical locations and in different environments. It is more amenable to study the compositions and structures of HSs when these have been isolated from the inorganic matrix. As analytical techniques continue to improve, it may be feasible to study OM in its natural state with no chemical interference (a holistic approach). However, until analytical techniques are refined for this approach, it is best to isolate different fractions of the OM for characterisation studies. The classifications and definitions of HSs, regardless of the phase of the sample (dissolved or gel/solid), are operationally based on the procedure used for their isolation, and no ideal system is currently available (Peuravuori et al., 2005). A solvent system has been developed by the Carbolea Research Group (University of Limerick), that provides a level of fractionation, a maximum recovery of organic components, and minimal creation of artefacts (Hayes, 2006). This solvent system has been successfully applied to both soils. The Carbolea methodologies can also extract HU components, a fraction that has traditionally been described as insoluble and difficult to isolate. The main focus of this chapter will be on the extraction and fractionation of HSs, on the extraction of HU and on the enrichment of the insoluble HU.

4.1 Extraction of Humic Substances

Based on the literature review, it is evident that the majority of the research to date has involved HSs extracted from soils. As a consequence, most of the extraction methods have been developed for soils. Fortunately, these methods can be applied to the extraction of HSs from sediments because these rely on the same principles. The extraction process seeks to isolate the organic components (HSs) from the inorganic components (clay, silt, sand). The isolates can then be characterised using a number of analytical approaches. According to Dubach and Mehta (1963) and Stevenson (1982) an ideal extraction method should meet the following criteria:

(1) the method should isolate unaltered material;
(2) the isolates should be free from inorganic components (e.g. clay);
(3) extraction should solubilise fractions from the entire molecular-weight range; and
(4) the method should be universally applicable.

In order to isolate OM, the solvent employed must be able to disrupt the forces that hold the molecules in their associations. These forces include: interparticle forces (e.g. multipole interaction); induction forces; dipole-induced dipole interactions; van der Waals forces; and hydrogen bond interactions (Clapp et al., 2005). Whitehead and Tinsley (1964) proposed that effective solvents for the isolation of HSs should comply with the following guidelines (Clapp et al., 2005):

(1) a high polarity and a high dielectric constant to disperse the charged molecules;
(2) a small molecular size to penetrate into the humic structures;
(3) the ability to disrupt the existing hydrogen bonds and to provide alternative groups to form humic-solvent hydrogen bonds; and
(4) the ability to immobilise metallic cations.

HSs are highly charged, mainly due to the presence of carboxyl and phenolic hydroxyl functional groups. HSs are sparingly soluble in water due to charge-neutralising hydrogen ions and divalent and polyvalent cations. These ions remain undissociated, or only weakly dissociated from the anionic functional groups, and a

cross-linking effect can occur from inter- and intra-strand hydrogen bonding, and from bridging in the cases of the cations (Clapp et al., 2005). The (strong) bridges formed between the polyvalent cations of the humic molecules and the inorganic colloidal components are important as they can increase the difficulty of achieving solubilisation (Clapp et al., 2005). To date most studies have used aqueous solvents; however, organic solvents have also been employed.

4.2 Aqueous Solvent Extraction

4.2.1 Alkaline Extraction

Alkaline extractions have proved most successful and are widely used since Achard (1786) used KOH to extract humic acid (HA) from peat. Following the successes by Achard, the classical methods for the isolation of OM components use aqueous base or neutral salt solutions, and combinations of aqueous base and pyrophosphate (Hayes, 2006). Repeated extractions are often necessary to obtain maximum recovery of the HSs. The first comprehensive study of the origin and chemical nature of HSs was carried out by Sprengel (1826) and from this research he introduced the operational definitions of soil OM based on their solubilities in aqueous media (Sprengel, 1837; Stevenson, 1994; Hayes, 2006).

Water can efficiently solvate HSs but to do so the acid groups of the humic molecules must be dissociated; the structures expand to allow solvation of the conjugate bases of the acidis groups and the polar moieties in the structures (Hayes, 1985). The strength of intermolecular hydrogen bonding in water inhibits its capability to form the required hydrogen bonds with the H^+–exchanged HAs (Hayes, 2006). Solvation of HSs is best achieved using aqueous alkaline solutions (Hayes, 1985).

The majority of HSs in soils and sediments are in aqueous insoluble forms. Therefore, the method of extraction must take into account the various ways in which OM is retained: (1), as macromolecular complexes; (2), as macromolecular complexes bound together by cations (Ca^{2+}, Fe^{3+}, Al^{3+}); and (3), in associations with clay minerals held together through bridging facilitated by polyvalent cations, and H-bonding (Stevenson, 1994). In the soil environment, soil colloids are considered to be composed of associations of clays, various hydroxides (especially Fe and Al), and humus material (mostly composed of HSs) and carbohydrate (Hayes, 1985). Calcium

and other polyvalent cations hold the OM in a flocculated and insoluble state; therefore OM solubilisation can be achieved using a medium that contains Na^+, K^+, or NH^+ ions (Stevenson, 1994). Reagents such as NaOH, $Na_4P_2O_7$, and KOH have been successful for the extraction of HSs. These salt solutions can form complexes with the polyvalent metals that neutralise the charges on HSs, and the polyvalent ions are replaced by sodium ions from the salt. The efficiency of each medium will depend on the extent to which the resident cations are exchanged and removed from the HSs (Hayes, 1985).

There are many examples for the use of neutral salt solutions e.g. $Na_4P_2O_7$ (Gerzabek et al., 1992, 1997; Novotny et al., 1999) as milder reagents for extracting HAs (Tatzber et al., 2007). $Na_4P_2O_7$ is a chelating agent that can solubilise OM which is precipitated with metallic elements. Neutral salt solutions have had some success (ca. 30% of soil OM) in the isolation of HSs, especially highly oxidised or transformed components, but weakly dissociated acidic functional groups are not ionized at neutral pH values (Hayes, 2006). Therefore, neutral salts are not suitable reagents for isolating OM from sediments, as the pH of the water in these environments is generally ca. pH 8, and humic components soluble at neutral pH values would remain in solution rather than in association with the sediments. Additionally, extraction of HSs using $Na_4P_2O_7$ can form artefacts due to incorporation of orthophosphate (PO^{3-}_4) in the low MW fractions, and pyrophosphate ($P_2O^{4-}_7$) in the high MW fractions (Francioso et al., 1998; Tatzber et al., 2007).

HSs are soluble in alkaline media because bonds holding organic material to inorganic components are disrupted, and acidic groups are converted to their soluble salt forms (salts of di- and trivalent cations are insoluble; Stevenson, 1994). Pre-treatment with dilute HCl (hydrochloric acid) prior to alkaline extraction increases the extraction efficiency by the removal of cationic species (Stevenson, 1982); however, the most soluble components of the HSs may be lost unless the filtrate is recovered.

Various concentrations of NaOH have been reported in the literature as suitable solvents for the extraction of HSs (e.g. 1 M, 0.5 M, 0.1 M) from coal, soil, and sediment (Song et al., 2008; Prentice and Webb, 2010; Orecchio and Mannino, 2010). The concentration of NaOH will impact the yield of the HSs extracted and the ash content of the isolates (Schnitzer and Khan, 1978; Tatzber et al., 2007). Aqueous

alkaline media cause the protons of the HSs in H^+ exchanged systems to dissociate, thus enabling water molecules to cluster around the negatively charged conjugate bases and results in the structures dissolving (Hayes, 2006). Media at a high pH facilitates full dissociation of the acidic groups in the molecules and the structure is fully expanded (swollen) due to the repulsion of charges. Oxidation reactions can occur at high pH values. Highly alkaline media and lengthy extraction times cause greater chemical changes (Bremner, 1950; Stevenson, 1994). Bremner (1950) showed that the uptake of oxygen by soil OM was influenced by the pH of the extract media (Hayes, 2006). The oxygen uptake is 18 times higher for a solution of 0.5 M NaOH than for the same system adjusted to pH 10.5, and oxygen uptake is negligible in a pyrophosphate solution at pH 7 (Hayes, 2006). Oxidation can be limited when extractions are carried out in an atmosphere of N_2 gas (Choudhri and Stevenson, 1957; Hayes, 2006), and by reducing the pH of the media following the extraction process.

The method used by the IHSS for the extraction of standard and reference samples involves pre-treatment with HCl to remove Ca^{2+} and polyvalent cations, followed by extraction with 0.1 M NaOH (Swift, 1996). This method only employs one extraction with the alkaline solvent; therefore, significant quantities of the OM will not be recovered. In order to achieve a level of fractionation of the organic components in soil, Hayes et al. (1996), Song et al. (2008; 2011), and Chang et al. (2014) employed extraction methods using aqueous media of increasing alkalinity. Hayes et al. (1996) used the following solvent sequence: (1), 0.1 M $Na_4P_2O_7$, adjusted to pH 7 (with H_3PO_4); (2), 0.1 M $Na_4P_2O_7$ (pH 10.6); and (3), 0.1 M $Na_4P_2O_7$ + 0.1 M NaOH. Song et al. (2008) and Chang et al. (2014) used the following sequence: (1), 0.1 M NaOH adjusted to pH 7 (with HCl); (2), 0.1 M NaOH adjusted to pH 10.6; (3), 0.1 M NaOH (pH 12.6); and (4) 0.1 M NaOH + 6 M urea. CPMAS ^{13}C-nuclear NMR spectroscopy revealed that relative quantities of components differ with increasing alkalinity and with the addition of urea to the media. HAs and FAs isolated at pH 7 had more aromaticity and acidic character than those isolated at the higher pH values. The isolates extracted at higher pH values were more enriched in carbohydrate and carbohydrate-related components, compared to the pH 7 isolates. Chang et al. (2014) reported that the HAs and FAs increased in aliphatic relative to aromatic functionalities as the pH of the media increased. Highly oxidised/transformed

materials are isolated at pH 7 (Song et al., 2008).

Despite the advantages of using aqueous alkaline media, there are a number of undesirable features associated with these solvents, as outlined by Stevenson (1994):

(1) silica and non humified plant components are soluble in alkaline media and therefore these can be co-extracted with the HSs;
(2) auto-oxidation of the humic components in the presence of air, at high pH values; and
(3) alkaline conditions can cause condensation between amino acids and the C=O group of reducing sugars or quinones to form humic-type compounds (Maillard reaction).

4.2.2 Urea

Urea is an excellent solvent for HSs due to its capability to break hydrogen bonds and the addition of high concentrations of urea to aqueous alkaline media greatly enhance the solvation of humic materials. Biochemists use urea to disrupt non-covalent bonds in proteins (Oh-Ishi and Maeda, 2002). Disaggregation of HSs has been achieved using urea prior to fractionation techniques such as polyacrylamide gel electrophoresis (PAGE), capillary zone electrophoresis, and high pressure size exclusion chromatography (HPSEC; Peuravuori et al., 2004; Zhang and Lu, 1987; Piccolo et al., 2002). Low concentrations of urea in aqueous solutions are poor solvents for H^+-exchanged HAs but almost complete solubilisation can be achieved in concentrated (5 M urea) aqueous solutions (Clapp et al., 2005). Song et al. (2011) reported that the addition of 6 M urea to aqueous alkaline media enhanced the solvation of HAs and FAs; the increased humic yields are attributed to the breaking of hydrogen bonding that can hold some humic components within the HU matrix. A mechanism by which the urea disrupts hydrogen bonding is proposed by Tobi et al. (2003): the C=O group in urea can compete with peptide C=O hydrogen bonded to peptide N-H groups; this results in the disruption of internal hydrogen bonding mechanisms causing changes in conformations, and humic components sterically trapped within, or hydrogen bonded in the peptide matrix are released into solution (Song et al., 2011).

Song et al. (2008) carried out exhaustive extractions using NaOH solutions at pH 7, 10.6, and 12.6, and subsequently used 0.1 M NaOH + 6 M urea media in order to achieve maximum recovery of the humic materials from a Mollisol. The NMR spectrum of the urea isolate was very similar to the spectrum of the isolate from the pH 12.6 (NaOH) extraction. The isolates from the 0.1 M NaOH + 6 M urea medium would not be classically regarded as HU; these isolates represent the last of the classically defined HAs and FAs (Song et al. 2008; 2011). Song et al. (2008; 2011) also exhaustively extracted a brown Podzolic soil, and a dark brown earth using the same solvent sequence and the NMR spectra of isolates from the alkaline-urea media were broadly similar to those isolated in alkaline media. Overall, it can be concluded that urea (6 M) enhances the abilities of 0.1 M NaOH to solvate humic materials by facilitating the release of additional HSs. Chang et al. (2014) utilised the alkaline-urea media for the extraction of HSs from a volcanic soil and reported that there was a greater increase in the amount of carbon extracted (relative to the aqueous alkaline media), and the increase was attributable to the release of tightly bound components. The additional aliphatic hydrocarbon components isolated in the alkaline-urea media may be attributable to biopolymers from higher plants that are difficult to dissolve in aqueous media (Chang et al., 2014).

4.3 Other Methods Available for the Extraction of Humic Substances

Aqueous solvents are routinely used for the isolation of HSs. However, there are a number of other options available; Patti et al. (2010) explored using ionic liquids to extract HSs from soil; Moredo-Pinera et al. (2004) used an ultrasound assisted extraction technique; Romaris-Hortas et al. (2007) employed a microwave assisted alkaline humic extraction technique; and a range of organic solvents were assessed by Hayes et al. (1975) and Hayes (1985) for the isolation of HAs. Problems encountered with methods other than aqueous alkaline solutions include low recovery of the HSs, difficulties recovering the humic components from the solvent, and the possible generation of heat. Organic solvents have been used only occasionally, due to the difficulties of separating the solvent from the humic components, as well as the possibilities of artefact creation. Hayes et al. (1975) conducted a comprehensive study using a series of organic and alkaline solvents (dimethyl sulfoxide, DMSO;

dimethyl formamide, DMF; ethylenediamine, EDA; pyridine; NaOH; $Na_4P_2O_7$) to extract HSs from an organic soil. The dipolar aprotic solvents (DMSO) did not alter the humic extracts and were regarded as mild reagents, EDA was shown to be as efficient as NaOH for the extraction of humic components, but it was found to alter the chemical nature and composition of the extracts. Hayes et al. (1975) concluded that aqueous NaOH represented the best reagents tested for isolating humic components.

4.4 Selection of a Suitable Solvent

Clapp et al. (2005) and Hayes (2006) carried out an extensive review of the literature in relation to desirable solvent properties for the isolation of HSs from soil. They suggest that a good organic solvent of HAs will meet the following conditions: EF (electrostatic factor) values > 140; a pK_{HB} (strength of a solvent to accept hydrogen bonding) values > 2; and dispersion (δ_p), hydrogen bonding (δ_h), and proton acceptor (δ_b) values (Hilderbrand multicomponent parameters) equivalent to, or greater than 6, 5, and 5, respectively. DMSO is a good solvent for HSs as it satisfies all three conditions. A good solvent for a non-electrolyte solute should have a solubility parameter (δ) value close to that of the solute; or a mixture of two solvents, one with a δ value above, and the other with a value below that of the solute is a more powerful solvent system for the solute rather than individual solvents (Hayes, 1985; Hayes, 2006). Dipolar aprotic solvents (DMSO, DMF, acetone) with the addition of 0.6 M HCl (8:2 v/v) were reported to extract HSs by breaking intermolecular hydrogen bonds (Piccolo et al., 1989). Owing to the long history of use, the efficiency, and the ease of use, aqueous alkaline solutions are generally favoured over organic solvents. Alkaline solvents remain the solvent of choice for HSs due to the low cost involved, the extensive background information available, control of the reaction conditions, and the simplicity of the extraction procedure; however, these are not suitable for the isolation of HU. Based on research by Hayes (2006), DMSO + concentrated sulfuric acid (H_2SO_4) has been successfully utilised for the extraction of HU from soils (Simpson et al., 2007; Song et al., 2008; 2011; Chang, 2014).

4.5 Dimethyl Sulfoxide (DMSO)

There is no ideal solvent system identified that is capable of extracting all of the HU materials in strong association with inorganic colloids (Hayes, 2006). Insolubility of HU is attributed to the hydrophobic nature of its compositions and its strong associations with mineral colloids. Hayes (1985) reported that DMSO is one of the best organic solvents for the dissolution of HSs. Law et al. (1985) showed that acidified DMSO can compare favourably with aqueous NaOH media for the extraction of HSs from soil. Metal cations will solvate readily in DMSO but it is a poor solvent for anionic conjugated bases (carboxylates and phenolates) unless they are neutralised with hydrogen ions (Martin and Hauthal, 1975; Hayes, 2006).

The interactions between DMSO-H_2O are stronger than the associations between water molecules. Therefore, it is of great importance that the solvent is anhydrous, and that water is not introduced during the extraction process. Exothermic reactions can be avoided by ensuring the sample (clay-sized fraction) and all the glassware are dry. DMSO is a good solvent for HSs as the DMSO interacts with carboxyl and phenolic hydroxyl groups (Figure 4.1; Hayes, 2006).

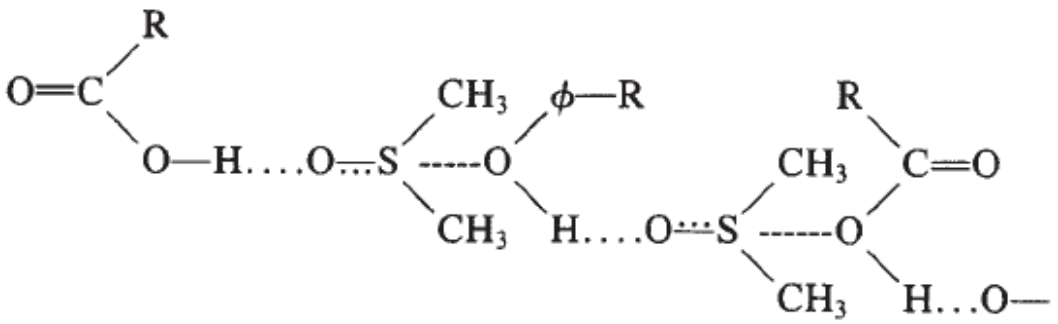

Figure 4.1. A hypothetical representation of associations between dimethyl sulfoxide (DMSO) and the carboxyl, phenolic, and other hydroxyl functional groups in humic substances. R represents the remainder of the humic structures stretching from the complexed carboxyl and phenolic or other hydroxyl functional groups; ϕ represents an aromatic functionality (Hayes, 2006, p. 993).

Hayes (2006) explains that DMSO can be considered to have both hydrophobic (CH_3 groups) and hydrophilic (the S=O functionality) regions (Figure 4.1). The hydrophobicity provided by the methyl groups complements the electron donor capabilities of the sulfur and oxygen, resulting in the solvation of hydrophobic and cationic species (Häusler and Hayes, 1996; Clapp and Hayes, 1996). Solvation of the hydrophobic components from humic materials in DMSO results in the isolates

having higher carbon contents compared to alkaline extracts (Hayes et al., 1975; Piccolo, 1988; Hayes, 2006). The extraction efficiencies are increased when concentrated H_2SO_4 (an excellent solvent on its own) is mixed with DMSO (94:6 *v*/*v*), because the acid protonates the conjugate bases of the HSs, increasing the solvation properties of DMSO (Hayes, 2006).

More recently, the DMSO-H_2SO_4 solvent system has been used for the isolation of HU from soils (Simpson et al., 2007; Song et al., 2008; 2011; Chang et al., 2014), rather than for the isolation of HAs as they can be successfully isolated using NaOH and NaOH-urea solvents. Simpson et al. (2007) used this DMSO-acid solvent to isolate a HU fraction following exhaustive extraction of the soil in aqueous alkaline and alkaline-urea media, resulting in the recovery of ≥ 70% of the traditional HU fraction. Using the same solvent system as Simpson et al. (2007), Song et al. (2011) found that 70-80% of the residual materials remaining after HSs extraction could be isolated using the DMSO-acid solvent. The NMR spectra of these DMSO-H_2SO_4 HU isolates had aliphatic hydrocarbon components associated with carbonyl functionalities, and with lesser amounts of carbohydrate and peptide and minor amounts of lignin-derived components. The recalcitrant HU materials extracted in the DMSO-H_2SO_4 are largely biological molecules, from plants and the microbial population (Song et al., 2011), and these molecules are more likely protected from degradation by their associations with hydrophobic moieties and by sorption to clays (Weiler and Mills, 1965; Knicker and Hatcher, 1997). Song et al. (2011) concluded that the major components of HU do not satisfy the classical definitions for HSs because the HU components are not adequately transformed by biological or chemical processes.

4.6 Other methods for the Extraction of Humin

The lack of research on the HU materials can be attributed to its insolubility (Rice, 2001). Several methods, involving aqueous and organic solvents, have been investigated in order to isolate HU (Rice and MacCarthy, 1989; Hayes, 2006; Song et al., 2008; 2011). Following extraction of HSs, Rice and MacCarthy (1989) used methyl isobutyl ketone (MIBK) as a mild solvent to separate the HU from the solid material remaining after alkaline extraction of HSs. Rice and MacCarthy (1989) used the MIBK method and the IHSS method for the isolation of HU from a soil sample

and concluded that the HU isolated by both methods exhibited similar chemical compositional features. Almendros and Sanz (1991) and Almendros et al. (1996) used ultrasonic disaggregation followed by flotation in a solution of bromoform-BF_3-ethanol (transesterification treatment) and partitioning in water-MIBK to isolate soil HU. This method isolated between 7-42% of the total C in the soil heavy fraction, and the HU samples isolated yielded a major series of alkyl products, including very variable amounts of alkanes, fatty acids and olefins.

Many methods regard the residue remaining after the extraction of HSs to be HU (IHSS method; e.g. Simpson and Johnson, 2006). However, this residue is predominantly inorganic to which the HU is strongly bound. High concentrations of HF or HF/HCl solutions are effective for the removal of the inorganic materials (metal oxides and aluminosilicates) (e.g., Huc and Durand, 1977; Hatcher et al., 1985; Ishiwatari, 1985; Preston et al., 1989). However, it should be noted that high concentrations of HF can lead to structural alteration of the organic components as well as loss of polysaccharides (Gélinas et al., 2001). A variety of chemical and/or physical procedures have been used to isolate kerogen (insoluble OM) from sedimentary rock and similar extraction methods could be utilised to isolate the most insoluble fractions of HU. Chemical methods are based on removing inorganic mineral matter using reagents such as hydrochloric acid, hydrofluoric acid, nitric acid, lithium aluminium hydride, sodium borohydride and zinc/hydrochloric acid (Saxby, 1970). However, the chemicals can bring about alterations to the functional groups of the OM (Saxby, 1970). Therefore, it is advantageous to use the DMSO-H_2SO_4 media as a mild solvent for the isolation of HU, and the DMSO insoluble HU can subsequently be isolated by dissolving the inorganic matrix using HF.

4.7 Fractionation of Humic Substances

Most often, HSs are isolated using aqueous alkaline media that solvates molecules with polar functionalities, and especially ionisable functionalities that give predominantly the conjugate bases of carboxyl and phenolic groups. However, non humic substances (NHS) can be co-extracted in the aqueous alkaline media. Fractionation procedures are based on differences in solubility which relate to polarity, charge characteristics, sizes and shapes, and the adsorption characteristics of the molecules (Clapp et al., 2005). When fractionating components with different

solubilities, less polar components can be precipitated from alkaline solutions. In principle, humic components with weakly dissociable acidic functionalities should be first to precipitate as the pH of the alkaline medium is lowered, and the strongest acids should be last to precipitate. There are a range of resins available for the isolation of HSs. However, HSs from waters have routinely been isolated by water chemists using XAD-8 and XAD-4 resins (Hayes et al., 1996), and these resins are now often used for fractionations of humic extracts from soils and sediments. XAD-4 resins (aromatic co-polymers of styrene-divinylbenzene) recover the hydrophilic components of the OM (XAD-4 acids). However, in sediments from aquatic environments, the majority of the hydrophilic components will remain in solution.

Malcolm (1991) outlined a number of important points to be considered when using resin technology for the isolation and concentration of OM: (1), resins must be cleaned thoroughly to remove impurities and avoid contamination of the isolated organic material; (2), sample volume should reflect the solute sorption capacity of the resin; and (3), resins should be used and stored correctly in order to achieve quality results.

4.7.1 Fractionation of HS using XAD-8 Resin Techniques

Chromatographic resins can separate HSs from non-covalently linked saccharides, peptides and other organic impurities (Hayes et al., 1996). The most popular resins used for HSs isolation are from the Amberlite XAD range. These are non-ionic macroporous resins with large surface areas (Aiken, 1985) that selectively sorb the HSs and the polar contaminants pass through the column. The humic components are retained on the XAD-8 resin through weak interactions such as hydrogen bonding and van der Waals forces. The sorbed HSs are subsequently recovered (desorbed) from the resin using an alkaline solution.

The XAD-8 resin technique is a chromatographic method for the concentration, isolation (of hydrophobic components) and fractionation of OM extracts. XAD-8 resin is an aliphatic polymer of methyl methylacrylate (Aiken, 1985). The properties of the resin include; a pore size of 250 Å**,** a surface area of 140 m^2 g^{-1}, and it has a slight polarity (Aiken et al., 1979)**.** XAD-8 resin was the most efficient of the XAD resins (Aiken et al., 1979), and high recovery (ca. 98%) of the humic components is achievable (Malcolm, 1991). The sorption of HSs on XAD-8

resins is based on the "hydrophobic effect" that is an important factor in hydrophobic column chromatography (Huque, 1989). Sorption of organic acids, such as HSs, on XAD-8 resin requires the humic components to be soluble in aqueous media and the pH of the media is important (Thurman et al., 1978; Aiken, 1985). When organic extracts are adjusted to a low pH weak acids are protonated and absorbed on the resin, whereas at a high pH, weak acids are ionized and desorption is favoured (Parsons et al., 2007).

4.8 Fractionation with other Resins

4.8.1 DAX-8 resin

Amberlite XAD-8 resin is no longer commercially available; therefore Superlite DAX-8 is the best substitute available for the isolation of HSs (Farnworth, 1995; Peuravuori et al., 2005). DAX-8 is also a poly(methylmethacrylate) resin and despite the small differences in the manufacture of the resin, the technical specifications (e.g. pore size and surface area) of DAX-8 are very similar to those of XAD-8 (Peuravuori et al., 2002a).

A number of studies have compared the isolation of aquatic humic samples using DAX-8 and XAD-8 resins (Lehtonen et al., 2000; Peuravuori et al., 2001). On average, DAX-8 resin has been reported to have a higher sorption efficiency compared to that of XAD-8 resin. Peuravuori et al. (2002b) demonstrated that DAX-8 resin can, optimally, isolate about 20% more hydrophobic organic species than XAD-8 resin; however, a major disadvantage is that certain failures can occur in the manufacturing of DAX-8 resin thus perverting the reliability of the isolation. DAX-8 is more efficient at sorbing the hydrophobic fraction but less efficient at sorbing the hydrophobic neutral fraction relative to the XAD-8 resin. Peuravuori et al. (2002a, b) compared the compositions retained by XAD-8 and by DAX-8 resins by analysing the isolates using pyGC/MS and scanning fluorescence spectroscopy. They concluded that the hydrophobic-hydrophilic sorption–desorption properties of the DAX-8 resin are more precise than those of the XAD-8 resin. Peuravuori et al. (2005) reported that some organic constituents (hydrophobic neutrals) cannot be eluted from the DAX-8 resin with alkaline media, but are eluted in methanol. Overall, it can be concluded that both resins have similar sorption capabilities and the compositions of the fraction recovered from the two resins are similar.

4.8.2 Diethylaminoethyl (DEAE) Cellulose Resin

Humic components are rich in acidic functional (mainly carboxylic) groups that confer polyelectrolyte properties. Therefore, the isolation of practically all humic-type solutes (aqueous phase) can be achieved in one step (no pH adjustments are required) using certain anion exchange resins (Peuravuori et al., 2005). Anionic resins are useful when isolation of HSs is necessary on site, rather than in the laboratory. DEAE (diethylaminoethyl) cellulose is the most routinely used anion exchange resin for HSs. The resin is a weak anion exchanger composed of a hydrophilic matrix to which tertiary amine functional groups are bound (-$OC_2H_4N(C_2H_5)_2$) (Peuravuori et al., 2005). Recovery of ionized organic humic solutes is high (ca. 80% - 100% of DOM in fresh water) as compared to the XAD resins (Packham et al., 1964; Miles et al., 1983; Petterson et al., 1989; 1994; Peuravuori et al., 1997; Peuravuori and Pihlaja, 1998) and pH adjustments are usually not necessary prior to sorption (Peuravuori et al., 2005). However, it is less efficient for marine HSs as the salinity decreases the retention of acidic solutes, especially lower molecular components (Pettersson and Rahm, 1996; Peuravuori et al., 2005). Another disadvantage is that desorption of the retained humic components is more difficult relative to that for the XAD resins (Hejzlar et al., 1994) and this can affect recovery of the organic materials. Hejzlar et al. (1994) reported that 98% of sorbed HSs could be recovered from the XAD-2 resin (using 1 M NH_4OH and methanol) whereas only 76% of the sorbed HSs were recovered from DEAE-cellulose (using 0.1 M NaOH). The DEAE cellulose resin is not specific for HSs as it can also adsorb pure polysaccharides, thus contaminating the recovered HSs (Boult et al., 2001).

4.8.3 Polyvinylpyrrolidone (PVP) Resin

The IHSS recommend the use of polyvinylpyrrolidone (PVP) resin when XAD/DAX-8 resin is not available, especially for water samples (DOM). PVP is a functional cross-linked resin (Peuravuori et al., 2005). PVP resin under acidic conditions (ca. pH 2) can retain aromatic carbon species, and phenolic, hydroxyl, and acidic functional groups (Peuravuori et al., 2005). The PVP resin retains the HSs because it can form strong hydrogen bonds with phenolic, hydroxyl, and carboxyl groups (Ciavatta and Govi, 1993), and so it differs from non-ionic macroporous

copolymers that sorb organic components based on hydrophobic–hydrophilic interactions, at a low pH (Peuravuori et al., 2005). For the same water sample, the adsorption capacity of PVP resin for HSs was very high; it isolated > 86% of DOM compared to DEAE cellulose resin (77%) and XAD-8 resin (65%) (Peuravuori et al., 2005). The main disadvantage of the PVP resin procedure is that the aqueous solution must be adjusted to pH 2 for protonating the acidic functional groups of the DOM (Peuravuori et al., 2005).

4.9 Instrumental Fractionation Techniques

The following techniques were not used in this study but are referenced because these can have uses in the fractionation of HSs. Early studies on the size and shape of HSs were misleading because, in the presence of an electrolyte, humic components can aggregate to give results suggestive of very large molecular sizes. Because HSs are polydisperse, their molecular weight (MW) has been reported to be 2,050 Daltons (Da) in the absence of a background electrolyte, but as high as 77,000 Da in the presence of an electrolyte (Flaig and Beutelspacher, 1968). Piccolo (2002) showed that HSs are supramolecular associations (using HPSEC), and Simpson et al. (2001) and Simpson (2002) showed (using diffusion ordered NMR spectroscopy) that FAs and HAs are aggregates of relatively small molecules that have colloidal properties when in the presence of metal ions in aqueous media. The HSs are held in aggregates through a complex combination of hydrophobic associations, charge interactions, hydrogen bonds, and metal bridging (Simpson et al., 2002). The work by Piccolo and Conte (2000) and Piccolo (2001) showed that the HSs are held in their supramolecular conformations due to hydrophobic forces and H- bonding. Reagents such as urea and DMSO can disrupt the humic associations to give more homogenous and chemically simpler samples. Modern instrumental fractionation techniques are often based on molecular size differences, and sorption characteristics of the organic molecules. Early size studies used the following techniques: ultracentrifugation, viscosity measurements, colligative property measurements, scattering techniques, gel chromatography, electrophoresis, and electron microscopy (Hayes et al., 1989).

A number of chromatographic techniques have been successfully utilised to provide additional information on the MW, compositions, and conformations of HSs.

Gel permeation chromatography (GPC), or low pressure size exclusion chromatography has been used extensively to determine molecular sizes, and to obtain homogenous size fractions of humic materials. Sephadex gels and (poly)acrylamide resins of different pore sizes are utilised. However, there are some issues with these methods because electrostatic repulsion occurs between the negative charges present on both the solute molecules and the gels, and hydrophobic interactions can take place between the solute and the stationary phase; both interactions can retard the chromatographic elution of the HSs. To overcome these issues non-zero ionic strength of the mobile phase will prevent electrostatic interactions, but if it becomes too high (> 0.5 M) it will drive hydrophobic associations of the molecules thus enhancing hydrophobic adsorption on the gel solid phase (Piccolo, 2002). Electrophoresis is widely used in biochemistry (for ionized or ionizable compounds) and can be employed to separate HSs (de Nobili et al., 1987; Dunkel et al., 1997). It separates components by applying an electric field to move negatively charged molecules through an agarose gel. A number of electrophoretic techniques are available such as: free solution capillary electrophoresis (FSCE); capillary gel electrophoresis (CGE); isoelectric focusing (IEF) in ultra-thin layers; micellar electrokinetic chromatography (MEKC); and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Dunkel et al., 1997). Piccolo (2002) employed both low- and high-pressure size exclusion chromatography (HPSEC) to investigate the supramolecular associations in HSs. HPSEC is favoured over the low pressure alternative as it has higher reproducibility, it is relatively faster, the column has a longer life, and it has higher sensitivity to chemical changes. HPSEC of HAs by Piccolo et al. (2002) showed that 10^{-3} M acetic acid disrupted supramolecular humic structure, and pyGC/MS characterisation revealed that the dilute acid treatment resulted in smaller molecular-sized molecules. The highest MW components were alkyl chains, suggesting the humic supramolecules are stabilised through weak interactions between alkyl chains and aromatic moieties.

Liquid chromatography can separate, fractionate, and characterise HSs by separating on the basis of the size, as well as the polarity of the molecules. The liquid chromatography can be hyphenated with analytical instrumentation for detections of the molecules. Such techniques include high performance liquid chromatography (HPLC), liquid chromatography mass spectroscopy (LC-MS), and liquid

chromatography nuclear magnetic resonance (LC-NMR) spectroscopy. It may be necessary to dissolve the sample in suitable media for these chromatographic techniques. The fractions from the chromatography can be detected by a UV detector in the HPLC instrument itself (however, due to the heterogeneity of the sample, it can be difficult to resolve peaks), or the fractions can be identified when coupled to a mass spectrometer (MS), or a nuclear magnetic resonance (NMR) spectrometer. Simpson et al. (2002) stated that no single experiment will describe the nature of HSs due to their inherent complexity. Therefore, he used LC-NMR, in conjunction with multidimensional NMR to characterise HSs. LC is utilised to fractionate the sample before identification using NMR spectrometry. Separation of HSs into well-resolved components is difficult using LC. Therefore, Simpson et al. (2002) used a time-slicing technique that involved pausing the LC at selected time intervals to yield separate eluted fractions on which NMR spectra could be obtained, and this technique showed that HSs are a complex mixture of relatively simple compounds.

4.10 References

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Chapter 5 Spectroscopic Techniques Utilised for the Characterisation of Organic Matter

There are a variety of analytical techniques available for the characterisation of humic substances (HSs) and humin (HU). The ideal analytical method would provide quantitative and qualitative information about a sample in its natural state (i.e. with no pre-treatments). However, this is difficult to achieve due to the predominance of inorganic components that result in organic concentrations being too low for adequate detection, as well as interferences from non-humic components and from inorganic species. To gain insights into the structure and composition of the components of natural organic matter (NOM) it is necessary to extract the OM from the inorganic colloids followed by a level of fractionation (as discussed in Ch. 4). Without fractionation, the number and variety of organic components in the sample are too great; therefore, meaningful interpretation of results proves difficult. Spectroscopic methods are popular for characterisation studies and these continuously evolve to provide more comprehensive data and additional levels of information. Spectroscopy is the study of the interaction of light (electromagnetic radiation) with matter (Smith, 2011). Analytical instrumentation and methodologies continue to improve but it is essential that quantitative methods are developed that can measure challenging biomolecules e.g. algaenans, and other macromolecules that are resistant to hydrolysis treatment (de Leeuw and Largeau, 1993, Hedges et al., 2000).

5.1 Ultraviolet-Visible Spectroscopy

The majority of organic molecules and their functional groups are transparent in the ultraviolet (UV) and visible (Vis) regions of the spectrum (190-800 nm), and therefore this type of spectroscopy has limited applications (Pavia, 2009). However, the information attained can be used in conjunction with spectroscopic or chemical characterisation data (Pavia, 2009). When radiation passes through a transparent material, a portion of the radiation is absorbed, causing atoms to pass from the ground state to an excited state, the energy required for this transition can be quantified. When using radiation in the UV-Vis region of the spectrum, a molecule

absorbs the energy and an electron is promoted from an occupied orbital to an unoccupied orbital of greater energy potential (Pavia et al., 2009).

Calculation of UV-Vis ratios has been used by researchers to characterise OM. According to Giani et al. (2010) the A_2/A_4 (272 nm/407 nm) ratio allows differentiation in the origin of the material, i.e. >3.4 for terrestrial origin, between 2.4 and 3.1 for coastal origin, and <1.9 for marine origin. Absorption of radiation at 260-280 nm and at 404 nm is attributed to double bonds of aromatic or unsaturated species (Agarwal et al., 2010), and pigment structures (Ishiwatari 1973; Povoledo et al. 1975; Cieslewicz et al., 2008), respectively. Small molecules absorb at 465 nm whereas larger molecules/macromolecules absorbance at 665 nm (You et al., 1999); therefore the E_4/E_6 (465 nm/665 nm) ratio is used to indicate of molecular size, condensation, and aromaticisation of the humic sample (Huang et al., 2008). The HAs are reported to have an E_4/E_6 ratio value of <5 (Kononova, 1966) and FAs have higher ratio values between 6-18.5 (Leenheer, 1980; You et al., 1999).

5.2 Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a popular characterisation technique because it is non-destructive to the sample, it is reagent-free, and is both a time and cost efficient analytical method. The IR spectrum provides information about bonding in the compounds in the sample. Fourier transform (FT) is an algorithm used to convert data points into an interpretable spectrum. This spectroscopic method passes mid IR (Table 5.1) through the sample and the absorbance of the radiation is subsequently measured. The wavelength of light/radiation is defined as "*...the distance travelled by a wave during a cycle; a cycle begins at zero amplitude and ends when the wave has crossed zero amplitude a third time"* (Smith, 2011). The FTIR spectrum is a plot of absorbance *versus* wavenumber (the number of cycles a wave undergoes per unit length, measured in units of cycles per centimetre (cm^{-1}; Smith, 2011)). IR passed through a sample absorbs energy that corresponds to the vibrational energy of atomic bonds. The functional groups in the sample absorb energy at specific wavelengths which are affected by the position of adjacent atoms. The FTIR spectrum provides a unique fingerprint of a compound (Howe et al., 2002); however, due to the vast mixture of compounds in HSs, there is overlapping of many functional groups.

Table 5.1. The region of the electromagnetic spectrum related to infrared spectroscopy (Smith, 2011).

>14,000 cm^{-1}	14,000- 4000 cm^{-1}	4000-400 cm^{-1}	400-4 cm^{-1}	< 4 cm^{-1}
Visible and UV Electronic Transitions	Near IR Molecular Vibrations	Mid-Infrared Molecular Vibrations	Far Infrared Molecular Vibrations	Microwaves Molecular Rotations

There are a number of advantages associated with using FTIR spectroscopy: (1), a wide variety of molecules absorb the mid IR region of the spectrum; (2), it provides structural information, and can provide quantitative analysis; (3), analysis time is short; and (5), good sensitivity can be achieved using small (ca. 1 mg) sample quantities (Smith, 2011). A major disadvantage associated with this spectroscopic method is that heterogeneous samples produce a complex spectrum that is difficult to interpret (MacCarthy and Rice, 1985; Smith, 2011). Additionally water interferes with the spectrum as it produces a number of bands in the spectrum (3300 to 3000 cm^{-1} and 1720 to 1500 cm^{-1}), especially when using potassium bromide (KBr) discs (Swift, 1996; Smith, 2011).

FTIR spectroscopy has been utilised extensively for the characterisation of natural OM, usually in a solid phase. KBr, an alkali halide salt, is used to make a transparent pressed pellet. Absorption occurs in a sample when (1), the radiation interacts with a molecule causing a change in dipole; and (2), the incoming IR photon has the necessary energy required to move to the next allowed vibrational energy state; no absorption will occurs if either of these conditions are not met (Eaton, 2007). Only vibrational bands (molecules stretching and bending) are observed for samples in a solid or liquid phase (MacCarthy and Rice, 1985). The spectrum can be interpreted because functional groups corresponding to a particular vibration occur at a specific frequency (MacCarthy and Rice, 1985).

Table 5.2. Fourier-transformed infrared (FTIR) bands in humic materials (adapted from Baes and Bloom, 1989; Swift, 1996).

Band	Assignment
cm^{-1}	
3330-3380	OH stretch of phenolic OH (inputs from aliphatic OH, H_20 and possibly NH)
3030	Aromatic CH stretch
2930	Asymmetric CH stretch of -CH_2-
2840	Symmetric CH stretch of -CH_2-
2600	OH stretch of H-bonded -COOH
1720	-C=O stretch of -COOH
1610	Aromatic C=C stretch and/or asymmetric -COO- stretch
1525	Aromatic C=C stretch
1450	-CH deformation of -CH_3 and -CH bending of –CH_2
1350	Symmetric –COO^- stretch and/or -CH bending of aliphatics
1270	-C-OH stretch of phenolic OH
1225	-C-O stretch and OH deformation of -COOH
1170	-C-OH stretch of aliphatic OH
1070	C-C stretch of aliphatic groups
830	Aromatic CH out of plane bending
775	Aromatic CH out of plane bending

Inorganic species are often co-extracted with OM and FTIR spectroscopy can also be utilised to identify these.

Table 5.3. Fourier-transformed infrared (FTIR) bands from inorganic components (Nayak and Singh, 2007).

Band	Assignment
cm^{-1}	
3696	– Al ---O–H stretch
3622 cm^{-1}	– Al ---O–H (inter-octahedral)
1033 cm^{-1}	– Si–O–Si, Si–O stretch
914 cm^{-1}	– Al---O–H stretch
790 cm^{-1}	–Si–O stretch, Si–O–Al stretch, (Al, Mg)---O–H, Si–O–(Mg, Al) stretch
693 cm^{-1}	– Si–O stretch, Si–O–Al stretch
538 cm^{-1}	– Si–O stretch, Si–O–Al stretch
468 cm^{-1}	– Si–O stretch, Si–O–Fe stretch

It is sufficient to measure absorbance of organic samples between 4000 and 400 cm^{-1} because the far infrared region (400-4 cm^{-1}) of the electromagnetic spectrum has absorbances from inorganic species (Smith, 2011). The IR spectra of HSs and HU appear to be simple as there are a small number of broad bands. The broadness of the bands is due to overlapping signals from the heterogeneous nature of the sample (MacCarthy and Rice, 1985).

The FTIR spectroscopy, in conjunction with other analytical techniques, has helped determine aspects of the chemical structures, functionalities, and reactivities of humic materials (Schnitzer, 1991; Rivero at al., 1998). FTIR spectroscopy is useful for the characterisation of soil (e.g. Haberhauer et al., 1998, 2000; Tatzber et al., 2007) and of sedimentary OM (Calace et al., 2006). FTIR spectra can track changes down a sediment core/soil profile.

Swift (1996) cautions that the FTIR spectra should be interpreted with caution and assignment of bands to particular groups can be tentative due to the complexity of the humic sample. Therefore, the results should be corroborated with other analytical data.

5.2.1 ATR-FTIR

ATR-FTIR (Attenuated Total Reflectance–Fourier Transform Infrared) is currently the best option for IR studies as it does not require sample preparation, and provides high resolution spectra. ATR-FTIR is often utilised over conventional FTIR spectroscopy, especially when the sample is difficult to prepare. The IR spectrum of both solids and liquids can be measured without dilution of the sample in a matrix such as Nujol (Schuttlefield and Grassian, 2008) or the preparation of KBr discs. This is a major advantage as KBr discs are hygroscopic, and Nujol absorbs radiation (2900, 1460 and 1375 cm^{-l}) which causes interference in a humic spectrum (Swift, 1996). The instrument has an internal reflection element (often diamond), on which the sample for analysis is placed. The spectrometer directs IR light onto the element at an angle (θ), greater than the critical angle (θ_c), resulting in the IR light undergoing internal reflection; each point of internal reflection produces an evanescent wave and this radiation can be absorbed by a sample (Figure 5.1; Schuttlefield and Grassian, 2008).

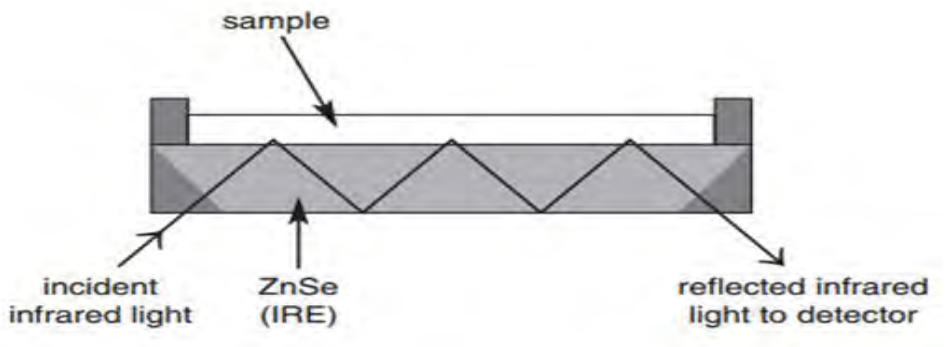

Figure 5.1. An illustration of the internal reflections through a high refractive index medium (e.g. ZnSe) used as the internal reflection element (IRE). At each reflection an evanescent wave is produced that decays exponentially into the medium above (Schuttlefield and Grassian, 2008, p. 279).

Comparison studies by Tanaka et al. (2001) concluded that ATR spectra are similar in most spectral regions to conventional FTIR using KBr discs. Conventional FTIR analyses use a KBr pressed-pellet as the medium for sample analysis. However, KBr is hygroscopic and results in interference from water bands (3300 to 3000 cm^{-1} and 1720 to 1500cm^{-1}) in the HSs spectrum (Swift, 1996). When studying a humic sample in aqueous phase, ionisation and protonation of COOH groups and OH groups with varying pH can be observed using ATR-FTIR (Tanaka et al., 2001).

5.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy has become an invaluable tool in the study of the composition and structure of HSs. Simpson et al. (2011) describes NMR spectroscopy as the most powerful analytical instrument for the determination of complex structures and interactions, from which their reactivities in the environment can be postulated. NMR spectroscopy provides an unrivalled level of molecular level information, and because the analysis can be carried out on samples that have had little or no pre-treatment this analytical tool is suitable for environmental samples (Simpson et al., 2011). Analyses can be carried out on samples in solid, liquid, or gaseous phases to provide information about the immediate environment of each types of atom. Structural information can be derived from the energy splitting of nuclei which is affected by the number and nature of chemical bonds, as well as the proximity of other atoms in space (Clapp et al., 2005).

A simple explanation of NMR spectroscopy is as follows: nuclei with odd atomic mass have a property called spin (^{1}H, ^{13}C ^{15}N, ^{19}F, ^{31}P), and when these

nuclei are placed in an external magnetic field, they line up with or against the applied field; these two different states have slightly different energy levels (spin state $+^1/_2$ or $-^1/_2$, Dixon and Larive, 1999). Spin relates to a rotation of a (charged) nucleus around a specific axis that produces a magnetic field (Knicker, 2011). Electromagnetic (EM) radiation (in the radio frequency region of the spectrum) with an energy corresponding to the gap between these two spin states can cause these nuclear magnets to flip from a low to a high energy state. As they drop back from a high to a low energy state (called relaxation), the nuclei of the atoms in different positions of the molecule give out energy at slightly different frequencies indicative of the structure of the molecule. The difference in frequency between the induced signal and the excitation frequency produces an FID (free induced decay) plot (Dixon and Larive, 1999). The FID is a time domain signal that contains all the amplitude and frequency information of each resonance in the NMR spectrum; however, this plot cannot be easily interpreted. Therefore, the Fourier Transform (FT) algorithm is used to convert the FID into a frequency domain (Keeler, 2013) and it separates out signals from atoms in different molecular environments and presents these as a spectrum that can be interpreted. To achieve a good signal-to-noise (S/N) ratio it usually is necessary to repeat the NMR experiment many times, especially for nuclei with low natural abundance (Keeler, 2013). It is not possible to provide a review of all the NMR experiments available; therefore, background theory on carbon (^{13}C) and (^1H) proton NMR will be provided as well as a description of the most relevant NMR experiments to the study of natural OM.

5.3.1 Basis of NMR Spectroscopy

Knicker (2011) has described the technical basis of NMR spectroscopy in detail. To provide an adequate understanding of NMR, the main points from Knicker (2011) are summarised here and illustrated in Figure 5.2. NMR spectroscopy is based on the interaction between the magnetic properties of the observed nucleus (with a magnetic moment (μ) and spin (I)) and an external magnetic field (B_0). The spin of the nucleus depends upon the gyromagnetic ratio (γ), a constant that is specific for each nucleus that is related to the nuclear charge number and mass. A nucleus with μ placed into a static magnetic field (B_0) will precess around its z-axis. Larmor frequency (ω_o) is the term used to describe the frequency (ν) of this movement which

is dependent upon the strength of B_0 and the size of γ (specific for each nucleus). In a static magnetic field a nuclear spin can inhabit $2I + 1$ possible energy levels; nuclei with $I = {}^1/_2$ arrange their orientations so that their magnetic moment vectors are parallel or antiparallel to B_0. To induce the movement of the spins between the two energy levels, energy must be provided that matches the energy difference between the two energy levels, i.e. the resonance condition ($\boldsymbol{\omega_0 = \gamma B_0}$) has to be fulfilled. This energy is provided by a pulse of a second oscillating electromagnetic radiofrequency (rf) field, B_1, aligned perpendicular to B_0 and with the required frequency (ν) (Figure 5.2a). The energy induces the net magnetisation on the *z*-axis to flip into the direction of the *y*-axis, at an angle related to the intensity and duration of the pulse. The transversal magnetisation continues to precess with ω_0 around the *z*-axis, and the magnitude of its *y* component is altering periodically; this alteration is detected as a voltage signal oscillating with ω_0 by a receiver, located in the *y* direction. Following the rf pulse, the excited spin system starts to relax to its thermal equilibrium and emits energy. The transversal magnetisation decreases with the spin–lattice relaxation time constant, T_1, while the net magnetisation along the *z*-axis is enhanced. Concomitantly, energy is rearranged among the spins with the spin–spin relaxation time constant, T_2. These relaxation mechanisms cause a reduction of the amplitude of the detected oscillating signal with respect to time (free induction decay, FID).

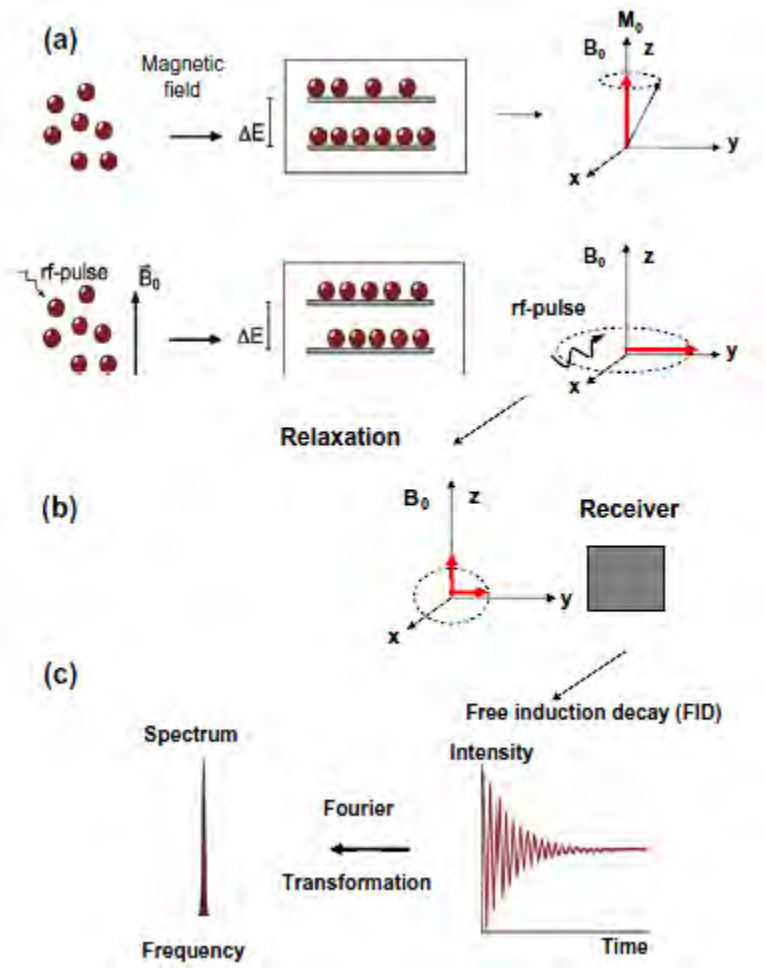

Figure 5.2. (a) Scheme of the resonance experiment with the corresponding vector model (B_0: strength of the static magnetic field; M_0: equilibrium magnetisation; rf: radiofrequency pulse), (b) detection of the free induction decay (FID) during relaxation, and (c) transformation of the data in the time domain into the frequency domain (spectrum via Fourier Transformation, Knicker, 2011, p. 869).

5.3.2 Chemical Shift

Nuclei resonate at different frequencies according to their local chemical environment (Balci, 2005) and their resonance position provides the chemical shift value. Chemical shifts values are expressed as δ values or as ppm. Electrons around the nucleus have their own local magnetic field that interacts with the external magnetic field. The local magnetic field opposes the external magnetic field, and the strength of the external magnetic field surrounding the nucleus is decreased, thereby lessening the net applied magnetic field that the nucleus is exposed to (Balci, 2005). This phenomenon is called shielding which causes the nucleus to precess at a lower frequency and results in the absorption of radiofrequency radiation at this lower

frequency (Pavia et al., 2009). Under certain circumstances the nucleus may be influenced by an increased magnetic field; this phenomenon is called deshielding (Balci, 2005). The extent of shielding will determine the resonance frequency of the nuclei. Shielding can occur for a number of reasons: diamagnetic shielding (circulation of the electrons at the nucleus); paramagnetic shielding (only applicable to heavier nuclei, electrons in the *p*-orbital do not have spherical symmetry and produce large magnetic fields); effect of neighbouring groups (substituents with electron withdrawing capabilities will decrease the electron density around the nucleus decreasing the shielding effect) and solvent shielding (generally these effects are small, but possible) (Balci, 2005).

The chemical shift of a nucleus is the difference between the resonance frequency of the nucleus and that of a standard. TMS (tetramethylsilane) is the standard most often used to calibrate the chemical shifts (resonance is set at 0 ppm). Originally TMS was used as a standard because its methyl groups protons are more shielded compared to those of most other known compounds (Pavia et al., 2009): as well as being inexpensive, TMS is readily available, and chemically inert (Balci, 2005).

5.3.3 ^{1}H NMR

^{1}H (proton) NMR spectroscopy is utilised to characterise the composition of humic samples and to measure the relative abundance of various HSs functional groups (Cardoza et al., 2004). Proton NMR experiments are generally carried out on samples in solution. Solution-state NMR is highly versatile because there are hundreds of experiments available, and new experiments are easily created to answer specific research questions (Simpson et al., 2011). Acquisition time is short due to the high natural abundance of protons and the high concentration of hydrogen atoms in organic samples. Short acquisition times are advantageous in the research laboratory and proton solution-state experiments can be acquired in a number of minutes. The narrow lines provided by the solution-state experiment are due to the motion of the molecules which cancel and average chemical shift anisotropy and dipolar interactions (both phenomena cause the broad resonances in solid-state NMR). Despite the small chemical shift of the nuclei (0-14 ppm), the narrow lines allow meaningful interpretation of the spectra. A major caveat of solution-state

experiments is that the sample must be soluble; thus it is highly applicable to dissolved OM but much more difficult for samples such as HU. There are a number of suitable solvents available for NMR spectroscopy, and the deuterated versions (D_2O, NaOD, D_2SO_4, DMSO-d_6) of the solvent are generally used to avoid interference from solvent signals with sample signals. DMSO is an attractive solvent for NMR studies of OM because it provides exceptional complimentary information, especially for peptide components, and it often produces spectra with better defined resonances (Simpson, 2001; Byrne et al., 2010). Efficient suppression of the solvent signal is necessary to allow correct interpretation of the structure and compositions of the sample (Zheng and Price, 2010; Adams et al., 2013).

The presence of water, in the sample or solvent, can cause broad resonances ca. 3.7 ppm, in the spectrum. It is important to have the sample as dry as possible and to reduce the exposure of the sample or solvent to the air. A number of techniques have been developed to suppress the water signal; pre-saturation is often employed, and if additional suppression is required sequences such as WATERGATE (WATER-suppression by GrAdient-Tailored Excitation) and PURGE are used. The simplest approach is to use pre-saturation which involves no additional delays (Redfield and Gupta, 1971; Simpson et al., 2011). WATERGATE is often used because signals from nuclei that exchange with the solvent are not suppressed (Adams et al., 2013). PURGE (Pre-saturation Utilizing Relaxation Gradients and Echoes) was developed by the Simpson Research Group (Simpson et al., 2011). This sequence uses very short relaxation delays (200 μs), in conjunction with echoes, gradients and pre-saturation which provides very effective and narrow suppression without suppressing the signals close to the water (Simpson et al., 2011). Diffusion edited experiments will also remove signals from water and solvent (e.g. DMSO) because all the signals from mobile molecules are removed from the final spectrum.

The ^1H experiment can employ diffusion editing (DE), a spectral editing technique, that removes all signals from mobile molecules and provides a spectrum of molecules that have limited diffusion. Spectral editing relating to molecular diffusion is based on the Hahn spin-echo experiment. The Pulsed Gradient Spin-Echo (PGSE) method was developed by Stejskal and Tanner (1965) for DE experiments and it is described in detail by Dixon and Larive (1999). The nuclei are

magnetised with a 90° pulse followed by a short delay (T_1) and a gradient pulse is applied which can encode the nuclear spins (Dixon and Larive, 1999). The nuclei are spatially encoding because they precess at distinct frequencies relative to their position of the applied magnetic gradient, and the resonance frequency changes with field strength ($\omega = \gamma B_0$) (Dixon and Larive, 1999). The magnetisation undergoes precession in the *xy* plane during the delay after the first gradient pulse and a 180° pulse is then applied along the *y*-axis to change the direction of precession (Dixon and Larive, 1999). A second gradient pulse, equal in phase and amplitude to the first gradient pulse can spatially decode the spins in the sample (Dixon and Larive, 1999). The spin-echo is formed during the first delay and the FID (free induced decay) is acquired at this time (Dixon and Larive, 1999). Movement of the encoded spins to another region of the sample results in the second gradient pulse not effectively decoding the spins, and these signal intensities are attenuated (Dixon and Larive, 1999). Large molecules diffuse slowly; therefore, their resonances are not attenuated.

Inverse diffusion editing (IDE) is a sub-spectrum of the total ^{1}H spectrum. The IDE experiment utilises PGSE based sequences along with post-processing difference spectroscopy to produce a spectrum that only shows the small mobile molecules in the sample, i.e. those that exhibit rapid diffusion (Courtier-Murias et al., 2012). The spectrum is obtained by subtracting the DE spectrum ("large molecules") from the unattenuated spectrum containing both small and large molecules (conventional ^{1}H spectrum). The result is a spectrum that contains only the most mobile/soluble/small components (Courtier-Murias et al., 2012). It is useful to obtain this spectrum in order to identify small mobile components in the sample that can be masked in the other ^{1}H spectra.

5.3.4 ^{13}C NMR

The diverse range of ^{1}H NMR experiments are useful for compositional studies, but analysis using ^{13}C nuclei is preferred because of the greater chemical shift dispersion (0-240 ppm) that decreases spectral overlap, and minor changes between samples are easier to identify (Cardoza et al., 2004). In general, one dimensional (1D) ^{13}C NMR experiments are carried out on samples in a dry, solid-state. Cross polarisation magic angle spinning (CPMAS) ^{13}C NMR is the most powerful experimental approach used to provide direct information on the structural and conformational

characteristics of humic components (Wilson, 1987; Preston, 1996; Piccolo and Conte, 1998; Conte et al., 2004). Solid-state CPMAS ^{13}C-NMR spectroscopy has been used to describe the composition of fractionated HSs and to help understand their genesis, transformation, and degradation (Baldock et al., 1997; Almendros at el., 2000; Lorenz et al., 2000; Lu et al., 2000; Spaccini et al., 2000; Lorenz and Preston, 2002; Conte et al., 2004). A major advantage of solid-state NMR is that no solvent is added; therefore samples are free from chemical interference, the sample does not need to be soluble, the spectrum does not have any resonances from solvent, and the sample is easily recovered. The disadvantages of the solid-state ^{13}C experiments include problems that relate to the broadness of the peaks (due to chemical shift anisotropy (CSA) and dipolar interactions), low natural abundance of ^{13}C (ca. 1.1%), difficulties in magnetising the carbon nuclei (due to low gyromagnetic ratio), and interference from paramagnetic species. The difficulties associated with solid-state NMR have been successfully overcome using a combination of high-frequency magic angle spinning (MAS) to remove CSA, broadband proton-decoupling to remove dipolar and scalar coupling, and cross polarisation (CP) for sensitivity enhancement (ca. a factor of 4; von Philipsborn and Müller, 1986; Simpson et al., 2011).

Conte et al. (2004) have provided a treatise of CPMAS ^{13}C NMR spectroscopy and its applications to natural OM. The main points have been summarised (5.3.5) to explain the basis of ^{13}C cross polarisation (CP) NMR spectroscopy.

5.3.5. Mechanism of Cross Polarisation

^{1}H nuclei have a much larger gyromagnetic ratio (Duer, 2002) than ^{13}C, and so this is exploited in CP, whereby the ^{1}H is easily magnetised and then this magnetisation is passed to the ^{13}C nuclei (Figure 5.3a). CP is achieved between ^{1}H and ^{13}C when an initial $90°_{x}$ pulse is used to produce a ^{1}H magnetisation along the *y*-axis, followed by applying a contact pulse on the proton along *-y* to spin-lock the ^{1}H and keep it relatively constant along this axis. At the same time, an analogous contact pulse is applied on ^{13}C and magnetisation transfers from ^{1}H to ^{13}C, when these contact pulses are applied. To achieve the best efficiency of magnetisation transfer the radio-frequency (rf) amplitudes of the contact pulses on ^{1}H and ^{13}C nuclei must fulfil the

Hartmann–Hahn matching condition: $\gamma_H B_1(H) = \gamma_C B_1(C)$ (where γ_H and γ_C are the gyromagnetic ratios of ^1H and ^{13}C and $B_1(H)$ and $B_1(C)$ are the rf fields of the two nuclei, Stejskal and Memory, 1994). To fulfil the Hartmann–Hahn condition the amplitude of the pulse of one of the spins (i.e. ^1H) is fixed and the amplitude of the other spin (i.e. ^{13}C) is slowly varied until a signal is obtained. The phase cycling used in the CP experiment observes only C signals generated from the CP of protons, and generally no signal will be observed from direct ^{13}C excitation (Duer, 2002). The strength of the dipolar interactions between ^1H and ^{13}C affect the rate of CP, i.e. the stronger the ^1H–^{13}C coupling and the shorter the inter-nuclear distances, the faster the rate of magnetisation transfer. Higher rotor spin frequencies have a negative effect on CP because it decreases the ^1H–^{13}C coupling.

Spin diffusion (dipolar interactions among the ^1H nuclei) is an alternative mechanism to CP (Scheler, 2002). ^1H can induce spin re-orientation on any other ^1H in close proximity, and may subsequently interact either with carbons or with other protons (Figure 5.3b).

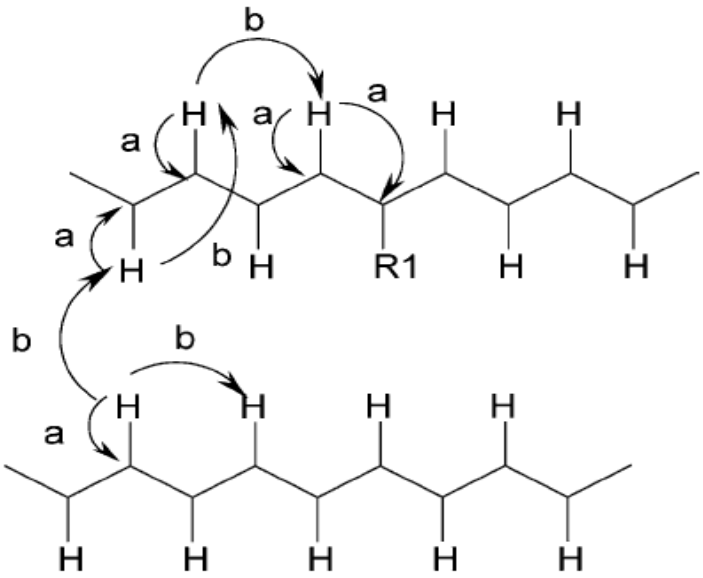

Figure 5.3. Mechanisms of magnetisation transfer. (a) Cross-polarization; (b) spin diffusion (Conte et al., 2004, p.219).

Ramped amplitude (RAMP) CPMAS technique is now routinely used for solid-state ^{13}C NMR, and this technique was created to overcome deficiencies associated with the Hartmann–Hahn conditions; these occur at high fields and from fast rotations of protonated samples (Fu et al., 1997; Cook and Langford, 1998; Kawashima and

Yamada, 1999; Preston, 2001). RAMP CPMAS NMR conditions are achieved when the amplitude of one of the rf fields (^{1}H or ^{13}C) is varied during polarisation transfer (Samoson et al., 2001).

When running single-contact-time CPMAS ^{13}C NMR experiments, the most important parameters are acquisition time (AT), and recycle delay (RD). The AT must be sufficiently long to avoid both signal loss and noise increase; and the RD must at a minimum be at five times larger than the T_1 value to allow the nuclei to reach equilibrium in magnetisation (Wilson, 1987). Contact time should be optimised to allow transfer of magnetisation between the ^{1}H and the ^{13}C nuclei to obtain reproducible and (semi)quantitative spectra.

5.3.6 Spinning Side Bands

Spinning side bands (SSB) are 'false' resonances positioned at chemical shifts equal to the spinning frequency of the sample and these appear at both the right and left side of the centre-band, and are not influenced by the strength of the applied magnetic field (Stejskal and Memory, 1994). These bands are easy to recognise by either measuring the distance between the resonance (SSB) and the centre-band, or by observing their intensities which will decrease and their positions will move if the rotor spinning rate is changed, but the position and intensity of the centre-band will remain at the same position (Conte et al., 2004). SSB become an issue in solid-state NMR if the rate of rotor spinning frequency is lower than the frequency of the CSA (Duer, 2002; Conte et al., 2004).

SSBs are undesirable because these have a negative effect on the qualitative and quantitative interpretation of spectra; such bands may be incorrectly interpreted as signals of non-existent functional groups, and misrepresent the relative intensities of some spectral regions. SSBs can be eliminated by spinning the sample at a high rotor spin velocity (c.a. 15–20 kHz), and using RAMP pulse sequence to maximise CP efficiency (Cook and Langford, 1998; Dai et al., 2001; Liu and Huang, 2002; Chefetz et al., 2002; Dria et al., 2002; Kiem et al., 2000; Dai et al., 2002). If the spectrometer has low spinning speeds, a pulse sequence called TOSS (Total Suppression of Side Bands) (Lorenz et al., 2000; Mao et al., 2000; Preston, 2001; Lorenz and Preston, 2002; Duer, 2002) can be utilised but these spectra may not allow for the C compositions to be quantified accurately (Preston, 2001).

Alternatively, a simple approach is to mathematically subtract the SSB, but that may not be accurate due to underestimation of hidden/unidentified SSBs. NMR spectra of natural OM can have SSB from carboxyl groups (Wilson, 1987). Aromatic structures have very small interference from SSB (Franz et al., 1992; Martinez et al., 1999; Duer, 2002), and aliphatic chains in amorphous materials do not generate SSBs (Duer, 2002).

5.3.7 Magic Angle Spinning

Magic angle spinning (MAS) was developed to counteract CSA and dipolar interactions. When the sample is rotated about an axis inclined at 54.7° relative to the direction of the applied magnetic field, all dipolar interactions are effectively removed allowing solid samples to behave in a similar way to solutions in NMR spectroscopy (Knicker, 2011). MAS can remove broadening of resonances from the solid-state NMR spectra and the narrower resonances result in finer features being visible (Andrew, 1981). Standard spinning frequencies range from 12 kHz - 35 kHz, and higher frequencies can remove intermolecular interactions. Coupling MAS with CP enables high-resolution NMR spectra to be obtained for ^{13}C nuclei (Andrew, 1981). To eliminate the broadening caused by the CSA/dipole interactions the spinning speed frequency has to be at least as high as the line broadening caused by these interactions (Knicker, 2011).

5.3.8 Direct Polarisation

Direct polarisation (DP) (also called Bloch Decay (BD) or Single Pulse Experiment (SPE)) NMR directly excites the ^{13}C nuclei allowing quantification of the C in the sample (Simpson et al., 2011). DPMAS experiments are based on the principle of suppression of ^{1}H-^{13}C dipolar interactions and reduction of anisotropy using MAS; this technique is then combined with a 90° pulse (Simpson et al., 2011). Experiment time is lengthy because of the low abundance of ^{13}C, but this technique is necessary for samples with low/no protonation, e.g. aromatic cores of coal (Franz et al., 1992; Jurkiewicz and Maciel, 1995; Kidena et al., 1996; Maroto-Valer et al., 1996; 1998; Freitas et al., 1999), and it has also been used for humic samples (Smernik and Oades, 1999; 2000a,b; Mao et al., 2000; Conte et al., 2004). DP of the ^{13}C nuclei with long relaxation delays is the only reliable method to prevent underestimation of

C content in the sample (Johnson et al., 2005; Berns and Conte, 2011). Unlike CPMAS, DPMAS can detect unprotonated carbons and can use high spinning frequencies to eliminate the presence of SSBs. Poor sensitivity is the main disadvantage of the DP experiments (Xing et al., 1999); experimental running times are lengthy because many scans are required to achieve good signal to noise ratios.

5.3.9 Paramagnetic Species

Paramagnetic species are undesirable in organic samples subject to analysis by solid-state NMR spectroscopy. Unfortunately, considerable quantities of OC are in strong associations with inorganic components that can have high ash contents and high concentrations of paramagnetic species (Gélinas et al., 2001). These paramagnetic species broaden NMR signals and produce spectra with strongly overlapping resonances. Additionally, these species can cause selective quenching of signal intensity by decreasing relaxation times of specific C functional groups, and OC in close associations to paramagnetic centres may be undetectable (Schmidt et al., 1997). To improve the sensitivity and resolution of the NMR spectrum it is strongly recommended to remove paramagnetic contaminants (Skjemstad et al., 1994; Schmidt et al., 1997; Conte et al., 2001; Conte et al., 2004).

The best method to decrease/remove inorganic and paramagnetic species is the use of HF and HF/HCl solutions to dissolve silica (HF) and to removes carbonates (HCl; Gélinas et al., 2001). Unfortunately, concentrated solutions of these acids can cause chemical alteration of the OM and loss of OC resulting from the hydrolysis and solubilisation of chemically labile biomolecules, such as carbohydrates (Schmidt et al., 1997; Gélinas et al., 2001). The harsh HCl/HF demineralisation methods were developed for de-ashing coal or kerogen and these methods have been applied to sediments and soils in recent years (Preston and Newman, 1992; Skjemstad et al., 1994; Schmidt et al., 1997; Gélinas et al., 2001). Alteration and OC losses can be more severe for OM in surface sediments because a large fraction of this OC can be composed of labile biomolecules that are preserved due to their close association with the mineral matrix (Mayer, 1994; Keil et al., 1994). Unfortunately, >50% OC can be lost when recently deposited marine sediments are demineralised using HCl/HF (Durand and Nicaise, 1980; Gélinas et al., 2001). The best approach to enhance the signal of organic sample for NMR

studies is to concentrate the OC by removal of sufficient quantities of inorganic and paramagnetic species while attempting to limit OC losses (Gélinas et al., 2001).

5.3.10 Chemical Shift Anisotropy

Chemical shift anisotropy (CSA) effects the relaxation of molecules in solid-state NMR experiments and can result in the spectra having very broad line shapes. CSA is not an issue in solution-state NMR experiments because the molecules continuously move and any directional differences in shifts within the molecule will be averaged to a single value (Claridge, 2009). In solid-state, the chemical shift depends on the orientation of the bond relative to the applied static magnetic field (Popov and Hallenga, 1991; Claridge, 2009). CSA relaxation is dependent on the square of the applied field, thus it has greater significance at higher field strength which creates broader line-widths; thus the potential benefits of greater dispersion and sensitivity provided by higher field strengths may be lost (Claridge, 2009).

5.3.11 Dipolar and Quadrupolar Interactions

Dipolar and quadrupolar interactions can generate very broad lines in an NMR spectrum (Popov and Hallenga, 1991). Dipolar interactions are the energy relationship between two magnetic moments, and these interactions are most relevant to many nuclei with spin $^1/_2$ (Claridge, 2009). When two dipoles are in close proximity they can attract or repel each other depending on their relative orientations, and this results in faster relaxation times (Claridge, 2009). The strength of the coupling is dependent on the gyromagnetic ratio, the distance between the nuclei, as well as their orientations (Laws et al., 2002). The spin–spin coupling interaction can be suppressed by decoupling the unobserved nuclei, which is mostly ^{1}H (Knicker, 2011). Quadrupolar interactions are purely electrostatic effects and are several orders of magnitude larger than dipolar interactions (Popov and Hallenga, 1991). These interactions only occur in nuclei with spin $>^1/_2$. These nuclei have an electric quadrupole moment which is a property similar to the gyromagnetic ratio, and this can interact with the electric field gradients (Schurko, 2009). Acquisition of NMR spectra of quadrupolar nuclei is challenging because of the rapid relaxation of the nuclei and generally very broad peaks are observed (Schurko, 2009).

5.3.12 Complete MultiPhase NMR

High resolution magic angle spinning (HR-MAS) ^{1}H NMR spectroscopy has been extended to study molecules in solution/swollen-state, but components in the solid phase are not observable (Shirzadi et al., 2008a,b) because the probe has relatively low power handling, thus preventing the applications of solid-state techniques (Courtier-Murias et al., 2012). Following on from the new insights provided using HR-MAS NMR spectroscopy, Simpson's Research Group (University of Toronto) developed a complete multiphase (CMP) probe that was introduced in a publication by Courtier-Murias et al. (2012). The probe was designed to study heterogeneous systems, such as soils and sediments which can be composed of numerous phases that determine their environmental properties (Courtier-Murias et al., 2012). Traditionally, each phase has been studied separately using NMR spectroscopy. However, changing the natural phases of the sample can remove important structural information (Courtier-Murias et al., 2012). Therefore, CMP-NMR was developed to study all bonds in all phases in whole unaltered natural samples (Courtier-Murias et al., 2012).

"The CMP-NMR probe is built with high power circuitry, Magic Angle Spinning (MAS), fitted with a lock channel, pulse field gradients, and is fully susceptibility matched" (Courtier-Murias et al., 2012). Therefore, the probe can be used to carry out all the experiments possible using a HR-MAS probe without compromising power handling to allow the full range of solution-, gel- and solid-state experiments (Courtier-Murias et al., 2012). This technology has provided new opportunities to study both structures and interactions independently in each phase as well as interactions between phases, within a heterogeneous sample (Courtier-Murais et al., 2012), and will provide new insights into natural OM.

The CMP-NMR probe was not developed with the intention to replace solution, solids, or HR-MAS probes (Maas et al., 1996) but to complement existing NMR experiments and to provide new possibilities to study *in situ* interactions at interfaces and conformations of structures (Courtier-Murias et al., 2012). This probe is in its infancy, and is currently not available commercially; however, it holds the key to more in-depth studies on whole samples. Future studies will allow assessment of organic samples with minimal pre-processing to provide insights to their composition and their reactivity in the environment.

5.3.13 ^{15}N NMR Spectroscopy

Nitrogen (N) NMR spectroscopy is a sensitive method for the study of natural OM because N is one of the major elements involved in the genesis of organic compounds, and N-containing functional groups are of great importance in organic and biochemical reactions (von Philipsborn and Müller, 1986). Traditionally the quadrupolar ^{14}N nucleus used because of its high natural abundance (99.6%), but it is not suitable for high resolution NMR because of its large nuclear quadrupole moment (Knicker et al., 1993). The ^{15}N isotope (spin ½) has low NMR sensitivity because it has low natural abundance (0.4%) and a small gyromagnetic ratio (Knicker et al., 1993) but the introduction of FT-NMR spectroscopy has allowed this nucleus to be utilised (von Philipsborn and Müller, 1986). Despite these sensitivity issues, this nuclei provide spectra that are easy to interpret because there are relatively few non-equivalent N atoms and the chemical shift differences are usually large (von Philipsborn and Müller, 1986).

5.3.14 2 Dimensional NMR Spectroscopy

To gain an overview of the bulk chemical composition of a sample it is useful to divide the spectrum into chemical shift regions assigned to the most likely chemical groups giving rise to these resonances (Knicker, 2011). However, due to the complex nature of OM samples it is difficult to identify clear boundaries that separate the different C groups from each other, and some overlapping can occur (Knicker, 2011). A solution to this problem is to study samples using multi-dimensional NMR spectroscopy. 2 dimensional (2D) NMR spectroscopy provides valuable information about heterogeneous samples due to the increased spectral dispersion, as well as additional connectivity information that allow detailed assignments of the chemical functionalities and of structural components present (Lam et al., 2007; Byrne et al., 2010). Conventional 1D NMR spectra are plots of intensity *versus* frequency, whereas in 2D the intensity is plotted as a function of two frequencies, usually called F1 and F2. The position of each peak is specified by two frequency co-ordinates corresponding to F1 and F2. A 2D NMR experiment is acquired in a similar way to a 1D experiment; the main difference being that following the 90° pulse, there is a variable evolution time prior to the next pulse (Balci, 2005).

Buddrus et al. (1989) were the first to report the application of 2D solution-state NMR spectroscopy. Since that time there have been phenomenal developments in relation to NMR spectroscopy experiments, many of which can be adapted for NOM studies. The 2D NMR experiments are based on bond interactions (homonuclear and heteronuclear), through space interactions, and diffusion. There is a variety of experiments available (refer to Simpson and Simpson (2009) for the "Top Ten" NMR experiments for the study of natural OM in solution), suitable for a variety of nuclei, in a number of dimensions. However, the ^{1}H-^{1}H and ^{1}H-^{13}C aspects will be reviewed as they apply to the research topic under study.

5.3.15 Homonuclear Experiments

5.3.15.1 TOCSY

TOCSY (total correlation spectroscopy, also called HOHAHA (homonuclear Hartmann-Hahn spectroscopy)) is an experiment which can help interpret the spectra of complicated molecules (Simpson, 2012), especially those with large interconnected networks of spin couplings. TOCSY provides information about protons that are in the same spin system (a continuous chain of spin-spin coupled protons, Simpson, 2001). Magnetisation from the first proton is transferred to the next proton, and so forth. The longer the mixing time, the greater the likelihood that a cross peak from a spin that is many bonds away will be observed (Simpson, 2012). Taking a slice from the TOCSY experiment can show ^{1}H 1D resonances in greater detail, thus aiding assignment of peaks.

5.3.15.2 NOESY

The nuclear Overhauser effect (NOE) relates to relaxation of one nuclear spin induced by a neighbouring spin, and it is observed as a change in intensity of one resonance when the intensity of a neighbouring resonance is perturbed (Gemmecker, 1999). The effect is strongly influenced by the internuclear distance (Williamson, 2006). NOESY is used to provide information about protons that are in close proximity (or any other nuclei with spin > 0) and have magnetic dipole interactions, but are not connected by chemical bonds (Balci, 2005). In order to determine the through space relationship between the protons, all the protons in a molecule must be irradiated one by one which is time consuming, and there can be issues arising from

protons whose chemical shifts are very close to each other (Balci, 2005). Any ^{1}H nuclei that may interact with one another through a dipolar relaxation process will appear as a cross peak in a NOESY spectrum. The cross peaks in NOESY spectra arise from spatial interactions, and therefore this type of spectroscopy is used to study the configurations and conformations of molecules (Pavia et al., 2009). Very large molecules have restricted mobility in solution which allows additional time for the NOE interactions to develop (Pavia et al., 2009). For natural OM samples, NOESY is useful for identifying groups such as Ar-O-CH_3 (methoxyl in lignin) that do not exhibit scalar through-bond couplings in TOCSY (Simpson et al., 2011), and for confirmation of protein resonances identified in 1D NMR experiments.

5.3.16 Heteronuclear Experiments

Heteronuclear correlation experiments are invaluable for environmental research, especially correlations with those involving ^{1}H-^{13}C nuclei (Simpson et al., 2011). These experiments decrease spectral overlap due to the additional dispersion provided by the ^{13}C dimension, allowing assignments that are not possible in the 1D NMR (Simpson, 2001). Unlike the 2D homonuclear experiments, heteronuclear spectra do not exhibit a spectrum diagonal. A ^{1}H-^{13}C bond will produce a resonance at the point of intersection of the ^{1}H and ^{13}C chemical shifts (Simpson et al., 2002). Early 2D heteronuclear experiments used the heteronuclear multiple quantum coherence (HMQC) experiment (Bax et al., 1983) and this has been instrumental in the study of HSs (Schmitt-Kopplin et al., 1998; Kingery et al., 2000; Simpson et al., 2001; 2002). More recently heteronuclear single quantum coherence (HSQC) has been developed and it has been shown to outperform HMQC for environmental samples (Simpson et al., 2002; Simpson et al., 2011). The HSQC experiment has one important advantage over HMQC; the ^{1}H–^{1}H multiples broaden the intersecting resonance in either dimension, providing increased sensitivity and resolution (Simpson et al., 2002). The HSQC experiment requires multiple refocusing periods and the experiment requires very precise pulse calibration to minimise artefacts and optimise sensitivity (Simpson et al., 2002).

5.3.17 2D NMR Applications to Humic Samples

There have been a number of 2D experiments which have provided new insights into natural OM. Kelleher and Simpson (2006) compared the multi-dimensional NMR of extracted HSs to that of biopolymers (e.g. bovine serum albumin, Kraft lignin, cutin) and found that nearly all the resonances in the HSs fractions could be assigned to intact or degrading polymers. HU (DMSO-H_2SO_4 extracts) was studied by Simpson et al. (2007) using conventional 1D ^1H and DE ^1H NMR, and 2D TOCSY and ^1H-^{13}C HMQC. The spectra showed that the major components in the HU isolate were peptides, aliphatic species, carbohydrates, lignin and some components from bacterial inputs (e.g. peptidoglycan and lipoprotein).

5.4 GC/MS

Chromatography is a physical separation method. Components of the analyte distribute themselves between two phases; a stationary phase and a mobile phase. The analyte must interact with the stationary phase causing the components to separate and subsequently they are identified based on their retention time on the column. Gas is the mobile phase and the stationary phase is a liquid coated on a solid support. Molecules that are more soluble in the stationary phase will travel slower and therefore, take longer time to elute.

GC has been combined with MS in order to identify and quantify volatile and semi-volatile organic compounds in complex mixtures by a spectral interpretation and comparing the chromatograms with reference spectra (Hites, 1997). The GC and MS instruments are compatible because for both techniques the sample is in the vapour phase, and small quantities of analyte are used. However, a pressure difference between the two systems causes some problems. The eluents from the GC are at a pressure of ca. 760 Torr, but the MS operates at a vacuum ca. 10^{-6} to $l0^{-5}$ Torr (Hites, 1997). There have been a number of approaches to solve the pressure incompatibility problem. The earliest approach was to split a small fraction of the GC effluent into the MS (Gohlke, 1959). Generally ca. 1-5% of the GC effluent was directed into the MS and the remainder was removed from the system (Hites, 1997), but this technique compromised the sensitivity of the analytical technique. Improved interfaces have decreased the pressure of the GC effluent, all of the analyte molecules from the GC can continue into the MS, and the carrier gas can be

separated from the analyte which increases the concentration of the analyte in the carrier gas stream (Hites, 1997).

Mass spectrometers have five main components: (1), the sample inlet; (2), the ion source; (3), the mass analyser; (4), the detector; and (5), the data system. The analyte (atmospheric pressure) is injected into the sample inlet (low pressure), and continues to the ion source where it is transformed into gas phase ions, and these are accelerated by an electromagnetic field (Pavia et al., 2009). The simplest method to convert molecules (from volatile samples) to ions is using electron ionisation (EI); a beam of high energy electrons is emitted from a filament (several thousand degree Celsius) that strike the stream of molecules (from the sample) removing an electron from the molecule, creating a cation (Pavia et al., 2009). The cation is then directed towards a series of accelerating plates by a repeller plate (positive electrical potential), and the large potential difference (1-10 kV) at the accelerator plates produces a beam of rapidly travelling cations (Pavia et al., 2009). Non-ionized molecules and anions are not accelerated towards the mass analyser. Large MW, non-volatile molecules require desorption ionisation (DI) techniques, such as matrix-assisted laser desorption ionisation (MALDI). This technique disperses the analyte in a matrix (non-volatile, relatively inert, and a reasonable electrolyte to allow for ion formation) and a beam of high intensity photons ionizes some of the analyte molecules which removes these from the matrix and these ions are then accelerated towards the mass analyser (pathway to mass analyser is similar to EI; Pavia et al., 2009). The ions are separated based on their mass-to-charge (m/*z*) ratio in the mass analyser. There are a number of variations of mass analysers available, e.g. quadrupole mass analysers, ion traps and time of flight analysers (TOF). Ion trap mass analysers are more sensitive than quadrupole instruments, while having similar resolution capabilities. A quadrupole ion trap mass analyser is composed of two hyperbolic endcap electrodes and a doughnut shaped ring electrode; an alternating current and an rf potential is applied between the endcaps and the ring electrode (Pavia et al., 2009). Sweeping the rf potential removes ions with increasing m/*z* ratios and these have an unstable trajectory that results in their removal from the trap in the axial direction towards the detector (Pavia et al., 2009). When the ions reach the detector they are counted and the current generated (proportional to the number of ions that strike the detector) is recorded and processed by the data system (Pavia

et al., 2009). The data are presented as a plot of the number of ions detected as a function of their m/*z* ratio. The high speed, resolution, and accuracy of MS make it an excellent instrument to resolve the components of complex molecule mixtures. Unfortunately, the challenge of resolving a variety of chemical details within large molecules has impeded specific structural assignments of natural OM, and there is a timely need for the establishment of well-defined reference materials (Hedges et al., 2000).

5.4.1 pyGC/MS

In contrast to the other spectroscopic methods available, pyGC/MS analysis is based on the heat destruction of the analyte and identification of the individual compounds liberated from the analyte, or of its altered pyrolysis products. The high temperatures break a compound into a number of volatile products that are characteristic of the original compound and the experimental conditions (Grob, 2004). The technique has advantages because small sample quantities are required (ca. 1 mg), run times are short, and chemical pre-treatments are not essential. Pyrolysis is a fast, sensitive, and reproducible procedure which releases structural units from large, non-volatile organic compounds (Larter and Horsfield, 1993; Hedges et al., 2000). The decomposition products from pyrolysis are separated in the GC column, and subsequently qualitatively and quantitatively analysed using MS. Very complex analytes produce some pyrolysis fragments that may not be possible to identify.

The scope and specificity of pyGC/MS can be enhanced by temperature programming pyrolysis filaments within the source of a MS, along with mild ionisation procedures (Boon et al., 1998; Hedges et al., 2000). There are a number of disadvantages associated with pyrolysis methods, such as: considerable alterations to the structures; low and unpredictable yields; and potential artefact formation from secondary reactions (Hedges et al., 2000). Thermochemolysis can improve the technique as it favours specific cleavage and/or the formation of volatile products, e.g. chemolysis mediated by tetramethylammonium hydroxide (TMAH) mediates concurrent chemolytic cleavage, and methyl derivatisation of refractory compounds, such as ether-linked lignins and carbon-linked coals (Hatcher and Clifford, 1997; Hedges et al., 2000). There have been pyGC/MS studies carried out on a range of macromolecules and natural OM in order to determine their compositions and

structures, e.g. lignin studied by Sáiz-Jiménez and De Leeuw, 1984; kerogen studied by van de Meent et al., 1980; soil OM studied by Sáiz-Jiménez and De Leeuw, 1986; HSs studied by Zhang et al., 1999; and HU studied by Fabbri et al., 1996.

5.5 References

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Chapter 6 Methodology – Sampling and Experimental Methods

This chapter will outline in detail the methods used in the laboratory to characterise the organic and inorganic components, as well as providing background information on sediment core sampling.

6.1 Field Sampling

The sediment samples were taken as part of cruise number CE09_04 on 28/Feb/2009 aboard the Marine Institute research vessel The *Celtic Explorer*. Dr. Catherine Dalton (a collaborator on the research project), and Fergus Melligan (a research student in the Carbolea Research Group), assisted in the sampling and labelling of the core samples. Four long core samples and four grab samples were taken at the same geographical position along a transect (Table 6.1, 6.2: Figure 6.1). The core samples were taken using a Vibrocorer (Geo-corer 6000). The grab samples were taken using a Day grab. The core positions were labelled A1001, B1002, C1003 and D1004 with A1001 being nearest the land and D1004 furthest from the land. The cores were sub-sectioned into 1 metre lengths and they were sealed in plastic core liners. Each core was labelled with cruise ship number CE09-04 followed by corer label VC (vibrocorer), site number and section number i.e. CE 09-04 Core 1 1/6. The top and bottom of each section was also labelled. Approximately 15 litres of surface sediment was collected in the Day grab. The grab samples were double bagged in ziploc® plastic bags and labelled with cruise number (CE09-04), Day Grab (D-Grab), site letter i.e. CE09-04 D-Grab A1001. A transect in the inner bay silt/clay depositional areas < 1.8 km from the coast was targeted for sediment extraction. This transect aimed to track the Corrib outflow freshwater plume while avoiding the main navigation channel (Dalton et al., 2010).

Table 6.1. Sediment coring positions.

Code/ Name	Latitude	Longitude	Northings	Eastings
VC-001/ Core 1	53°15 22.21’	09° 02 14.40’	223340.704	130817.056
VC-002/ Core 2	53°14 57.20’	09° 02 33.41’	222572.676	130453.368
VC-003/ Core 3	53°14 47.30’	09° 03 48.30’	222287.072	129060.248
VC-004/ Core 4	53°14 15.44’	09° 05 21.54’	221328.181	127316.336

Table 6.2. Environmental parameters, water depths and core lengths (practical salinity units, psu).

Code/ Name	Water Temperature	Salinity	Water Depth	Core Depth
VC-001/ Core 1	7.83 ^{o}C	29.220 psu	11.2 m	5.35 m
VC-002/ Core 2	7.96 ^{o}C	29.158 psu	12.4 m	5.59 m
VC-003/ Core 3	7.85 ^{o}C	27.802 psu	13.4 m	5.42 m
VC-004/ Core 4	7.85 ^{o}C	26.956 psu	13.5 m	4. 78 m

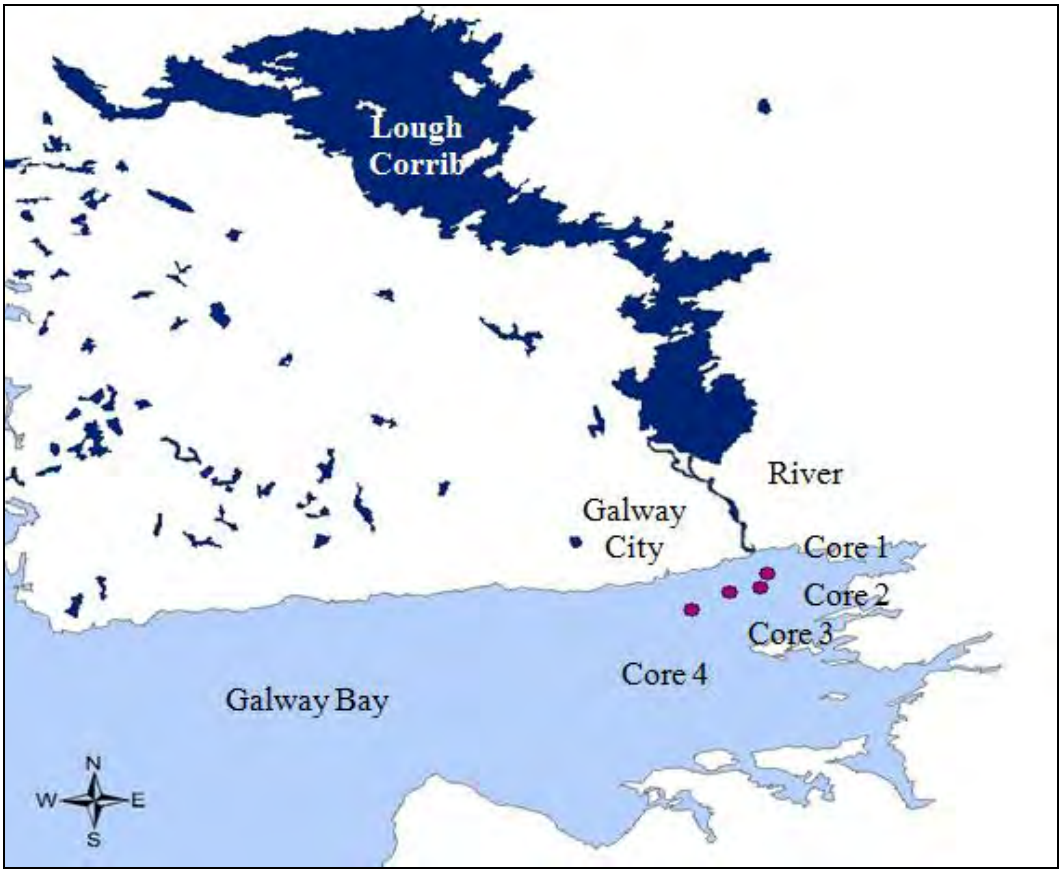

Figure 6.1. Map of coring positions in Galway Bay.

The River Corrib flows from Lough Corrib through Galway City and into Galway Bay. Lough Corrib has an area of 165 km^2 making it the second largest lake in Ireland and the Corrib river has annual mean discharges of ca. 95 m^3 s^{-1} (Goddijn and White, 2006). The current in Galway Bay flows in an anti-clockwise direction (Lei, 1995). There is a waste water treatment plant located on Mutton Island; the facility is approximately 1 km off-shore (53°15'16N, 09°03'16W). Secondary

treated effluent is discharged into the bay ca. 400 m to the south of the island via an outfall pipe (equipped with diffusers to increase dilution).

6.2 Choice of Samples for Analysis

The grab samples were analysed initially and these preliminary characterisation studies are reported in Ch. 7. The in-depth characterisation of organic matter (OM) was focused on the isolates from the sediment cores. Organic carbon (OC) was measured at 25cm intervals throughout all four cores (see appendices, 1-1). Based on this data it was decided that it would be necessary to bulk up the samples in order to extract adequate quantities of humic substances (HSs) to acquire sufficient material for analyses and characterisation. Core 1 and Core 4 were chosen for characterisation studies to illustrate the difference between the more terrestrially influenced sediments (Core 1) and the marine influenced sediments (Core 4) and their organic matter (OM) compositions. The top of the cores were targeted for extraction to provide information on recent sediments and the base of the cores were extracted to investigate humic compositions from older sediments. Core 2 was omitted from the study because of its close proximity to Core 1 (approx. 0.5 km distance, Figure 6.1) and Core 3 was omitted from the study due to its proximity to the waste water treatment plant, and due to the time constraints of the research period. Originally, it was planned to extract samples from the middle sections of Core 1 and 4; however, the OC concentrations were low and ^{14}C radio-carbon dating showed little variation in the ages of the sediments between the mid- and base core sediments. Additionally radiocarbon dating demonstrated a complex sediment chronology (see appendices, 1-3. Therefore rather than analyse a broad range of spatial and temporal samples, it was decided to focus on in-depth analysis of a just a few samples.

6.3 Extraction of Organic Matter from Bulk Sediment

Extraction of HSs follows the methodologies of Hayes et al., 1996; Song et al., 2008; 2011; Chang et al., 2014).

6.3.1 Sample Preparation

The sediment was placed in a solution of 1 M HCl and left to stand, allowing the HCl to react with the carbonates and for cation exchange to occur. Upon completion, the

sediments were washed thoroughly with distilled water to wash away the chloride ions. The supernatant was tested with silver nitrate, to ensure all the Cl^- was removed. The sediment was dried and weighed.

6.3.2 Sequential Exhaustive with Aqueous Solvents

(1) NaOH solvent (0.1 M) was added to a known weight of sediment. The extraction work reported here was carried out in an N_2 atmosphere to avoid oxidation.
(2) The sediment was mixed continuously in the alkaline media for 4 h to allow maximum contact between the sediment and the media.
(3) The pH of the media was lowered using 6 M HCl and the suspension was allowed to settle for 2 h.
(4) The supernatant was decanted and centrifuged at 1700 *g* for 19 min to remove solids.
(5) The supernatant was collected from the centrifuge tubes.
(6) The supernatant was filtered through 142 mm diameter, 0.2 μm cellulose acetate membrane filters, under 10 psi air. Filter pads were cleaned twice daily and the residue was returned to the sediment for further extraction.
(7) Repeated extraction was carried out with 0.1 M NaOH until the colour of the supernatant was negligible.
(8) The sediment was then extracted using a 6 M urea + 0.1 M NaOH solution. The procedure was carried out as described for the 0.1 M NaOH extraction.

6.3.3 Extraction of Humin using an Organic Solvent

The humin (HU) was isolated by following the method used by Song et al. (2011). HU components tend to concentrate in the clay/silt fractions. Therefore, following centrifugation of the sediment after the alkaline-urea extraction process, the recalcitrant HU remains concentrated in the dark clay/silt surface layer (Clapp and Hayes, 1999).

(1) The clay-sized fraction was isolated (using wet sieving and sedimentation, see 6.3.4) and air dried.
(2) The clay-sized fraction was mixed with DMSO + (98%) H_2SO_4 (94:6 *v/v*) using a clay-sized fraction/solvent ratio of 1:10.

(3) The clay-sized fraction suspension was shaken for 12 h in a glass bottle.
(4) The suspension was centrifuged (1700 *g*, 19 min) to remove solids.
(5) This process (steps 2-4) was repeated until the supernatant was virtually colour free.
(6) The extract (following centrifugation) was diluted with distilled water to pH 2.
(7) The precipitate which forms (DMSO-HU) was isolated by centrifugation, washed with distilled water, dialysed, and freeze dried.
(8) The DMSO-HU was de-ashed using HF.

6.3.4 Isolation and Preparation of the Clay-Sized Fraction

The clay-sized fraction was collected from the filter pads from the aqueous alkaline-urea extracts. The clay-sized fraction was washed with distilled water to remove residual urea. The clay-sized fraction was also isolated from the bulk sediment that had not been extracted (for unextracted clay-sized fraction samples), and also following the exhaustive aqueous extractions (for HU extraction). The sediment was wet sieved and the filtrate was transferred to graduated cylinders. The clay-sized fraction was collected based on the sedimentation of particles (Stokes' law).

Stokes' law relates the terminal settling velocity of a smooth, rigid sphere in a viscous fluid of known density and viscosity to the diameter of the sphere when subjected to a known force field (Foth, 1984).
The equation is as follows:

$$V = \frac{2gr^2(d_1 - d_2)}{9\eta} \qquad \textbf{Equation 6.1}$$

Where:
V = velocity of fall (cm sec^{-1})
g = acceleration due to gravity (980cm sec^{-2})
r = "equivalent" radius of particle (10^{-4} cm for clays)
d_1 = density of particle (gram cm^{-3})
d_2 = density of medium (gram cm^{-3})
η = viscosity of medium (dyne sec cm^{-2})

(1) Air dried sediment was weighed, and the weight was recorded.
(2) The sample was sonicated in a bath at 25°C for 30 min at 40 KHz.
(3) The sediment was wet sieved using 125 μm and 63 μm sieves, and distilled water. The fraction < 63μm was placed in a graduated cylinder and left to settle.
(4) It takes 8 hr for the coarse clay-sized fraction to settle 10 cm, according to the calculation of Stokes law (Equation 6.1).
(5) 10 cm of liquid was siphoned off (containing the fine clay-sized fraction in suspension) every 8 hr.
(6) The graduated cylinder was agitated and the suspension was allowed to settle for another 8 hr. This process was repeated until all the clay-sized fraction had been isolated.
(7) The clay-sized fraction was freeze dried to remove water.

6.3.4.1 Isolation of the Clay-Sized Fraction Sample for Characterisation

The clay-sized fraction (clay) was isolated from bulk sediments that had not been subjected to any chemical pre-treatment, following the method outlined in 6.3.4.

6.3.4.2 Isolation of Demineralised Clay-Sized Fraction

(1) A subsample of the clay-sized fraction was taken from the whole clay-sized fraction sample (see 6.3.4).
(2) The clay-sized fraction was reacted with 10% HF to remove the silicates. This procedure was repeated 10 times to ensure complete dissolution of the silicates.
(3) The demineralised clay-sized fraction was washed thoroughly with distilled water, and the sample was dialysed against distilled water until the conductivity was < 200 μS.
(4) The clay-sized fraction was air dried and ground.

6.3.4.3 Isolation of Insoluble Humin

A 10% HF solution was prepared from a 40% HF reagent and contained in plastic bottles.

(1) The clay-sized fraction sample (remaining following DMSO-H_2SO_4 extraction) was weighed and placed in a polyethylene bottle.

(2) 100 ml of solution was added and the sample was shaken for 16 hr.
(3) The supernatant was removed using a syringe and the procedure (steps 2-3) was repeated.
(4) The sample was then washed with distilled water a number of times (conductivity < 200 μS) to remove all the residual HF.
(5) The sample was dialysed and freeze dried.
(6) This sample is classified as DMSO-insoluble humin (DIHU).

6.4 Fractionation of Organic Matter

6.4.1 Choice of Resin for Fractionation

(1) Amberlite XAD-8, an acrylic ester based resin; white beads, 20-60 mesh size.
(2) Amberlite IR-120, a cation exchange resin; orange/brown beads, 16-50 mesh size.

Amberlite^{TM} XAD-8 resin is no longer commercially available; Superlite^{TM} DAX-8 has been substituted for it in recent studies. Fraction of the organic extracts using XAD-8 resin follows the method outlined by Hayes et al., (1996).

6.4.2 Cleaning of Resin

6.4.2.1 New Resins

New resins must be cleaned thoroughly prior to use. Cleaning requires three consecutive Soxhlet extractions using ethanol, and acetonitrile; the process follows the sequence:

(1) Wash with 0.1 M NaOH (over 2 weeks);
(2) Rinse with water;
(3) Rinse with hot ethanol;
(4) Rinse with hot water;
(5) Rinse with hot acetonitrile;
(6) Rinse with water;
(7) Wash with 0.1 M NaOH;
(8) Rinse with water;
(9) Rinse with hot ethanol; and
(10) Store in ethanol to prevent biological growth in the pores.

The XAD resin was packed into a glass column (up to 80% capacity). Distilled water (75–100 column volumes) was pumped through the newly packed columns to remove any residual ethanol.

6.4.2.2 Cleaning and Regenerating used XAD Resins

After XAD-8 resins are used for a number of samples, the sorptive capacity may decrease because of sorption of neutral fractions into pores of the resins. Hot ethanol is used to regenerate the resin.

(1) Soxhlet extraction with ethanol;
(2) Rinse with water;
(3) Rinse with ethanol;
(4) Rinse with water;
(5) Wash with 0.1 M NaOH;
(6) Rinse with 0.1 M HCl;
(7) Wash with 0.1 M NaOH;
(8) Rinse with 0.1 M HCl; and
(9) Rinse with 0.01 M HCl (pH 2).

6.4.2.3 Routine Cleaning and Regeneration of XAD Resin

(1) Back elute the column with 0.1 M NaOH until the eluate is clear;
(2) Pump 0.1 M HCl down through the column until the pH of elution is close to 2; and
(3) Pump 0.01 M HCl down through the column (one column volume) before pumping on any sample.

6.4.2.4 Regenerating of the Ion-Exchange Resin

A 2 L column was packed with Amberlite IR-120 resin (ca. 60% capacity), and 4 L of 1 M HCl was passed through the resin at a drop-wise rate. The column was then flushed with distilled water until the conductivity of the effluent is close to that of the distilled water (<10 μS).

6.4.3 Cleaning Visking Dialysis Tubing

(1) Dry membranes should be stored in a cool, dark place with a minimum relative humidity of 35%. No drying out should occur before use.
(2) Glycerine from the manufacturing processes must be removed from the membranes before use. This can be removed by washing with distilled water.
(3) After cleaning, the membrane can be stored (fully immersed) in 1% formaldehyde at 4 °C for a long period of time or stored in distilled water for a short period of time.

Visking dialysis membranes are resistant to a wide range of chemicals but may be damaged by some chemicals at certain concentrations. Damage can cause losses of materials, or contamination of samples. The pH range tolerance of the membrane ranges from 2 to 12. Cellulose acetate membranes have good tolerance for acid (HCl <5%) and urea. They have a poor tolerance for HF solution (25%), DMSO, and high concentrations of sulfuric acid. Therefore, these membranes should not be used for undiluted DMSO-H_2SO_4, and for undiluted HF.

6.4.4 Fractionation of the Humic Extracts using Resin technology

(1) The extract was diluted with distilled water and was adjusted to pH 2.0 (HCl), and left to stand overnight.
(2) The extracts were applied to XAD-8 resin [(poly)methylmethacrylate] (Rohm and Haas, Philadelphia).
(3) Two column volumes of 0.01 M HCl were pumped through the column to ensure that the entire sample had passed through the column.
(4) The resin was desalted with distilled water until the effluent conductivity was < 100 μS cm^{-1}.
(5) Back elution was carried out using 0.1 M NaOH, and the eluate was adjusted to pH 1.0.
(6) The eluates were left to stand overnight at 4 °C, and the HAs that had precipitated were isolated by centrifugation (1700 *g*), dialysed until chloride free, and freeze dried to give the HA fraction.
(7) The supernatant (FA at pH 1.0) was desalted by pumping it back on the XAD-8 resin and passing distilled water through the column until the effluent conductivity was <100 μS cm^{-1}. The column was then back eluted using 0.1 M

NaOH. The effluent was H^+-exchanged (Amberlite IR-120, H^+-form; Rohm and Haas, Philadelphia) and the eluate was freeze dried to give the FA fraction.

6.4.5 Recovery of DMSO-Humin

(1) Distilled water was added to the DMSO-H_2SO_4 extract until the extract reached pH 2.

(2) A precipitate was allowed to form at 4°C for 12 - 16 h and the medium centrifuged (1700 *g,* 30 min) to isolate the DMSO precipitate (DMSO HU).

6.4.6 Deashing of Humic Fractions

(1) A 0.3 M HF/ 0.1 M HCl solution was carefully prepared in a plastic bottle with distilled water.

(2) Humic fractions were deashed by adding 100 ml of the HF/HCl solution and shaking for 12 - 16 h.

(3) The mixture was allowed to settle down for at least 4 h and decanted. The supernatant was centrifuged (1700 *g*, 30 min) to separate the precipitate (or the supernatant was removed with a syringe).

(4) The HF digestion was repeated one more time.

(5) The de-ashed humic fractions were washed with distilled water and centrifuged a number of times before being transferred to clean Visking dialysis tubing. The humic fractions were dialysed until the conductivity was >200 μS cm^{-1}, and the fractions were freeze dried.

6.5 Percent Organic Carbon (Walkley Black method)

The organic carbon (OC) content of the sediment was measured using the Walkley Black Method (Walkley and Black, 1934; Allison, 1965). This is a titrimetric method in which excess potassium dichromate ($K_2Cr_2O_7$), in the presence of sulfuric acid, is measured following oxidation of the OC in the sample. Excess dichromate is back titrated with ferrous sulfate ($FeSO_47H_2O$). The amount of OC oxidised was measured by calculating the amount of dichromate reduced. However, the recalcitrant OC is not determined; therefore a correction factor of 1.33 is used in the calculation as shown in Equation 6.2.

6.5.1 Reagents

(1) 0.167 M Potassium dichromate ($K_2Cr_2O_7$);

(2) Sulfuric acid (98%);

(3) *o*-phenanthroline-ferrous complex indicator;

(4) 0.5 M Iron (II) ($FeSO_4.7H_2O$) solution,:

6.5.2 Titration Procedure (Allison 1965)

(1) Grind the sample (to particle size <500 μm).

(2) Transfer a weighed sample, containing 10 to 25 mg of organic-C, but not in excess of 10 g of sample, into a 500 ml wide mouth conical flask;

(3) Add 10 ml of 0.167 M $K_2Cr_2O_7$, and swirl the flask gently to disperse the clay-sized fraction/sediment;

(4) Rapidly add 20 ml of concentrated H_2SO_4. Immediately swirl the flask gently until the clay-sized fraction/sediment and reagents are mixed, and then swirl more vigorously for 1 min. Allow the flask to stand for 30 min;

(5) Add 200 ml of distilled water to the flask;

(6) Add 3-4 drops of *o*-phenanthroline indicator and titrate the solution with 0.5 M $FeSO_4.7H_2O$.

(7) As the endpoint is approached, the solution takes on a greenish cast and then changes to dark green. At this point add the ferrous sulfate drop by drop until the colour changes sharply from dark green to dark red.

(8) Make a blank determination in the same manner, but without clay-sized fraction/sediment, to standardise the $K_2Cr_2O_7$.

Calculate the results according to the following formula, using a correction factor f=1.33.

$$\frac{\left(moles\ K_2Cr_2O_7 - \frac{moles\ FeSO_4.7H_2O}{6}\right) \times 18 \times f \times 100}{sample\ (g), on\ a\ dry\ weight\ basis} \qquad \textbf{Equation 6.2}$$

Dry combustion of sediment is also cited in the literature as a method for determining OC content (Cummins, 1962). These methods involve measuring weight loss, or collection of the CO_2 evolved. However, the Walkley Black method is

preferred due to its simplicity and ease of obtaining results. Loss on ignition techniques are considered unreliable due the removal of constitutional water from clays and silts (Sherman, 1951; Cummins, 1962). The Walkley Black method is subject to inaccurate results if there is chloride or iron (II) present in the sample; this causes over-estimation of OC concentration. However, when the sample is dried, iron (II) is oxidised to iron (III), thus the effect of iron (II) is minimal (Combs and Nathan, 1998).

6.6 Moisture Determination

(1) A sample (ca. 20 mg) was weighed, to four decimal places, into a clean, dry, porcelain boat of known weight.
(2) The boat was placed in an oven at 105 °C for 24 hr.
(3) The boat was removed from the oven and cooled in a dessicator. The boat was reweighed and the moisture content calculated using the equation.

$$\%\, Moisture = \frac{(mass\ of\ wet\ sample - mass\ of\ dry\ sample) \times 100}{mass\ of\ wet\ sample}$$ **Equation 6.3**

6.7 Ash Determination

(1) A sample, previously dried at 105 °C for 24 hr, was weighed, to four decimal places, into a clean dry porcelain boat of known weight.
(2) The boat was placed in a furnace and heated to 550 ° C using a temperature controlled programme.
(3) The boat was removed from the furnace and placed in a dessicator to cool.
(4) The boat was reweighed and the ash content calculated using the equation.

Equation 6.4

6.8 Particle Size Separation

(1) Bulk sediment was wet sieved using 125 and 63µm sieves.
(2) The material retained in the sieves was regarded as coarse sand/shell debris and fine sand respectively.
(3) The material which passed through the 63µm sieve was transferred to graduated cylinder and agitated. The suspension was allowed to stand and the clay-sized fraction was isolated based on the calculation of Stokes law. The cylinder was agitated and allowed to settle a number of times in order to isolate the entire clay-sized fraction. The silt particles collect at the base of the cylinder.
(4) The clay-sized fraction suspension was centrifuged at 1700 *g* for 20 min to collect the clay-sized fraction and the excess water was discarded.
(5) The clay-sized fraction was freeze dried.

6.9 Elemental Analyses

The C, N and H contents of humic fractions were calculated on moisture and ash-free samples. The samples were analysed at The Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand and in the University of Limerick, Ireland.

6.9.1 University of Otago

Each sample was precisely weighed (using a Mettler UMT2 microbalance) into lightweight tin capsules. The elemental analyser (Carlo–Erba EA 1108) maintained a constant stream of helium in the combustion tube during the analyses. Samples were analysed in duplicate and the average measurements were recorded.

6.9.2 University of Limerick

The instrument used for analyses was an Elemental Vario el Cube analyser. A sulfanilic acid/vanillin standard was used to calibrate the instrument. The method and details of the instrument were outlined by Melligan (2012).

(1) The sample (5-10 mg) was accurately weighed in an aluminium boat that was sealed and placed in the auto-sampler.
(2) The samples were dropped into the combustion tube, which was held at 1150 °C, oxygen was then purged into the combustion tube; additional oxygen was

supplied by WO_3 (prevents formation of non-volatile sulfates and binds alkali elements), to ensure complete combustion.

(3) The product gas then passed through a reduction tube (packed with copper wire) in order to convert NO_x and SO_3 into N_2 and SO_2, respectively. Volatile halogen compounds were trapped in silver wool at the top of the reduction tube. The CO_2, H_2O and SO_2 were then trapped in individual thermal adsorption columns. Water is removed from the CO_2, SO_2 and N_2 stream using Sicapent (P_2O_5) adsorption tubes. N_2 is not adsorbed in an adsorption column and is therefore, the first component to enter the thermal conductivity detector (TCD). Once all the nitrogen has passed through the detector the adsorption columns are heated individually to desorb the gases at different times, and these gases are quantified using the TCD. The maximum capacity of the adsorption columns is 40 mg C, 3 mg H, and 6 mg S for the CO_2, H_2O and SO_2 columns respectively.

(4) Each sample was run in triplicate to generate average C, H, N, S values.

6.10 $\delta^{13}C$ and ^{15}N Analyses

$\delta^{13}C$ and $\delta^{15}N$ isotope signatures were measured at the Water Research Centre, Monash University, Victoria, Australia. The organic fractions were analysed using an ANCA GSL2 elemental analyser coupled to a Hydra 20-22 isotope ratio mass-spectrometer (Sercon Ltd., UK). The precision was 0.1‰ for both ^{13}C and ^{15}N. The standard used was Pee Dee Belamnite limestone. Results of the isotope analyses are expressed in terms of δ values (‰), for example:

$$\delta^{13}C = \left[\frac{(Rsample)}{(Rstandard)} - 1\right] \times 1000 \qquad \textbf{Equation 6.5}$$

Where R = ratio of $^{13}C/^{12}C$.

6.11 Sugar Analyses

The method used for sugar analyses was developed and outlined by Hayes (2011).

6.11.1 Acid Hydrolysis of Sugars

(1) Add ca. 300 mg (+/- 10 mg) of sample to a pressure tube and record the weight to the nearest 0.1 mg.

(2) Tare the scales and add 3.00 ml (4.92 g) of sulfuric acid (72%) using an automatic titrator. Record the volume dispensed and the weight on the scales (to the nearest 0.1 mg).
(3) Using a glass rod, mix the acid and sample for 30 sec.
(4) Place the pressure tube in the water bath (30 °C). Stir the sample every 10 min over the next 60 min.
(5) After 60 mins, take the pressure tube out of the water bath and place it on the scales. Add 84 g of deionised water.
(6) Seal the pressure tube and invert a number of times before placing it into the autoclave rack.
(7) Turn on the autoclave (121 °C for 60 min).
(8) When the autoclaving is complete, remove the rack and allow the hydrolysates to cool slowly to near room temperature.
(9) Filter the hydrolysate using a pressure filter and collect the filtered hydrolysate for ion chromatography.

6.11.2 Ion Chromatography

All analyses conducted on D-fructose, D-glucose and sugar derived derivatives were carried out on an Ion Chromatography Ion exchange -3000 system (Dionex Corp., Sunnydale, CA) equipped with an auto sampler with dual pump column oven and integrated flow detector (AS,10 μL sample loop, Dionex Corp., Sunnydale, CA). The analyses of standards and samples were carried out at 18 °C by isocratic elution with deionised water (18.2 MΩ.cm) at a flow rate of 1.1 ml/min using a Dionex CarboPac PA1 carbohydrate column. The column was reconditioned using a mixture of 0.4 M sodium hydroxide and 0.24 M sodium acetate after each analysis. For ion chromatography analysis, a 25 mg sample of the reaction media is diluted with 1g of deionised water. The sample was then was filtered before the sample was analysed.

6.12 Fourier Transform Infrared spectroscopy

6.12.1 Conventional FTIR

6.12.1.1 Reagents and Equipment

Infrared spectra were recorded on a Bomen MB spectrophotometer with a resolution of 4 cm^{-1}, from 4000 to 400 cm^{-1} (Swift, 1996). Pellets were prepared using 1 mg of HA to 100 mg of KBr (spectroscopic grade) (MacCarthy and Rice, 1985). The mixture was pressed to obtain a transparent pellet. Thirty two scans were averaged to decrease noise. Background scans are taken before analysis to increase the accuracy of the spectra obtained.

6.12.1.2 Procedure

(1) The KBr was dried in an oven at 105°C, and left in a desiccator prior to use.
(2) The sample (ca. 1 mg) was placed into a quartz mortar with a stainless steel spatula and mixed with the KBr powder (ca. 100 mg), quickly to avoid moisture uptakes. The mixture was ground sufficiently using a quartz pestle.
(3) The KBr pellet was pressed for one min under a pressure of 10 tonnes.
(4) The KBr pellet was placed into the sample holder and the FTIR spectrum was recorded by scanning from 4000 to 400 cm^{-1}, averaging 32 scans at 1.0 cm^{-1} interval, and with a resolution of 4.0 cm^{-1}. The spectrum is processed and analysed by OMNIC E.S.P 5.1 software package (Nicolet Corp.).
(5) The background spectrum was subtracted from the analyte spectrum to eliminate bands from atmospheric CO_2.

6.12.2 ATR-FTIR

6.12.2.1 Reagents and Equipment

ATR-FTIR spectra were recorded on a Varian Scimitar FTS 1000 Fourier Transform Infrared (FTIR) spectrometer fitted with a Specac MKII Golden Gate single reflection ATR cell and ZnSe optics. Freeze dried samples were desiccated in the presence of P_2O_5 under vacuum, prior to analysis. The samples were scanned 256 times to decrease noise.

6.12.2.2 Procedure

(1) Dry, powdered samples were placed on the diamond (internal reflection surface).
(2) The FTIR spectrum was recorded by scanning from 4000 to 600 cm^{-1}.
(3) The background of air was subtracted before the FTIR spectrum for each sample was recorded. This eliminates the peaks caused by atmospheric CO_2.

6.13 UV-Vis Spectroscopy

UV-Vis spectra were recorded using a Varian Carey 3000. The analysis was carried out by dissolving 5 mg of the humic isolate in 10ml of 0.05 M $NaHCO_3$ solution (Giovanela et al., 2010). The absorbances were recorded at 270nm and 407nm (A_2/A_4) (Giani et al., 2010); and 465nm and 665nm (E_4/E_6) (Chen et al., 1977).

6.14 Nuclear Magnetic Resonance (NMR) Spectroscopy

Solid-state NMR spectra were acquired in Monash University with assistance from Dr. Katherine Narin, and solid-state, solution-state and swollen-state NMR spectra were acquired in the University of Toronto with assistance from Prof. Andre Simpson and Dr. Andre Sutrisno.

6.14.1 Solid-State NMR Spectroscopy – Monash University

A 4 mm MAS probe was used. Dry solids were packed into 4 mm o.d. (outside diameter) zirconia rotor and sealed with a Kel-F cap. ^{13}C ramped-amplitude cross polarisation magic angle spinning (^{13}C RAMP CP-MAS) spectra were acquired using a 300 MHz Bruker NMR spectrometer (7T magnetic field), with a 1 s recycle delay, using a RAMP CP pulse sequence, with a 1 ms contact time, 8 kHz spin speed. The number of scans acquired varied depending on the signal-to-noise (S/N) of the spectrum. 100 Hz line broadening was applied to all the spectra acquired.

6.14.2 Solid-State NMR Spectroscopy – University of Toronto

A 4 mm BB ^1H–X probe was used. Dry solids were placed directly in the 4 mm o.d. zirconia rotors and sealed with Kel-F caps. ^{13}C RAMP CP-MAS spectra were acquired using a 500 MHz (11.7T magnetic field) Bruker Avance III NMR spectrometer acquiring between 4096-80920 scans (dependent on the (S/N) ratio per

unit time from each sample). In general, the well fractionated samples with high organic C contents were run for less scans and the clay-sized fraction (non-HF treated) sample was run for the longest time. All data were acquired with a 1 ms contact time and a spinning speed of 13 kHz. T_1 times were measured using the standard inversion recovery approach and the recycle delay was set at 5 times the measured T_1 value. Spectra were processed using a zero filling factor of 2 and an exponential function corresponding to a line broadening of 50 Hz.

6.14.3 1D Solution-State NMR Spectroscopy

Samples were analysed using a 500 MHz Bruker Avance III NMR spectrometer equipped with a ^{1}H-^{19}F-^{15}N-^{13}C 5 mm, quadruple resonance inverse probe with actively shielded z-gradient (QXI). Samples (ca. 20 mg) were dissolved in 800 µL of DMSO-d_6 (deuterated dimethyl sulfoxide). 1D solution-state ^{1}H NMR spectra were obtained with 128 scans, a recycle delay of 2 s, 8192 time domain points, and an acquisition time of 0.27 s. Water suppression was achieved using PURGE (Simpson and Brown, 2005). Spectra were apodized through multiplication with an exponential decay corresponding to 1 Hz line broadening, and a zero filling factor of 2. Diffusion-edited (DE) spectra were obtained using a bipolar pulse pair longitudinal encode-decode (BPP-LED) sequence (Wu et al., 1995). Scans (1024) were collected using a 2 s delay, 2.5 ms at 49 gauss/cm, sine-shaped gradient pulse, a diffusion time of 200 ms, 8192 time domain points, 0.27 s acquisition time, and a sample temperature of 298 K.

6.14.4 2D NMR Spectroscopy

^{1}H TOCSY spectra were acquired in the phase sensitive mode, using a mixing sequence with rotor synchronized constant adiabatic WURST–2 pulses within an X_M16 mixing scheme (Peti et al., 2000). Scans (512) were collected for each of the 128 increments in the F1 dimension, and 2048 data points were collected in F2, and the mixing time used was 100 ms. Both dimensions were processed using sine-squared functions with a $\pi/2$ phase shift and a zero filling factor of 2.

2D NOESY spectra were acquired in phase-sensitive mode, using time proportional phase incrementation; 256 scans and 2048 data points were collected for each of the 196 increments in the F1 dimension at a mixing time of 200 ms. Both

dimensions were processed using sine squared functions with a π/2 phase shift and a zero filling factor of 2.

$^{1}H–^{13}C$ HSQC spectra were collected in phase sensitive mode using Echo/Antiecho-TPPI gradient selection. Scans (1400) were collected for each of the 128 increments in the F1

dimension; 1024 data points were collected in F2 dimension and a ^{1}J $^{1}H–^{13}C$ of 145 Hz. The F2 dimension was processed using an exponential function corresponding to a 15 Hz line broadening in the transformed spectrum whereas the F1 dimension was processed using a sine-squared function with a π/2 phase shift. Both dimensions were processed with a zero filling factor of 2.

6.14.5 Swollen-State NMR Spectroscopy

The sample (ca. 25mg) was placed in a 4 mm o.d. zirconia rotor and filled with DMSO-d_6 (75μL) both to swell the sample and to act as a lock solvent. After mixing with a stainless rod, the rotor was sealed using a top insert made from Kel-F, a Kel-F sealing screw and Kel-F cap. All NMR measurements were carried out at room temperature on a 500 MHz Bruker Avance III NMR spectrometer at a spinning speed of 6666 Hz using a prototype CMP-NMR MAS 4 mm $^{1}H–^{13}C–^{19}F–^{2}H$ probe fitted with an actively shielded Z gradient (Bruker BioSpin). All experiments were locked on DMSO-d_6, and the lock was maintained for all experiments, including solid-state experiments. Standard ^{1}H NMR spectra were recorded using pre-saturation utilizing relaxation gradients and echoes (PURGE) (Simpson and Brown, 2005). The 90° pulse was calibrated for each sample in the study. A spectral width of 20 ppm was used, 128 scans were acquired, and 8192 time domain points. T_1 relaxation times were measured using the standard inversion recovery approach, and the recycle delay was set at 5 times the measured T_1 value. Spectra were processed using a zero filling factor of 2 and an exponential function corresponding to a line broadening of 1 Hz in the transformed spectrum.

Diffusion edited proton spectra were carried out using a bipolar pulse pair longitudinal encode-decode (BPP-LED) sequence (Wu et al., 1995). When required, pre-saturation was also used in combination with diffusion editing to attenuate extremely large water signals in some samples. This results in the area from 3.5-4 ppm being slightly attenuated in certain cases. Scans (1024) were collected using

encoding/decoding gradients of 1.8 ms at 50 gauss/cm and a diffusion time of 180 ms. Inverse Diffusion Edited (IDE) spectra were created via difference from the appropriate controls as previously described (Courtier-Murias et al., 2012). One dimensional ^{13}C NMR spectra were recorded with a spectral width of 400 ppm using inverse gate decoupling. Scans ranged from 2K - 12K and 16 K time domain points. ^{13}C ramped-amplitude cross polarisation magic angle spinning (^{13}C RAMP CP-MAS) spectra were acquired with 1024 scans, and a 1 ms contact time. T_1 times were measured using the standard inversion recovery approach and the recycle delay set at 5 times the measured T_1 value. Spectra were processed using a zero filling factor of 2 and an exponential function corresponding to a line broadening of 50 Hz.

6.15 PyGC/MS

pyGC/MS analyses followed the method outlined in Mosse et al. (2012).

6.15.1 Thermochemolysis

Approximately 1 mg of the dried solids (weighed to 0.01 mg) was loaded into a quartz tube and spiked with the internal standard n-decylbenzene (50 ng) and 3 µL of 25% (wt/wt) tetramethylammonium hydroxide (TMAH) dissolved in methanol before insertion into the platinum coil of a Chemical Data Systems (CDS) 1000 coil pyroprobe unit. This unit was connected with a CDS 1500 interface directly to the GC/MS. Particulates were subjected to a single thermochemolysis treatment (1 °C/msec ramp with 40 sec dwell time at 720 °C) under helium.

6.15.2 Flash Pyrolysis

The experimental procedure was as per above for thermochemolysis (ca. 1 mg pyrolysed at 720 °C) except that no TMAH reagent was added. A set of the samples were also subjected to sequential thermal treatments under helium. The initial treatment involved desorption by heating to 340 °C to evolve volatile or guest compounds. Then additional internal standard was added to the product residue (from this 340 °C treatment) prior to its further sequential flash pyrolysis at 720 °C to produce fragments from the pyrolytic cleavage of its residual macromolecules.

Following pyrolysis, the remaining sample in the quartz tube was weighed to quantify the weight loss of the sample during the pyrolysis step. The remaining

sample was then subjected to heating to 1200 °C (repeated three times) to record the ash content of the sample.

6.15.3 Running Conditions

The GC/MS (CP8400 GC and Saturn 2200 ITMS; Varian Inc.) was equipped with a 1177 split/splitless GC injector. The injector operated at 300 °C and its split vent was open 1:40 during injection, and reduced to 1:15 after 3 min. A Varian FactorFour capillary column VF-5ms 30 m × 0.25 mm ID and 0.25 μm film thickness was used for separation using helium carrier gas programmed to a constant flow (1 mL/min). The column oven was held at 0 °C for 3 min then ramped to 80 °C at 6 °C/min; 200 °C at 8 °C/min, and finally 325 °C at 10 °C/min with a 15 min hold time. The transfer line to the mass spectrometer was heated to 170 °C and the trap was operated at 150 °C. In MS mode, the scan range was 29 m/z to 200 m/z between 1 and 10 min; then 29 m/z to 380 m/z to 25 min, and finally 50 to 450 m/z for the rest of the acquisition with 0.61 sec/scan.

Where certified reference materials were unavailable, tentative identities were assigned to compounds based on their retention time and mass spectral data. Mass spectra were compared to the NIST/EPA/NIH 2005 library (Gaithersburg, MD, USA) with all computer spectral matches (minimum R2>85%) checked manually.

6.16 Scanning Electron Microscopy – Energy Dispersive X-Ray Spectroscopy

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) characterisation was carried out in the Material and Surface Science Institute (MSSI) in the University of Limerick. A Hitachi FE SU-70 SEM coupled with an EDX (Oxford system) was used to measure the major elements in the clay-sized fraction (clay) and the demineralised clay-sized fraction (DCF) samples. This is a multipurpose, high resolution, SEM with a thermal field emission source. A solid sample is bombarded with a focused beam of electrons and the X-ray spectrum emitted is characteristic of the elemental composition (Reed, 1995). The samples were in powder form and small quantities were mounted onto carbon tabs and placed in the instrument for analysis.

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Chapter 7 Extraction of Organic Components from Grab Samples and from Core 1

7.1 Introduction

Four grab samples and four core samples were collected in Galway Bay. The four grab samples were obtained at the surface of the sea bed at the same geographical positions as the sediment cores (Figure 7.1). Studies of the organic colloidal components associated with the sediments at different depths can give indications of changes that have occurred with time to the compositions of the materials transported to the Bay.

This is the first time the solvent sequence method developed by the Carbolea Research Group (0.1 M NaOH; 0.1 M NaOH + 6 M urea; and DMSO + H_2SO_4 (94:6 *v/v*)) has been applied to the extraction of organic components from sediments. The efficiency of the extraction solvent system will be reported along with characterisation of the humic substances (HSs) and humin (HU, from Core 1) using elemental analyses, Fourier Transform Infrared (FTIR), and Ultraviolet-Visible (UV-Vis) spectroscopy. The data will provide an initial insight into the nature and the associations of the OM in sediments from an estuarine environment.

7.2 Regional Setting

Details of sediment coring and grab sample collection are outlined in Ch. 6, Section 6.1. Grab samples are labelled A1001, B1002, C1003, and D1004, and are from the same geographical positions as Core 1, Core 2, Core 3, and Core 4, respectively.

Figure 7.1. Map of Galway Bay showing coring positions.

7.3 Methodology

7.3.1 Extraction Procedure

The methods for the extraction of OM from sediments involve an initial exhaustive extraction using 0.1 M sodium hydroxide (NaOH), followed by exhaustive extraction using 0.1 M NaOH + 6 M urea, and extraction of HU from the clay-sized fraction using dimethyl sulfoxide (DMSO) + (98%) sulfuric acid (H_2SO_4) (94:6 *v/v*). Amberlite XAD-8 resin was used to separate the HSs from non-covalently linked saccharides, peptides and other organic impurities (Hayes et al., 1996). Extractions followed the methods outlined in Ch. 6; 6.3.2 (HSs), 6.3.3 (HU), and fractionation followed the method outline in 6.4.4.

7.3.2 Chemical and Spectroscopic Characterisation

The ash contents were measured by the method outlined in Ch. 6, 6.7. Elemental analysis was conducted as the University of Otago; the method is outlined in Ch. 6, 6.9.1. Organic carbon determinations were carried out on dry sediment/ clay-sized fraction samples, of known weight. This provided a basis for selection of samples to be extracted based on % OC, and to estimate approximate weight of sample

necessary for extraction; the method is outlined in Ch. 6, 6.5. FTIR spectra were recorded from 4000 to 400 cm^{-1} using KBr pellets by the method outlined in Ch. 6, 6.12.1. UV-Vis absorbance was measured by dissolving the HSs in alkali solution and recording the absorbances at 270 nm and 407 nm (A_2/A_4); and at 465 nm and 665 nm (E_4/E_6); the method is outlined in Ch. 6, 6.13. The isolates were not pre-treated with HCl or with HCl/HF solutions prior to freeze drying.

7.4 Results

7.4.1 Elemental Analyses and $\delta^{13}C$ and $\delta^{15}N$ Isotopes

Elemental and isotope data help to identify the origin of the humic materials retained in the sediments. The urea isolates have higher C compositions compared to the alkaline counterparts (Table 7. 1). Additionally, these isolates also have elevated N contents, possibly indicating residual urea was not completely removed from the isolate, or it could reflect the enhanced solubilisation of peptide structures. The C/N values vary considerably between the HAs and the FAs. The HAs range between 8.49 and 10.20, the HA urea isolates range between 7.49 and 7.98, and the FA isolates range between 15.46 and 17.56. High C/N values (>20) suggest terrestrial OM inputs such as cellulose, lignin, and tannins, whereas values < 10 are indicative of non vascular plants (He et al., 2008; Giovanela et al., 2010).

The $\delta^{13}C$ values for the isolates from A1001 (closest to the mouth of the river) are close to the range for soil HAs and FAs (-25 to -26 ‰, Nissenbaum and Kaplan, 1972). The D1004 HA urea isolate also has a $\delta^{13}C$ signature close to that of soil HSs, despite this isolate being the sample furthest from terrestrial influence. However, the alkaline-urea media have been shown to enhance the solubility of lignin components and therefore it may be solubilising the more recalcitrant terrestrial OM. The isolates from the intervening transect samples are closest to the marine HAs range (-22 to -23 ‰, Nissenbaum and Kaplan, 1972).

The $\delta^{15}N$ isotopes range between 3.1 and 6.7 for the isolates from all the grab samples. The urea isolates have the lowest values. Values for the isolates from B1002, C1003, and D1004 (5.0-7.0‰, omitting the D1004 HA urea isolate) are higher than the A1001 isolates (4.1-5.7‰) which may reflect the influence of sewage (4-19‰, Xue et al., 2009) from the waste water treatment plant on Mutton Island. Overall the values fit in the ranges reported for seawater nitrate (5-7‰, Hedges et

al., 1997), coastal sediments (0-6‰), and coastal particulate OM (3-9‰, Kuramoto and Minagawa, 2001). The value for D1004 HA urea (3.1‰) is close to the range reported for soils (0-3‰, Kuramoto and Minagawa, 2001). Urea isolates having lower values can either be due to solubilisation of terrestrial OM or due to the influence of the reagent itself as it has a depleted $\delta^{15}N$ signature (-2.3‰, Choi et al., 2002).

Table 7.1. Elemental composition, atomic ratios, and isotope signatures of the grab samples collected in Galway Bay.

Sample	Ash %	% C	% H	% N	C/N	$\delta^{13}C$ (‰)	^{15}N (‰)
A1001 HA	7.33	47.96	5.43	4.75	10.1	-24.6	5.7
A1001 HA urea	31.91	48.99	6.43	6.38	7.68	-24.9	4.1
A1001 FA	6.82	48.08	5.05	3.11	15.46	-25.0	5.6
A1001 FA urea	63.77	-	-	-	-	-	-
B1002 HA	25.94	49.62	5.93	5.82	8.49	-23.5	7.0
B1002 HA urea	35.35	-	-	-	-	-	-
C1003 HA	10.55	48.95	5.71	4.80	10.20	-23.4	6.6
C1003 HA urea	27.17	-	-	-	-	-	-
D1004 HA	24.02	48.14	6.03	5.25	9.17	-23.3	6.7
D1004 HA urea	17.26	51.59	6.19	6.89	7.49	-24.3	3.1
D1004 FA	27.49	48.63	5.84	2.77	17.56	-23.8	5.0
D1004 FA urea	0.63	-	-	-	-	-	-

7.4.2 Extraction Efficiency

The efficiency of the extractions is illustrated in Figures 7.2 and 7.3. No HU was extracted from the grab samples. It is evident that the quantity of OC isolated increases in progressing through the sequence of solvents. In relation to the grab samples (Figure 7.2), the 0.1 M NaOH media isolates ca. 50-65% of the OC, and the NaOH-urea media isolates an additional ca. 5-25% OC. This highlights the benefits of carrying out the NaOH-urea extraction in order to maximise HA/FA yields and to ensure maximal removal of HSs prior to HU extraction. It is evident (Figure 7.1) that

the samples taken closest to the mouth of the river had the highest OC, and the % OC decreases significantly with distance from the estuary.

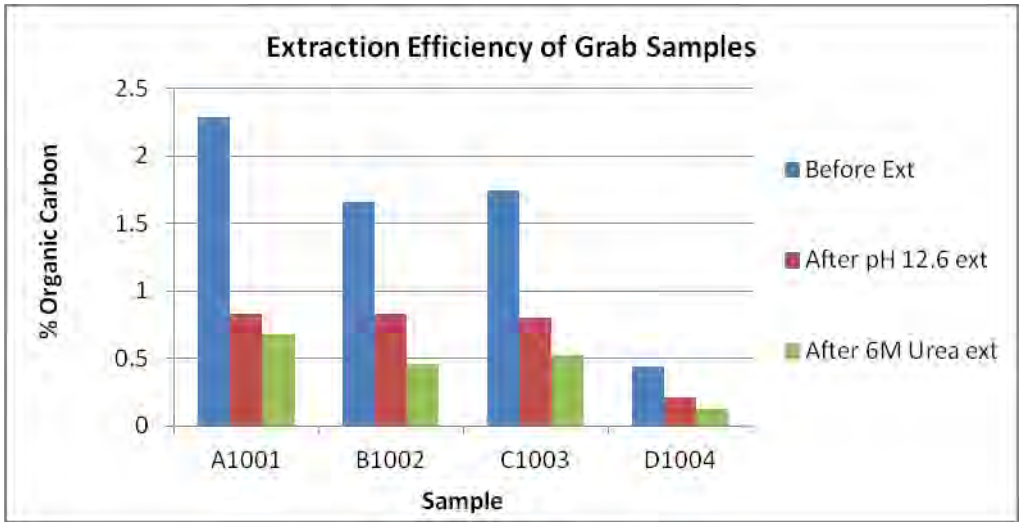

Figure 7.2. Organic carbon content of the sediment following extraction using different solvent systems.

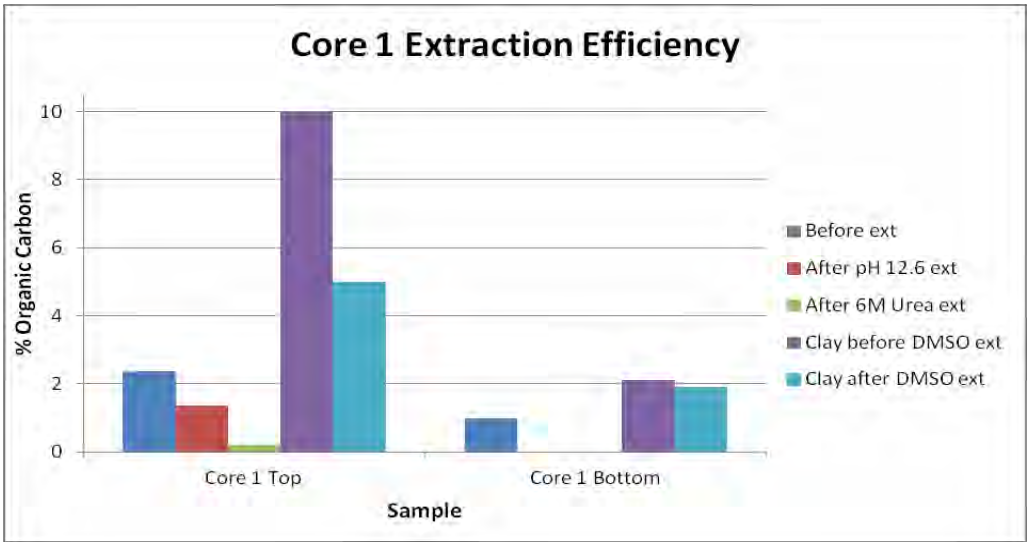

Figure 7.3. Organic carbon content of Core 1 fractions following extraction using different solvent systems.

In relation to the extraction of OM from the sediment core (Figure 7.3), the 0.1 M NaOH media removed ca. 40 % of the OC, and the NaOH-urea media removed an additional 50 % OC. Extractions were carried out on sediment from the base of the core using both NaOH and NaOH-urea, however, the colour of the extracts was negligible. Therefore, only HU was isolated from the base of the core. Following HSs extraction, the clay-sized fraction was isolated and dried prior to the % OC

measurements. This appears as a higher value (relative to the values for the whole sediment sample) in Figure 7.3, because the removal of the inorganic sand and silt concentrates the OC in the sample. The DMSO-H_2SO_4 medium removed 50 % OC from the clay-sized fraction in the top of the core and only 10 % OC from the clay-sized fraction from the base of the core. It is evident that the DMSO-H_2SO_4 medium is capable of removing the refractory OC from the clay matrix that is not isolatable using aqueous alkaline media. This DMSO-soluble HU fraction would remain unextracted following traditional extraction methodologies. In relation to the clay-sized fraction from the base of the core, it is possible that the extraction efficiency is decreased because the organic materials are in very strong associations with the clays and these are difficult to break. Thus, it is possible that the majority of the OC at the base of Core 1 are highly refractory and strongly protected; this fraction must be isolated using 10% HF.

There was a low recovery of FAs from the grab samples as it has the most polar functionalities of the organic fractions and it could therefore remain in solution in the aquatic environment and become re-mineralised rather than being retained in the sediments. Due to the low quantities of FAs isolated, a greater emphasis is given to analyses of the HAs.

7.4.3 FTIR Spectroscopy

Infrared (IR) spectroscopy is an analytical technique that has been routinely used for the characterisation of natural OM. Absorption bands were interpreted based on literature (Ch. 5, Table 5.2 and 5.3) assignments. The broadness of the spectral bands arises from the fact that humic isolates are a complex mix, resulting in severe over-lapping of absorbances.

The FTIR spectra for both the HAs and the HA-urea isolates are broadly similar. The major absorbances are in the regions of 3700-3000 cm^{-1} (OH stretching, N-H stretching, aromatic CH stretch), 2850-2890 cm^{-1} (aliphatic -CH_2- stretching), 1680 cm^{-1} (aryl-C=C conjugated with C=O and /or COO^-, carboxylates, amides/ketones; MacCarthy and Rice, 1985), 950-1170 cm^{-1} (carbohydrates/Si-O-Si; Badis et al., 2009). A noticeable difference between the urea isolates (Figure 7.5) is the presence of bands at 1400 cm^{-1} (A1001) and 1380 cm^{-1} (C1003) assigned as O-H bending of alcohols and/or carboxylic acids (MacCarthy and Rice, 1985), not present

in the alkaline isolates. A1001 and C1003 urea isolates have very broad bands centering at 3400 cm^{-1}. This could be attributed to the N-H from protein or phenolic OH from lignin components which are more soluble in the alkaline-urea media than the alkaline media alone. Sample B1002 HA (Figs. 7.4 and 7.5) has less pronounced bands compared to the other samples, especially at 3300-3500 cm^{-1} and 2850-2890 cm^{-1}, suggesting a lower carboxyl/amine/aliphatic hydrocarbon composition. The aliphatic hydrocarbon bands are much more pronounced in the B1002 HA-urea isolate (Figure 7.5) suggesting that the alkaline-urea medium has enhanced abilities to solubilise these aliphatic hydrocarbon species that are more resistant due to their chemical composition and structure. All isolates, except for A1001 urea, have absorption bands at ca. 1220 cm^{-1} indicative of carboxylic acids. All the HAs show strong resonances between 600 and 400 cm^{-1} and 1038 cm^{-1} attributable to silicate minerals; this coincides with high ash contents (Table 7.1). The bands at 1038 cm^{-1} are not as strong for A1001 and for C1003 reflecting their lower ash composition compared to B1002 and D1004. The HAs (0.1 M NaOH isolates) all have strong absorbances at 470 cm^{-1} for Si-O/Si-O-Fe, at 538 cm^{-1} for Si-O/Si-O-Al, and at 1038 cm^{-1} for Si-O-Si/Si-O. There are very broad absorption bands between 3700 and 3000 cm^{-1} for all samples, and it is not possible to identify if there are any Al-O-H species present in this region. The HA urea isolates have much broader absorbances compared to the 0.1 M NaOH HA isolates, suggesting that inorganic colloids are more likely to be co-extracted when 6 M urea is included in the alkaline media. This is also reflected in the higher ash contents (Table 7.1). All samples, except A1001, have absorption at 470 cm^{-1} for Si-O/Si-O-Fe and at 1033 cm^{-1} for Si-O-Si/Si-O (Figure 7.5). A1001 has a very broad strong absorbance between 600 and 500 cm^{-1} which would include absorption from Si-O/Si-O-Al. Additionally, A1001 has a strong, sharp absorbance at 790 cm^{-1} indicative of Si-O/Si-O-(Mg, Al)/(Al, Mg)-O-H. Absorption for all samples between 3700 and 3000 cm^{-1} are extremely broad so it is difficult to identify if there are Al-O-H species present.

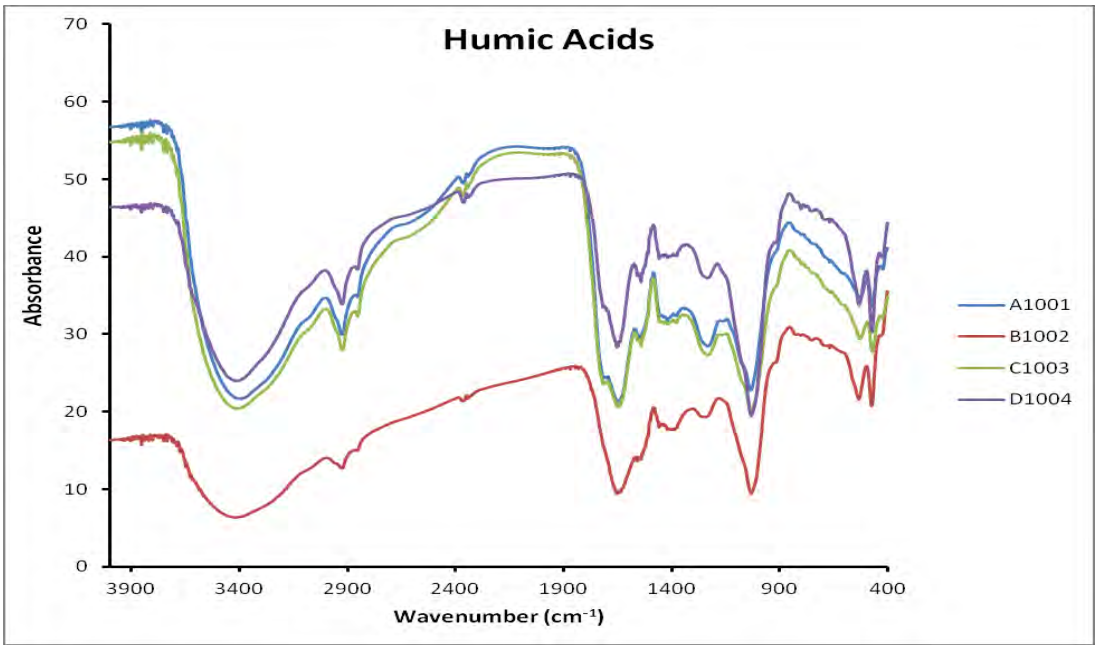

Figure 7.4. FTIR spectra of humic acids (HAs, extracted in 0.1 M NaOH media) isolated from grab samples.

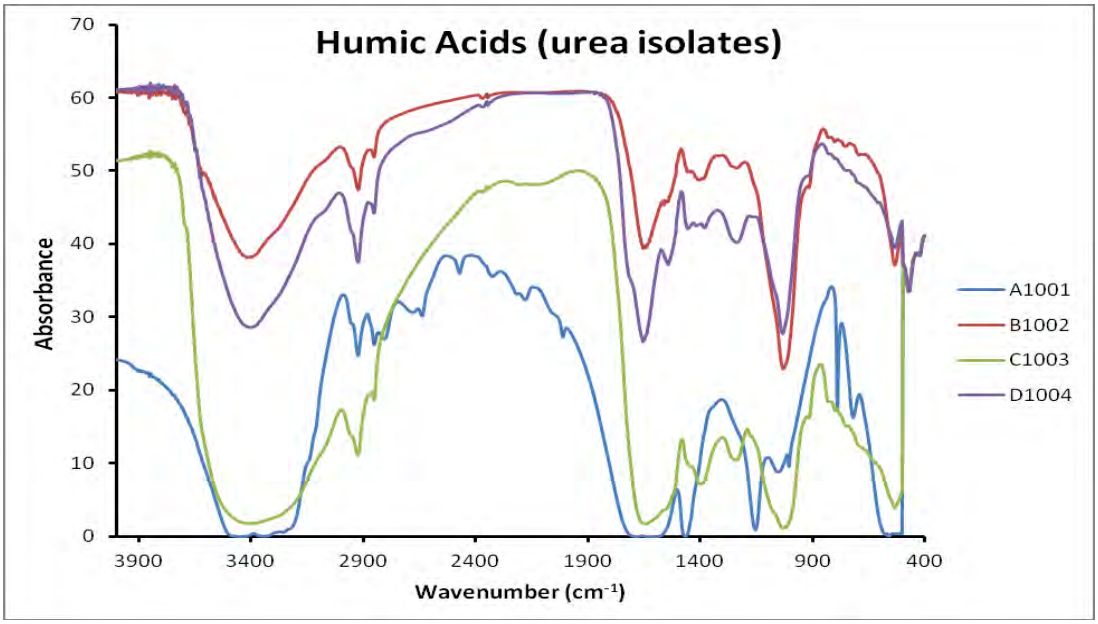

Figure 7.5. FTIR spectra of humic acids (HAs, extracted in 0.1 M NaOH + 6 M urea media) isolated from grab samples.

FAs were isolatable from all samples, however only FAs from A1001 and D1004 are illustrated in Figure 7.6 as those isolates showed the most compositional differences. There are broad resonances between 3500 and 3000 cm^{-1}. The aliphatic C-H bands are decreased in the FAs compared to the HAs, with A1001 urea isolate having the most definition in this region. There are strong absorbances between 1690 and 1600 cm^{-1}, most likely from aryl-C=C conjugated COO^{-}. The isolates from A1001 have a

sharp band at 1510 cm^{-1} corresponding to C=C stretching. D1004 urea has a strong absorbance at 1460 cm^{-1} indicative of bending of aliphatic $-CH_2$ deformations and at 1159 cm^{-1} indicative of C-OH stretching of aliphatic OH, indicating that urea enhances the solubilisation of aliphatic components. There is a broad resonance between 1410 and 1380 cm^{-1} corresponding to carboxylic and phenolic groups (MacCarthy and Rice, 1985) in the FAs (alkaline isolates), particularly in the D1004 sample. All samples except D1004 urea have a band at 1230 cm^{-1} from -C-O stretching and OH bending of carboxylic groups. There are strong absorbances between 1100 and 400 cm^{-1} which indicate Si-O and carbohydrate components. The FAs have broad resonances below 700 cm^{-1}, but due to poor resolution it is difficult to assign the peaks. It is possible that these contain an overlap of Si-O and Si-O-X (X = Fe/Al) but bands are not evident at the positions indicative for those silicates minerals. The urea isolate from D1004 has a strong absorption at 790 cm^{-1} indicative of Si-O/Si-O-(Mg/Al)/(Mg/Al)-O-H, and this sample also has a very high ash content. The absorption bands between 3700 and 3600 cm^{-1} are small suggesting low concentrations of aluminium hydroxides.

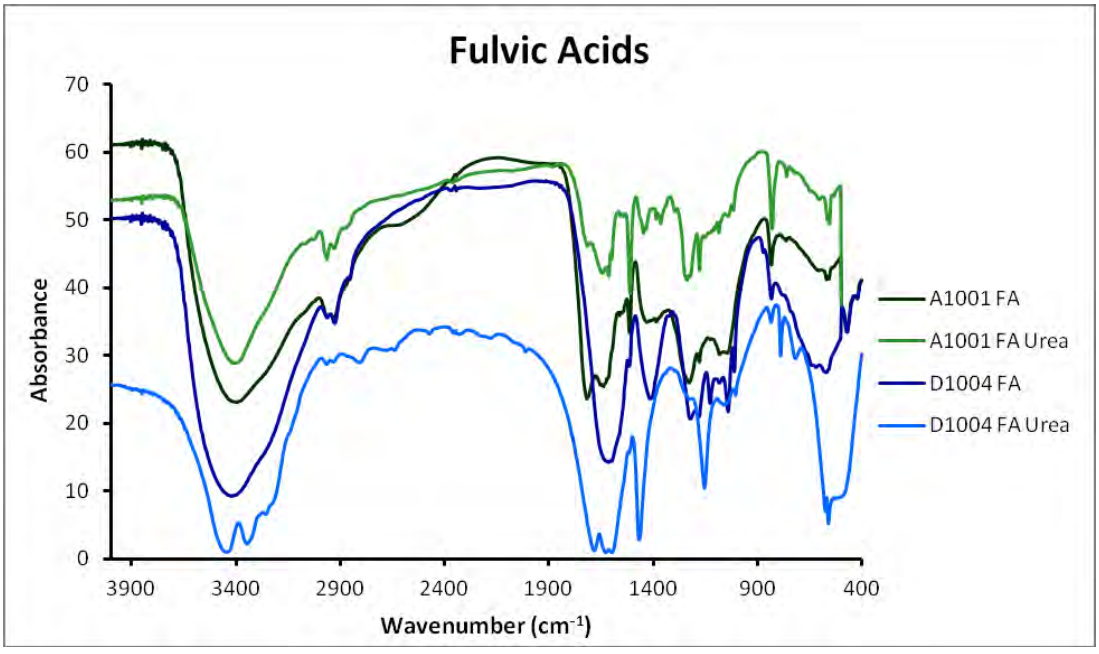

Figure 7.6. FTIR spectra of fulvic acids (FA, extracted in 0.1 M NaOH) and FA urea (extracted in 0.1 M NaOH + 6 M urea media) isolated from grab samples.

The spectra of the humic fractions from the surface sediments of Core 1 are comparable to those of the grab samples as expected. The HA isolate does not have

strong resonance for OH/NH groups (3400 cm^{-1}) (Figure 7.7). The sample has three well defined bands: 1650 cm^{-1} (C=C/COO^-/C=O), 1040 cm^{-1} (aryl-C-OH/C-O-C stretching), and 540 cm^{-1} (Si–O/Si–O–Al stretch). The HA spectrum highlights the limitations of FTIR and much more compositional data can be gained by studying the sample with NMR or pyGC/MS (Ch. 8). The other humic isolates show strong absorbances in the 3400 cm^{-1} region. Aliphatic C-H stretching bands are evident in all samples, but are most defined in the HA urea isolate. All samples have strong absorbances between 1660-1630 cm^{-1} (aryl-C=C conjugated with C=O and /or COO^- **)**. The FAs have a strong absorbance ca. 1230 cm^{-1} indicative of C-O stretching and O-H bending of carboxylic groups. There is also a small absorbance at 1510 cm^{-1} in all samples, except the HA isolate, suggesting peptide contributions (N-H deformation and C=N stretching/aromatic C; Senesi et al., 2003), coupled with a small band at 1420 cm^{-1} in the HA urea (C=C stretching from primary amides; Senesi et al., 2003). The isolates do not show strong absorbances for carboxyl functionalities (1725-1710 cm^{-1}, 1620-1600 cm^{-1}, 1400-1380 cm^{-1}) however; this may be due to overlapping of bands and strong absorbances from silicate species retarding the absorbance of other functionalities in the samples. All samples show strong resonances between 1100 and 400 cm^{-1}, most likely from Si-O (corresponding to their high ash contents), but may also be from carbohydrates. The strong absorbance in the HA at 538 cm^{-1} is assigned as Si-O or Si-O-Al, as well as a shoulder at 914 cm^{-1} and a small band at 3625 cm^{-1} indicative of Al-O-H. The 538 cm^{-1} absorbance is present in the HA, the HA urea, and the FA isolates, but absent in the FA urea isolate. The HA and FA urea isolates have a strong absorbance at 470 cm^{-1} indicative of Si-O or Si-O-Fe. Both HAs have strong absorbances for Si-O/Si-O-Fe at 470 cm^{-1}, at 538 cm^{-1} for Si-O/Si-O-Al, and at 1038 cm^{-1} for Si-O-Si/Si-O, as well as weak absorbances between 3700 and 3600 cm^{-1} for Al-O-H. The spectrum for the HA urea isolate has poor signal-to-noise (S/N), and overall the resolution of the bands is poor.

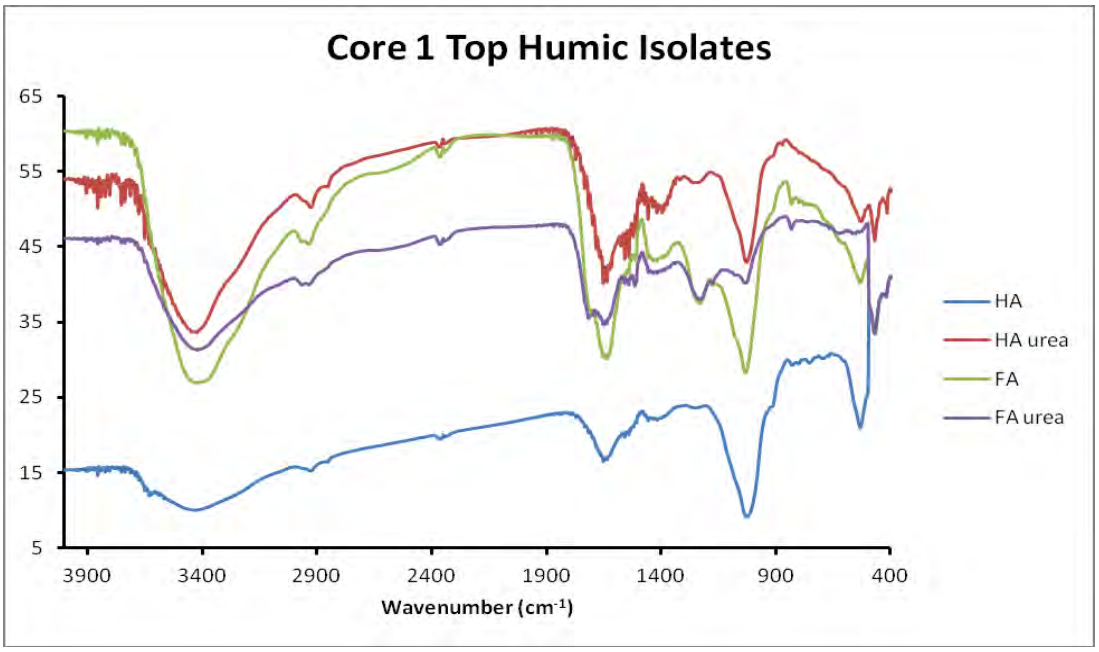

Figure 7.7. FTIR spectra of isolates from Core 1 (surface sediment). HA (humic acids, 0.1 M NaOH isolate); HA urea (0.1 M NaOH + 6 M urea isolate); FA (fulvic acid, 0.1 M NaOH isolate); and FA urea (0.1 M NaOH + 6 M urea isolate).

The two HU samples have very similar spectra (Figure 7.8), and the major differences relate to the absorbance. These isolates have very sharp resolution for aliphatic CH stretching, especially the isolate from the base of the core. There are also absorbances for C-H deformation between 1460 and 1440 cm^{-1} corresponding with the strong absorbances for aliphatic CH. The absorbances at 3400 cm^{-1} could be assigned to O-H from cutin and/or lignin, and N-H from peptide. Both HU samples have strong absorbances at 1650 cm^{-1} for aryl-C=C conjugated with C=O/COO^{-}, and a smaller band at 1240 cm^{-1} indicative of C-O stretching and O-H bending of carboxyl groups. These acidic functionalities can form complexes with cations such as Fe^{2+}, Fe^{3+} and Mg^{2+}. It is also possible that there is absorbance for C=C from amides at 1420 cm^{-1}. The absorbance and at 950 cm^{-1} (HU top) suggests Al-O-H clay minerals. It would not be expected that there would be a significant carbohydrate composition in these recalcitrant samples. Both HU samples have a strong absorbance at 1033 cm^{-1} indicative of Si-O-Si/Si-O, and absorption bands between 3700-3400 cm^{-1}, HU bottom has a sharp band at 3690 cm^{-1}, for Al-O-H. HU from the bottom has a band at 538 cm^{-1} for Si-O/Si-O-Al. Both samples have an absorption band at 473 cm^{-1} indicative of Si-O/Si-O-Fe. The Al-O-H and Si-O-Fe colloids may form strong associations with the OM, thereby protecting it from degradation as well as making the extraction process difficult (Chang et al., 2014). Similar to the isolates

from the other samples the resolution is generally poor and there is severe over-lapping of absorption bands.

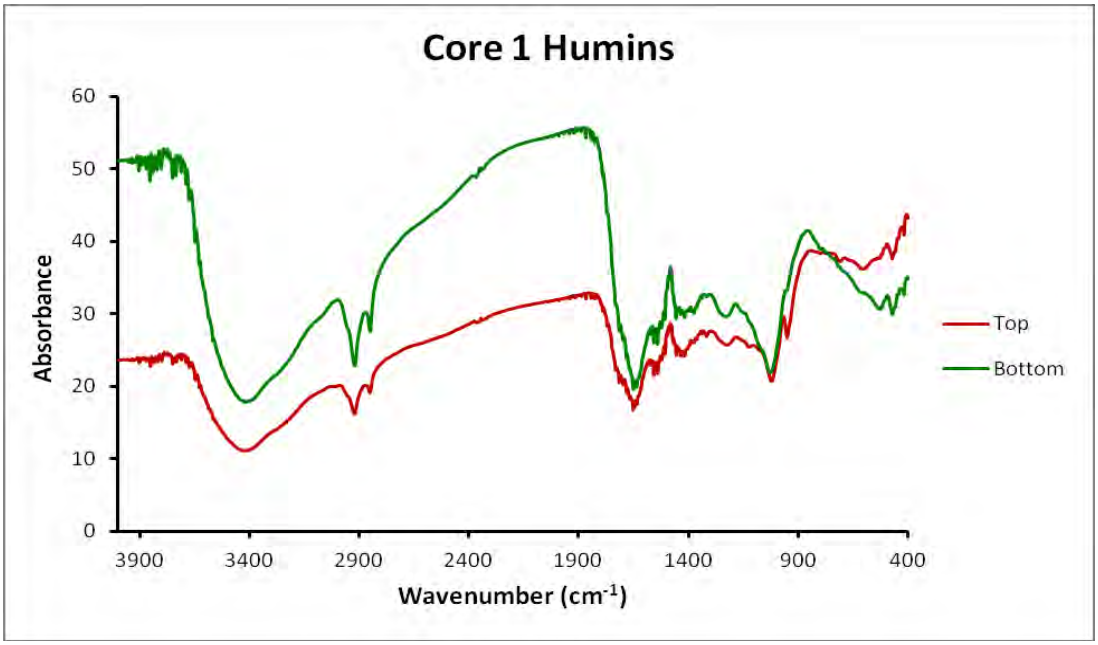

Figure 7.8. FTIR spectra of humin from the top and the base of Core 1.

7.4.4 UV-Vis spectroscopy

A_2/A_4 and E_4/E_6 ratios were calculated for the HSs from the grab samples using the absorption data from UV-Vis spectroscopy analyses. These ratios provide additional characterisation of the organic isolates; the A_2/A_4 ratio allows differentiation in relation to the origin of the material (Fooken and Liebezeit, 2000; Giani et al. 2010) and the E_4/E_6 ratio is indicative of molecular size (Leenheer, 1980; You et al., 1999; You et al., 1999; Huang et al., 2008).

The A_2/A_4 values are higher for the FAs than the HAs (Table 7.2, Figure 7.9). The alkaline extracted FAs have values ranging between 3.45 and 6.60 whereas the alkaline extracted HAs have lower values between 2.37 and 2.79. The alkaline-urea extracted FAs range between 3.55 and 9.28, and the alkaline-urea HAs range between 2.84 and 2.91 (Figure 7.9). The values for FAs would suggest that the origin of the OM is from terrestrial sources whereas the values for the HAs are in the range reported for coastal OM. There is no obvious trend for the E_4/E_6 ratios and the values obtained for the FAs do not correspond with the ranges reported in the literature (6-18) (Table 7.2, Figure 7.10). It would be expected that the FAs would have a higher

E_4/E_6 value than the HAs due to their smaller molecular size; however, in many cases the FAs have lower values than the HAs. However, in agreement with the literature the alkaline-urea extracts have a lower E_4/E_6 value due to their enhanced solubility for macromolecules (with the exception of D1004 isolates which have similar values). The assumptions that the E_4/E_6 ratio can indicate the size of the molecules may be obsolete in light of the research which shows HSs to be supramolecular associations that are dependent on sample concentration and its compositions.

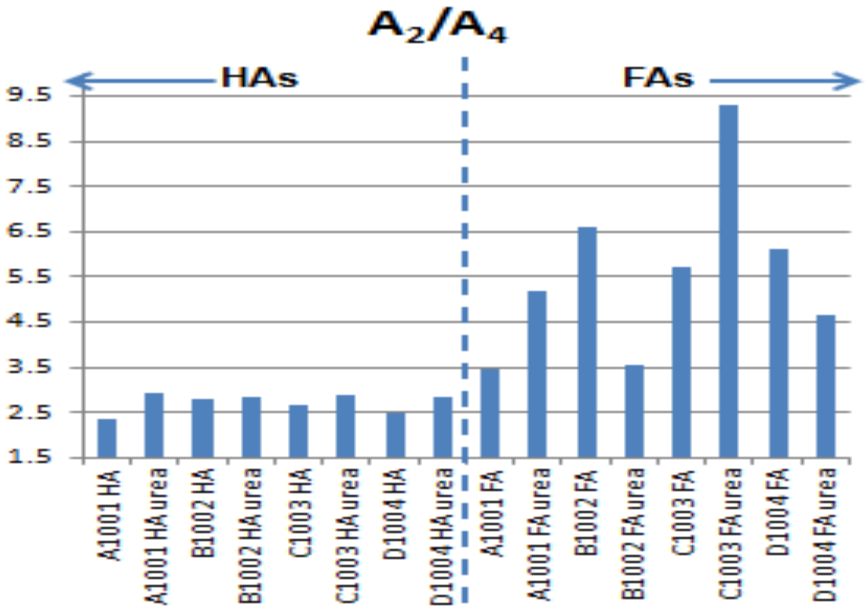

Figure 7.9. A_2/A_4 (270/407 nm) ratios for the humic and fulvic acids isolated from the grab samples.

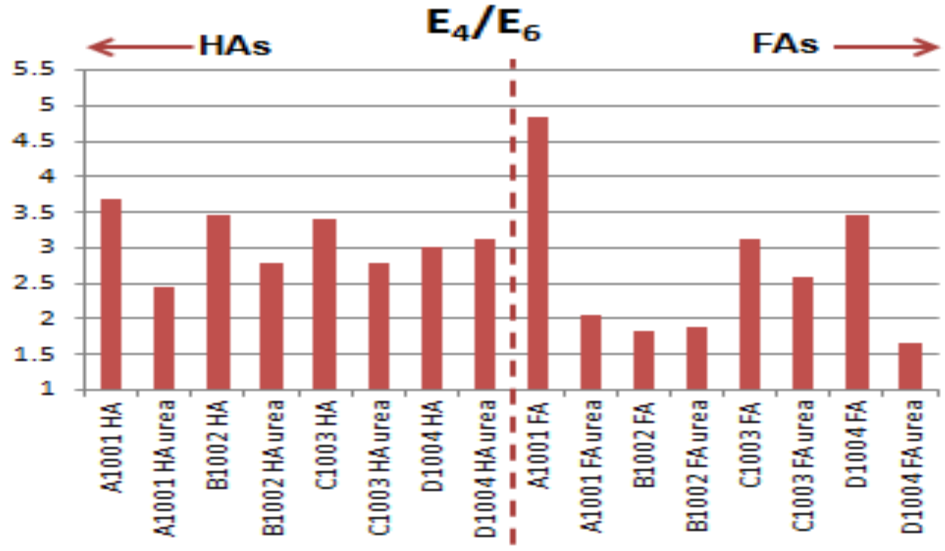

Figure 7.10. E_4/E_6 (465/665 nm) ratios for the humic and fulvic acids isolated from the grab samples.

Table 7.2. UV-Vis absorbance values and ratios for the isolates from the grab samples.

Sample	280 nm	404 nm	A_2/A_4	465 nm	665 nm	E_4/E_6
A1001 HA	3.5435	1.4972	2.37	0.8588	0.2319	3.70
A1001 HA urea	1.3516	0.4644	2.91	0.2951	0.1200	2.46
A1001 FA	3.5530	1.0300	3.45	0.5210	0.1076	4.84
A1001 FA urea	0.8754	0.1684	5.20	0.1109	0.0539	2.06
B1002 HA	3.2599	1.1677	2.79	0.6756	0.1961	3.45
B1002 HA urea	2.6323	0.9263	2.84	0.5473	0.1967	2.78
B1002 FA	0.8374	0.1268	6.60	0.0846	0.0461	1.84
B1002 FA urea	0.4726	0.1331	3.55	0.0883	0.0469	1.88
C1003 HA	2.6044	0.9781	2.66	0.5728	0.1683	3.40
C1003 HA urea	2.7237	0.9488	2.87	0.5811	0.2086	2.79
C1003 FA	2.2748	0.3978	5.72	0.2253	0.0724	3.11
C1003 FA urea	2.7890	0.3007	9.28	0.1762	0.0679	2.60
D1004 HA	1.5498	0.6174	2.51	0.3622	0.1204	3.01
D1004 HA urea	2.1938	0.7720	2.84	0.4811	0.1535	3.13
D1004 FA	2.8257	0.4635	6.10	0.2158	0.0622	3.47
D1004 FA urea	0.5083	0.1089	4.67	0.0694	0.0420	1.65

7.5 Discussion

It is evident that the % OC decreases with distance from the mouth of the river Corrib, suggesting that the river has a considerable influence on the OM compositions and/or the region close to the estuary has the highest autochthonous OM productivity. Extractions carried out on the grab sample sediments and Core 1 sediment (top) show that the solvent system is efficient in removing the HSs from the sediments. The urea improves the removal of OC from the inorganic matrix and enhances the solubility for macromolecular components such as aliphatic hydrocarbons, lignin, and protein. A higher C/N value would have been expected for the HAs isolated in the alkaline-urea media due to the enhanced solubilisation of lignin. However, the N levels are elevated, possibly from the urea reagent, and from enhanced protein solubilisation resulting in lower C/N values. The DMSO-H_2SO_4 medium is an effective solvent for HU extraction. However, the extraction efficiency of the DMSO-H_2SO_4 medium is significantly decreased when the organic components are associated with inorganic colloids, especially Fe and Al hydroxides (Wiseman and Püttmann, 2006; Chang et al., 2014). The major aliphatic hydrocarbon composition of HU bottom (strong aliphatic $(CH_2)_n$ bands in the FTIR spectrum) can explain the insolubility of this fraction in DMSO-H_2SO_4; the hydrophobic domains are difficult for the solvent to effectively penetrate. The solvent sequence, as utilised in this study, allows for subsequent detailed characterisation of HAs, FAs, and HU.

The IR spectra of the grab samples illustrate the heterogeneous nature of the HSs. There are broad bands due to overlapping of absorption from different bonds. The high concentration of silicates and hydroxides increase the broadness of the bands and hinder the identification of carbohydrate components. The isolates were not pre-treated with HCl or HCl/HF solutions prior to analyses, and as a consequence there was a high concentration of mineral matter in the isolates. The $\delta^{13}C$ provides valuable insights into the origins of the OM. The grab samples nearest the land have terrestrial signatures, whereas the samples further out in the Bay are closer to the marine range. The urea isolates have signatures closer to the terrestrial range which suggests that the urea is solubilising plant materials. The enrichment in $\delta^{15}N$ suggests that there may be an influence on the organic isolates from a waste water treatment plant (53°15'16N, 09°03'16W) located in the Bay adjacent to

samples B1002 and C1003. The low value for D1004 HA urea isolate is either due to the solvation of terrestrial OM or that residual urea is lowering the ^{15}N isotope signature. The C/N ratios and the A_2/A_4 ratios indicate that the FAs are composed of OM from terrestrial sources, whereas the HAs have their origins from coastal/marine OM. The urea isolates have small E_4/E_6 values, possibly due to the ability of urea to solvate macromolecules. The FAs have low E_4/E_6 values which do not correlate with values reported in the literature (Kononova, 1966; Leenheer, 1980; You et al., 1999). However, the FTIR spectra show the FAs to have aliphatic C-H compositions that are not as pronounced in the IR spectra of soil FAs. Therefore, the FAs in the aquatic environment may be larger in molecular size and more hydrophobic.

The HSs isolates from Core 1 (surface sediments) have poor resolution in the FTIR spectra due to the high ash contents. The main absorption is from OH/NH groups (3400 cm^{-1}) and C=O stretching (1660-1630 cm^{-1}), as well as bands from inorganic mineral matter. The FAs from Core 1 have a strong absorbance at 1250 cm^{-1} indicative of C-O stretching of aryl ethers and phenols. A small absorbance at 1510 cm^{-1} (N-H deformation and C=N stretching/aromatic C) in the isolates suggests peptide contributions. There were no HSs isolated at the base of the core suggesting that the majority of these organic components have become re-mineralised with time. The HU isolated from the base of the core has very strong IR absorbances for aliphatic CH (Figure 7.8). This highly aliphatic composition would not solvate in the alkaline, alkaline-urea medium, but it was sparingly soluble in the DMSO-H_2SO_4 medium. It is evident that a fraction of the organic components was not solubilised using the DMSO-H_2SO_4 medium (Figure 7.3). Therefore, the remaining organic materials are in strong associations with the inorganic colloids, or the HU has highly ordered aliphatic hydrocarbon structures which cannot be penetrated by the solvent. This organic material was concentrated by dissolving the silicates in HF acid.

Overall, FTIR is useful as it provides an overview of the compositions of the organic isolates. However, it is an unsatisfactory method of analyses for the in-depth characterisation of a sample because of the broad bands and the over-lap of absorbances from different chemical species. For example, it is difficult to definitively identify carbohydrate because it absorbs in the same region as some of the silicate species; the absorbance from OH groups is very broad and it is difficult to identify the chemical environment from which these OHs arise; and peptide

contributions are also difficult to assign. However, FTIR is a suitable analytical technique to assess compositional differences between samples. It is evident from the spectra that the alkaline-urea medium is a good solvent for HSs and it enhances the solvation of aliphatic hydrocarbons, amides, and lignin; however it also co-extracts a large amount of Si-O-Si, Si-O-Fe and Al-O-H species. The FAs from both the alkaline and alkaline-urea isolates show more defined absorbances for aliphatic $-CH_2-$ than is characteristic for the spectra of soil FAs; for example, a study by Chang et al. (2014) provided FTIR spectra for FAs isolated from a volcanic soil and almost no absorption in the 2840-2950 cm^{-1} region, with the exception of the FA urea isolate but this was poorly defined. It is probable that the FAs isolated from an aquatic environment are more hydrophobic relative to soil FAs, in order to enhance their retention and due to the origins of their compositions from aliphatic materials such as algaenan and aliphatic biomolecules from higher plants. The isolates from Core 1 were treated with HF and their IR spectrum was recorded using ATR-FTIR in an attempt to acquire spectra with better resolution and S/N (discussed in Ch. 8).

E_4/E_6 ratios indicate the degree of humification of the HSs (Schnitzer, 1971, Giani et al., 2010). UV-Vis spectroscopy has little value for studying functionality in HSs (MacCarthy and Rice, 1985). One should proceed with caution when interpreting UV-Vis spectroscopy data. The data should only be used to support other data sets. There is controversy about how useful this method of characterisation is for HSs. Chen et al. (1977) dismissed the theory that the E_4/E_6 ratio is indicative of the presence of aromatic rings because he did not find any direct relationship between the E_4/E_6 ratio and the concentration of condensed rings in HAs and FAs (Schnitzer, 1978). Therefore, it would be better to use alternative methods for molecular weight analyses (e.g. HPSEC), and assessments of aromatic, aliphatic and carboxyl composition (FTIR, NMR, pyGCMS). The A_2/A_4 ratio may be more useful than the E_4/E_6 ratios. The C/N and $\delta^{13}C$ concur with the A_2/A_4 ratio for the FAs which indicate that they are of terrestrial origin. The $\delta^{13}C$ for the HAs are close to the marine range and the A_2/A_4 values suggest that the HAs are from coastal OM.

7.6 Conclusion

The solvent system used was efficient in the isolation of organic components associated with sediments. Inclusion of urea in 0.1 M NaOH media solubilises additional HSs that have higher concentrations of C and N in their compositions. DMSO-H_2SO_4 is a suitable solvent for HU, however, it cannot break strong associations between the organic colloids and the silicate minerals containing Fe and Al. FTIR spectroscopy is useful for characterisation of both organic and inorganic components of the humic samples. However, detailed compositional information cannot be obtained due to the poor resolution and over-lapping bands. UV-Vis spectroscopy is a simplistic method of characterisation and A_2/A_4 ratios are reliable when coupled with elemental and isotopic signatures. The E_4/E_6 data should be interpreted with caution, and alternative analytical methods should be employed to study molecular size and degree of condensation. It is recommended to demineralise the organic isolates prior to characterisation because these isolates had high ash contents and there were strong absorbances of silicate species and aluminium hydroxides in the FTIR spectra.

7.7 References

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Chapter 8 Isolation and Characterisation of Recalcitrant Organic Components from Core 1

8.1 Introduction

When the hydrogen bonds that hold the low molecular weight humic molecules in aggregate form are broken or weakened, components of the humic substances (HSs) associations are dissolved in the aqueous media. It is more difficult to transform/degrade the organic components of humin (HU), and this organic fraction is insoluble in aqueous media at any pH.

This chapter will provide analyses of the chemical compositions of HSs and of HU from Core 1. The top 100 cm and the bottom 100 cm of sediment from the core were chosen for the study to highlight the compositional differences with time between the organic components. The data acquired are not interpreted in relation to a stratigraphic context as it was necessary to bulk the 100 cm depth of sediment in order to extract sufficient quantities of organic materials for characterisation studies. Three radiocarbon dates were obtained for the top 100 cm of sediment and one date were obtained for the bottom 100 cm of sediment (Dalton et al., 2010). The top 100 cm of sediment represents ca. 2,260 years before present (BP) and bottom sediments represent 9,770 years BP.

8.2 Materials and Methods

8.2.1 Regional Setting

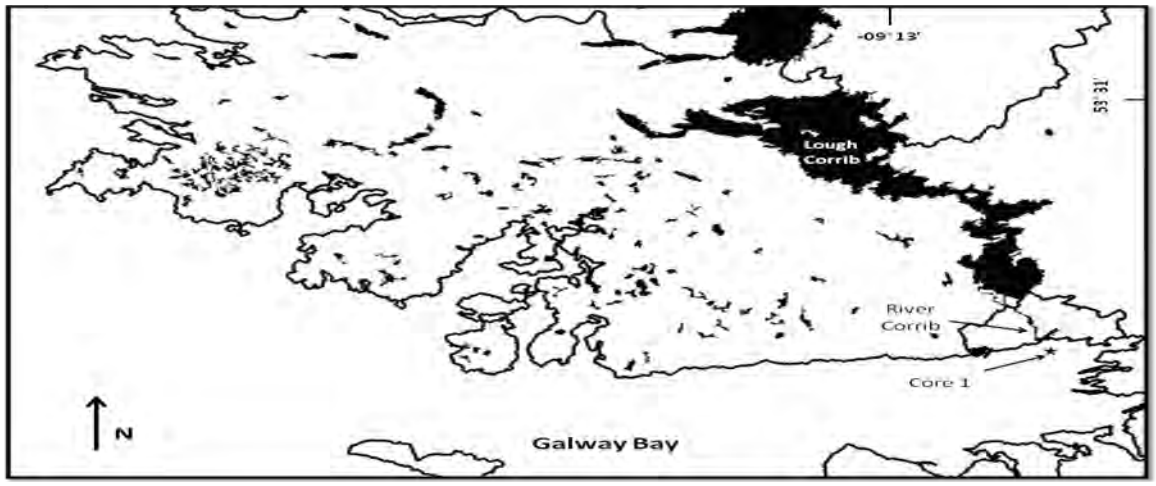

Figure 8.1. Map of the sediment coring position (53° 15'22.21N, 09° 02'14.40W) in Galway Bay, Ireland.

The site chosen for this study is influenced by terrestrial, lacustrine, and anthropogenic inputs from the River Corrib, and by marine inputs from the Atlantic Ocean. The core (535 cm) was taken in close proximity to the mouth of the River Corrib (53° 15'22.21 N, 09° 02'14.40 W, Figure 8.1). Soil types in the Lough Corrib and Galway Bay region include shallow brown earth, lithosols, blanket peats, and basin peats (Gardiner and Radford, 1980). Run off from the surrounding soils are washed into the Bay and will contribute to the OM retained in the sediments along with aquatic biomass from the river, lake, and the ocean.

8.2.2 Extraction Methodology

8.2.2.1 Isolation of Humic acids and Fulvic acids

Extraction methods involved exhaustive extraction using sodium hydroxide (0.1 M NaOH), followed by exhaustive extraction with 0.1 M NaOH + 6 M urea; the methods are outlined in Ch. 6, 6.3.2. HAs and FAs from the alkaline solvent were labelled HA and FA, respectively. HAs and FAs isolated in the alkaline-urea solvent were labelled HA-urea and FA-urea, respectively. The extracts were fractionated using XAD-8 resin column; the method is outlined in Ch. 6, 6.4.4.

8.2.2.2 Isolation of Humin

The HU was extracted using DMSO + (98%) H_2SO_4 (94:6, *v/v*) (Hayes, 1985, 2006; Song et al., 2011). The dry clay-sized fraction was mixed with DMSO-H_2SO_4. The HU extract was diluted with distilled water to pH 2; a precipitate formed which was isolated by centrifugation, washed with distilled water, dialysed, and freeze-dried**.** The method is outlined in detail in Ch. 6, 6.3.3.

8.2.2.3 Demineralised Clay-Sized Fraction

The clay-sized fraction was isolated from the top and base of the core, and subsequently demineralised using 10% HF. The method outlined in Ch. 6, 6.3.4.2. This organic fraction was hydrolysed to analyse for the sugar content.

8.2.3 Chemical Analysis

The % OC of both the bulk sediments and the clay-sized fraction were measured using the Walkley Black method, outlined in detail in Ch. 6, 6.5. The C, H, N compositions of the organic isolates were measured at The Campbell Microanalytical Laboratory (University of Otago), outlined in Ch. 6, 6.9.1. The $\delta\ ^{13}C$ and $\delta\ ^{15}N$ of the organic isolates were measured at the Water Studies Centre (Monash University, Victoria), outlined in Ch. 6, 6.10. Sugar analysis was carried out at the University of Limerick, as per the method outlined in detail in Ch. 6, 6.11.

8.2.4 Spectroscopic Characterisations

Prior to each spectroscopic analysis, the isolates were desiccated in the presence of P_2O_5, under vacuum. The IR spectra were acquired using a Varian Scimitar FTS 1000 Fourier Transform Infrared (FTIR) spectrometer fitted with a Specac MKII Golden Gate single reflection ATR cell and ZnSe optics. The spectra were acquired following the method outlined in detail in Ch. 6, 6.12.2. 1D RAMP-CPMAS ^{13}C NMR solid-state NMR spectra were acquired using a Bruker 300 MHz NMR at Monash University (Clayton, Victoria), following the method outlined in detail in Ch. 6, 6.14.1. 2D ^{1}H–^{13}C HSQC NMR spectra were acquired at the University of Toronto (Scarborough, Ontario), following the method outlined in detail in Ch. 6, 6.14.4. PyGCMS, flash and thermochemolysis, data were acquired at Monash

University (Gippsland, Victoria), following the methods outlined in detail in Ch. 6, 6.15.

8.3 Results

8.3.1 Extraction and Fractionation

The OC content of the bulk sediment from the top of the core (0-100 cm) was 2.29%, and the concentration decreased with depth to a value of 0.99% at the base of the core. The sediments were bulked (100 cm from the top and from the bottom of the core) to give sufficient quantities of the organic components for characterisation studies. The solvent system efficiently removed the organic components from sediments at the top of the core (Figure 8.2); HAs, HU, and FAs were isolated in decreasing amounts. It is clear that urea facilitated the isolation of additional materials that satisfy the solubility criteria for HAs and FAs. It could be argued that the inclusion of urea might cause the additional extracts to be outside of the criteria for HAs and FAs, but Hayes et al. (2012) have shown that such extracts in the alkaline-urea system were similar to those extracted in 0.1 M NaOH. The quantity of FAs isolated was very low (Figure 8.2), which may suggest that the majority of the FAs had remained in solution in the aquatic environment. Alkaline and alkaline-urea extractions were carried out on the sediments from the base of the core but no HAs or FAs were recovered.

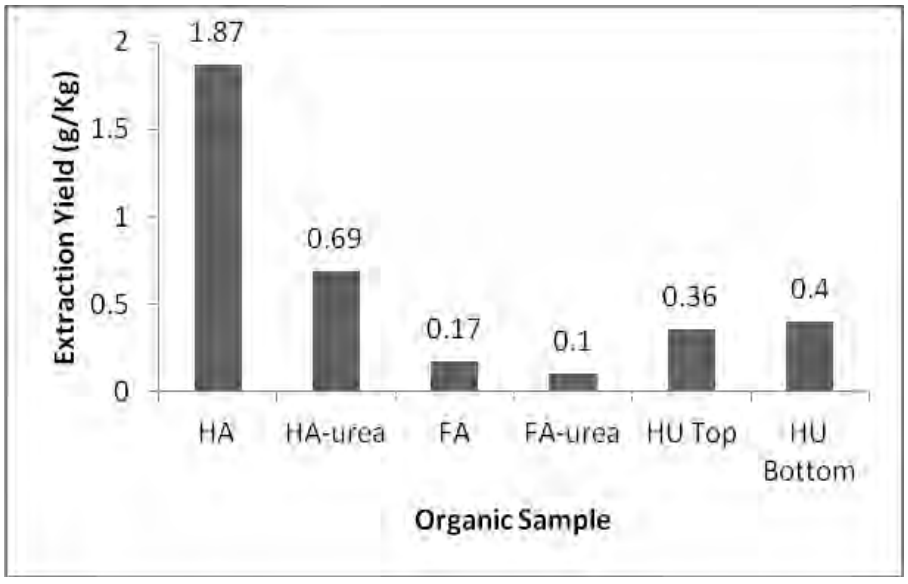

Figure 8.2. Extraction yields (on a dry, ash free basis) of organic fractions isolated from sediments from Galway Bay.

8.3.2 Chemical Characterisation

Elemental compositions and atomic ratios data for the organic fractions are given in Table 8.1. The HUs had the highest C contents, especially the HU-bottom, due to the predominance of aliphatic hydrocarbons in the compositions of these isolates. The ash content was high in all samples, especially in the HAs. We attribute the inordinately low C content of the HA sample to the very high silicate composition indicated in the FTIR spectrum. The % N ranged from 3.62 to 5.45. It is possible that the N content of the HA-urea isolate was influenced by interactions between urea and reactive functional groups in the humic structures, as discussed by Hayes and Swift (1978). However, the FAs, the most reactive components, have no evidence for N enrichment in the FA-urea isolate. That would indicate negligible interactions between the urea and the functional groups of the FAs. Therefore, the % N can be attributed to the enhanced solubilisation of peptides in concentrated urea solutions. In relation to the HUs, the high % N indicates inputs from microbial necromass and suggests that protein components are recalcitrant or protected.

The isotope data provide an insight into the origins of the OM; for example the $\delta^{13}C$ values indicate whether the OM is composed of terrestrial C_4 or C_3 plants, or of aquatic biomass. Differentiation is aided by the fact that the enrichments in ^{13}C in marine sources are different from those of terrestrial plants (Nissenbaum and Kaplan, 1972). The data (Table 8.1) show the range for $\delta^{13}C$ values for the different organic fractions isolated. The values (interpretation based on Table 2.2) for the HSs are closer to the marine $\delta^{13}C$ humic matter range, whereas the signatures for the HUs suggest terrestrial influences, especially for the HU-top. As the HU is the most recalcitrant fraction, the signatures for both HUs indicate that the terrestrial OM inputs are selectively preserved or inherently more recalcitrant than the marine OM. The $\delta^{13}C$ of the HSs are at the end of the marine $\delta^{13}C$ range, which would suggest the OM inputs to the humification/transformation process were from a mixed source, unlike the HUs which appear to be predominantly terrestrial. This marine signature does not carry through to the HU and consistent with the contemporary belief that HUs are not part of the HSs but rather a separate entity composed of mainly plant waxes and long chain hydrocarbons. However, HSs may become trapped in the hydrophobic domains of the HU in which they are preserved from remineralisation.

^{15}N isotopes are influenced by the parent source of OM (Kuramoto and Minagawa, 2001). These provide information about the types of N present in the organic materials. All organic fractions are in the range reported for soil nitrogen (0-8‰, Xue et al., 2009), except in the case of FA (19‰) which is strongly enriched in ^{15}N. Manure and sewage have ^{15}N nitrate values ranging between 5 and 25‰ and 4 and 19‰, respectively (Xue et al., 2009). Nitrate is soluble in water, hence it is concentrated in the FA isolate. The enrichment in ^{15}N may be explained by the proximity of the sampling site to the waste water treatment plant (discussed in Section 8.4.2.3).

Sugar analysis was carried out on HA from the top, and the demineralised clay-sized fraction (DCF, demineralised using 10% HF) from the top and bottom of the core. The DCF consists of fresh and transformed OM. Analyses were not carried out on any other samples due to limited quantities of the samples. The [MAN + GAL)/ (ARA + XYL)] ratio values for HA-top, DCF- top and DCF-bottom were 2.09, 2.07, and 2.02 respectively, indicating that the humic/organic composition has significant contributions from microbial sugars.

Table 8.1. Elemental composition (on a dry, ash free basis), atomic ratios, ^{13}C and ^{15}N isotopes data, and ash values for the organic fractions (HA = humic acids, FA = fulvic acids, HA urea and FA urea refer to the HAs and FAs isolated in 0.1 M NaOH + 6 M urea, HU = humin). Sugar analysis for HA isolate.

Sample	%C	%H	%N	C/N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	Ash	Sugar Ratio [(MAN + GAL) / (ARA + XYL)]
HA	37.29	5.01	3.62	10.30	-22.4	5.9	49.11	2.08
HA urea	52.59	5.93	5.45	9.65	-22.5	5.7	45.63	-
FA	50.45	5.76	4.85	10.40	-22.8	19.0	32.82	-
FA urea	48.28	5.00	4.19	11.52	-23.4	4.7	11.76	-
HU Top	53.71	7.46	4.29	12.52	-25.1	4.6	22.48	-
HU Bottom	59.33	7.56	4.90	12.11	-24.3	5.1	30.39	-

8.3.3 Spectroscopic Characterisation

FTIR provides useful indications of the gross chemical/structural compositions of the fractions. The ATR-FTIR spectra are comparable to those acquired using conventional FTIR (Ch. 7, Figure 7.7). The CO_2 absorbances (ca. 2300 cm^{-1}) are absent for the ATR-FTIR spectra. The HU material is dominated by aliphatic hydrocarbon absorption (2920 and 2860 cm^{-1}). All samples show high mineral content between 850 and 1100 cm^{-1} (Si-O, Si-O-Si, Al-O-H), especially the HA isolate as evidenced by both conventional and ATR-FTIR. Additionally the HA isolate has a sharp band at ca. 3650 cm^{-1} for Al-O-H and bands ca. 720 and 650 cm^{-1} for Si-O, Si-O-Al, Al-O-H. In all samples, especially the HU top, there is a broad, overlapping band between 1800 and 1500 cm^{-1} indicative of C=O stretching in carboxylic acids (1710 cm^{-1}), amides (1650 cm^{-1}), and N-H deformation/C≡N stretching/aromatic C=C (1550 cm^{-1}). In addition to the small band/shoulder at 1710 cm^{-1}, there is a band at 1220 cm^{-1} for O-H bending and C-O stretching from carboxyl groups. All the isolates have a band at ca. 1640 cm^{-1} for C=C and at ca. 1520 cm^{-1} for aryl-C=O/COO^- stretching, especially in the cases of the HU and HA isolates. All the isolates have a band at 1370 cm^{-1} indicative of O-H bending of alcohols and/or carboxylic groups, especially the HU. It is difficult to make definitive interpretations from the spectra because of the overlap of bands.

The ^{13}C CP/MAS NMR spectra provide additional information about the compositions of the HSs and of the HU. However, the high ash content lowered the concentration of OC that could be packed into the NMR rotors, and therefore, the sensitivity of the experiments is lessened. Additionally, there may have been some interference from paramagnetic ions that have a negative impact on the sensitivity and resolution of the spectra, as evidenced by identification of Si-O-Fe in the FTIR spectra (Figure. 7.7 and 8.3). Differentiation between highly-ordered and amorphous structures was not possible in the ^{13}C NMR due to lower resolution in the spectra, as these samples had not been pre-treated with HF/HCl. Samples were pre-treated prior to 2D NMR experiments to remove silicates and paramagnetic ions.

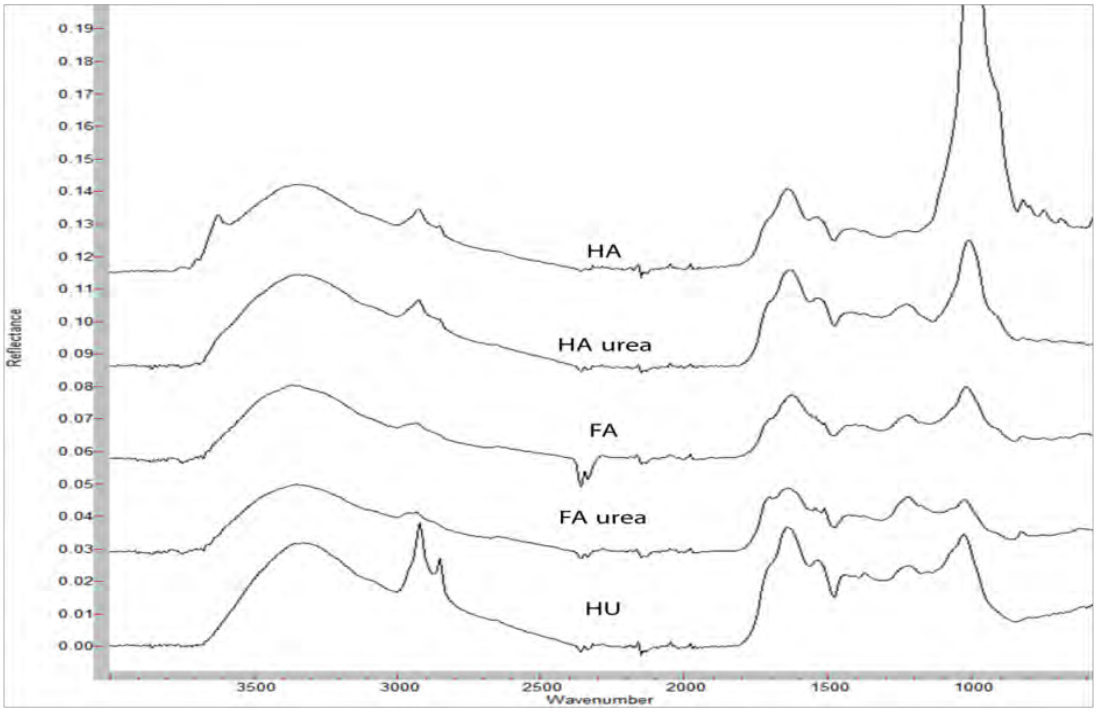

Figure 8.3. Attenuated Total Reflectance (ATR) Fourier Transform Infrared (FTIR) spectra of the HA (humic acid), FA (fulvic acid), and HU (humin) organic fractions isolated from sediments from Galway Bay. (HA urea and FA urea refer to the HAs and FAs isolated in 0.1M NaOH + 6 M urea).

The spectra of the HAs (alkaline and alkaline-urea extracts) are broadly similar (Figure 8.4A). The spectra are dominated by aliphatic functionalities (20-40 ppm). Because there is no clear evidence for O-aromatic functionality (140-155 ppm) characteristic of lignin components, the band at 50-60 ppm is likely to be attributable to amine/peptide-type structures. The resonances in the 50-60 ppm region are stronger in the HA-urea isolate, as urea is a good solvent for peptide structures. The resonances at 70-90 ppm and the weak resonance centred at 105 ppm (anomeric C) would typically suggest carbohydrate components in the HA; however, the anomeric C is difficult to identify, especially in the HA-urea isolate. The resonances at 70 ppm may also arise from alkyl-OH groups. The evidence for aromaticity (120-140 ppm) and carboxyl/ester/amide (165-185 ppm) is stronger for the HA-urea isolate, due to the increased protein (amide) and aliphatic hydrocarbon (CO_2H in fatty acids) compositions. The HSQC for the HA (Figure 8.6A) shows a weak resonance for methoxyl C from lignin, but it gives no evidence for aromatic structures that could be attributed to lignin. There are also weak resonances for peptide components and carbohydrate. The aliphatic region has the strongest resonances consistent with the

non-polar compositions of HAs. There is evidence for peptidoglycan which suggests that microbial biomass forms part of the HAs.

The spectra for both HUs are dominated by aliphatic functionalities (Figure 8.4B), and with some evidence for O-alkyl and CO_2H groups. There are a number of differences evident for the ^{13}C NMR spectra for the HUs from the top and from the bottom. The HU-bottom spectrum has stronger resonances for 56 and 130 ppm (possibly lignin), and 72 and 105 ppm (generally assigned as carbohydrate). The strong resonances at 172 ppm, coupled with resonances in the 50-60 ppm region, can be attributed to amide in protein, and the 172 ppm resonance, coupled with the strong aliphatic resonances, and alkyl-OH resonances at 70 ppm indicate CO_2H from fatty acids and esters in waxes such as cutin. Deshmukh et al. (2005) assigned the peak at 73 ppm to methylenes attached to ester-linked mid-chain hydroxyls, and the peak at ca. 40 ppm as methines in α-branched carboxylic acids/esters found in cutin/cutan. Lorenz et al. (2000) attribute resonances at 144 and 154 ppm specifically to tannins, along with resonances at 105 ppm (quaternary C). Therefore the peak at 105 ppm in this instance can be attributable to tannins rather than anomeric carbon. The resonance at 105 ppm in HU is unlikely to be anomeric C because the evidence for carbohydrate in the HSQC is weak. The HSQC spectrum confirms the protein assignment (Figure 8.6B). There are also strong resonances for aromatic amino acids and weak resonances for α- protons in peptides, confirming contributions from protein. There are no visible resonances for anomeric C, but there are weak resonances for CH and CH_2 from carbohydrates, suggesting small carbohydrate contributions. There is a methoxyl C resonance, but the aromatic lignin resonances are absent, indicating that the contributions of lignin to the HU are small. It is also possible that there is a resonance for peptidoglycan, from bacterial cell walls.

There is evidence for aliphatic hydrocarbon resonances in the FAs (Figure 8.4), providing the polar FAs with hydrophobic domains which may prevent their re-mineralisation by microorganisms. The FAs have stronger resonances for aromatic structures (125-160 ppm), especially the FA-urea isolate, compared to the HAs and HUs. The aromatic structures are most likely from phenolic units in lignin, tannins and cutan. There is evidence for carbohydrate, especially in the FA (alkaline isolate), because of the large O-alkyl C peak at ca. 70 ppm, but the anomeric C (105 ppm) resonances are broad. The FA-urea isolate has lower carbohydrate signals coupled

with a higher content of aliphatic hydrocarbons, illustrating the enhanced hydrophobic nature of this isolate. There is evidence for lignin derived materials in the FA-urea isolate due to a strong resonance centred at 130 ppm, a small band at 148 ppm (O-aromatic) and at 153 ppm (syringyl units), although the resonance at 56 ppm does not display the sharp peak characteristic of methoxyl. The resonance between 50 and 60 ppm is more defined in the urea isolates due to the ability of the urea to solvate peptides and H- bonded components, e.g. lignin structures. There is a strong resonance between 100 and 120 ppm in the FAs. Resonances in this region can arise from an array of aromatic structures including the C_2, C_5, and C_6 from guaiacyl lignin units (Wikberg and Maunu, 2004), C_6 in ferulic acid type structures, and alkenyl C atoms in terpenoids (Schuhr et al., 2003; Forte et al., 2006). The resonances ca. 107, 160, 171 and 182 ppm are indicative of aromatic structures in cutin/cutan (Mc Kinney et al., 1996; Deshmukh et al., 2005), and that is supported by the C_{16} and C_{18} fatty acid methyl esters identified in the pyGC/MS pyrograms. In contrast to the HA and HU, the HSQC of FA (Figure 8.5) have stronger resonances for carbohydrate, protein, and lignin. The resonances from anomeric C and CH and CH_2 confirm carbohydrate inputs. The presence of lignin is also confirmed with a strong OCH_3 and aromatic resonances from lignin units. Protein is also shown to make a significant contribution to the FA with strong resonances for aromatic amino acids and α- protons from peptides. The peptidoglycan resonance (coupled with the strong proteins resonances) is clearly evident, suggesting that microbial necromass make contributions to the compositions of the FAs.

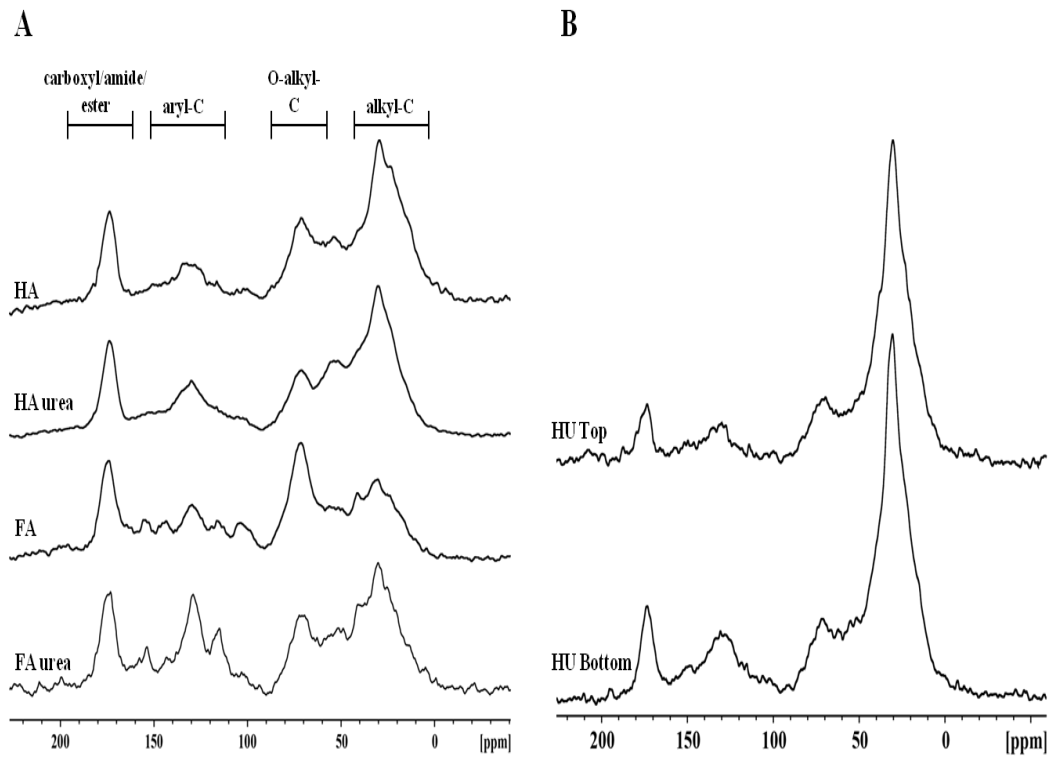

Figure 8.4. Solid-state ^{13}C CPMAS NMR spectra of: (A), HA (humic acids), HA urea. FA (fulvic acids), and FA urea; (B), HU (humin, top and bottom), isolated from sediments from Galway Bay (HA urea and FA urea refer to the HAs and FAs isolated in 0.1M NaOH + 6 M urea).

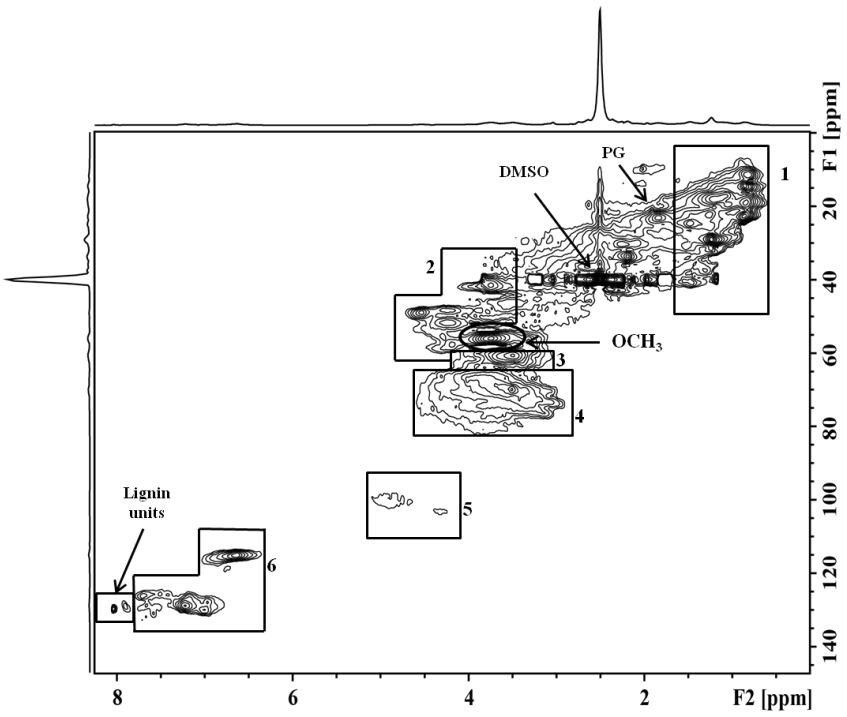

Figure 8.5. HSQC 2D NMR spectrum of fulvic acid (FA; top of core). Assignments are as follows: (1), aliphatic CH_2 and CH_3, including signals from hydrocarbons, and from protein side-chains; (2), α protons in peptides; (3), CH in carbohydrate; (4), CH_2 in carbohydrate; (5), anomeric carbon in carbohydrate; (6), aromatic amino acids (Phe and Tyr). PG= peptidoglycan, DMSO = dimethyl sulfoxide (solvent); OCH3 = methoxyl.

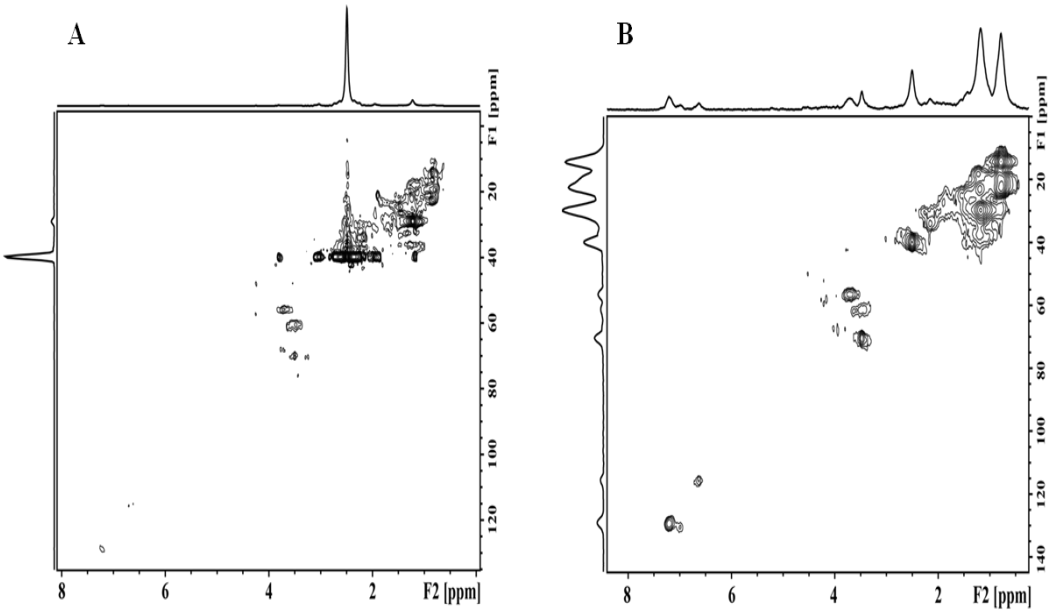

Figure 8.6. HSQC 2D NMR spectrum of: (A) humic acid (HA; top of core); and (B), humin from the base of the core (HU bottom).

8.3.4 PyGCMS

The sequential pyrolysis at 340 °C provided assessments of compounds that were not part of the humic structure, followed by pyrolysis at 720 °C to release the components of the humic suprastructures. Fewer compounds were evolved at 340 °C compared to 720 °C (Table 8.2), except in the case of the FAs. It was possible to evolve high molecular weight compounds at 340 °C (Figure 8.7), and a number of compounds from carbohydrate, proteins, lignin, tannins, and plastic contaminants were released. However, this temperature is not sufficient to cleave the macromolecular components and release the building block compounds, and compounds entrapped in the humic matrix. The carbohydrate and protein compounds that are released at higher temperatures are most likely protected from degradation by their associations with aliphatic hydrocarbons. Benzene, toluene, and ethylbenzene compounds were not formed at 340 °C. These compounds are not part of the humic materials but are reformation products following the liberation of aromatic compounds at high temperatures. All contaminants that will be discussed in Section 8.4.2.3 were also identified in the 340 °C pyrolysis. The single pyrolysis at 720 °C evolves the majority of the compounds in humic structures allowing for complete compositional characterisation.

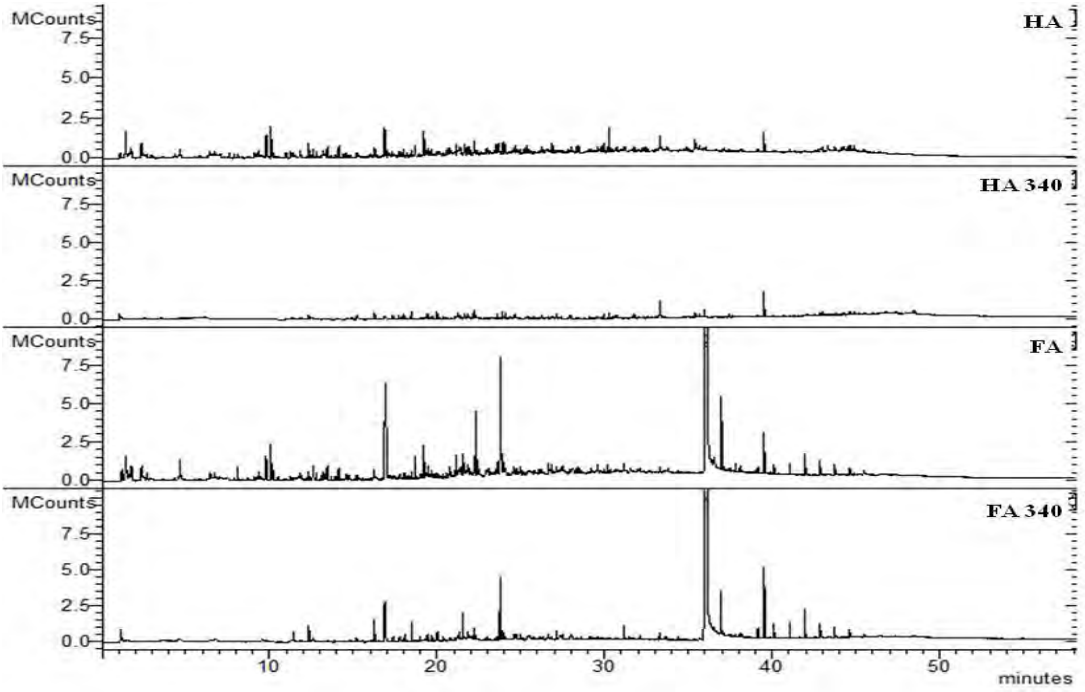

Figure 8.7. Pyrograms of the humic isolates from the Galway Bay Core 1 sediment using flash pyrolysis at 720 °C and 340 °C (HA= humic acid (720 °C), HA 340 (340 °C), FA = fulvic acid (720 °C), FA 340 (340 °C)).

Compositional differences between the fractions from the pyGC/MS analyses are illustrated in Figure 8.8A. In many instances the data complement those from ^{13}C NMR spectroscopy. FAs produce the highest yields of lignin derived compounds, especially in the FA-urea isolate. A number of lignin derivatives (i.e. guaiacol, methoxyphenols) were identified in the pyGC/MS, especially in the FAs (Table 8.2). There are also strong lignin signals in the HUs, especially HU bottom. The highest yields of aliphatic compounds were contained in the HUs, because the HU is composed primarily from lipid and waxy materials. Similar quantities of furan compounds were identified in the HAs, FAs and HUs. However, the quantities of furans were lower in the HA-urea and FA-urea fractions, indicating that compounds that gave rise to the furans were extracted in the 0.1 M NaOH extractions. N-containing heteroatoms are highest in the HAs and HU-bottom. Furans and N-containing heteroatoms (such as pyrroles and pyridine) are derived from carbohydrates and proteins, respectively (Kaal et al., 2008b, c). Sterol contributions to the humic isolates are shown to be small, and the identified sterols are outlined in Table 8.2. The presence of cholesterol (and cholesta-3,5-diene), an animal sterol, is present in the FA fraction. This is of note because this fraction was enriched in $\delta^{15}N$, possibly from sewage/manure. Cholestanol was identified in the HA and the HU from the thermochemolysis. Ergosterol methyl ester, an indicator for fungal inputs (Weete, 1980; Young, 1995), was present all the isolates from the top of the core but the highest concentrations were in the HUs. Hopanes, microbial type sterols that are not present in higher organisms, were also identified (Table 8.2) in the organic fractions. Hopanes are a minor part of the lipid membrane and the total cell biomass of some bacterial cells (Greenwood et al., 2006), indicating that cellular debris is contained within the organic fractions of the sediments. The presence of ergosterol and hopanes suggest an important microbial input to the lipid fractions of organic materials.

Key biomarkers were targeted in the pyGC/MS (Figure 8.8B) to help to determine the origins of organic materials. Phenol is the dominant compound common to all of the organic fractions. It is postulated that the majority of phenols in the organic fractions are derived from lignin, a terrestrial biomarker. The FAs are richest in phenols, and especially the FA-urea isolate, which also has the strongest spectroscopic evidence for lignin-derived substances. Additionally, the alkaline-urea

isolates are more enriched in phenols than the alkaline isolates, suggesting that the urea assisted in the isolation of these aromatic materials. Due to the weak resonances of aromatic lignin resonances in the 2D NMR (Figure 8.5, 8.6), it is likely that there are phenol sources other than lignin, e.g. from cutan (based on the resonances indicative of cutan in the ^{13}C NMR consistent with those identified by Deshmukh et al. (2005)) and from tannins (Lorenz et al., 2000). The quantities of isoprene ketones, indicators of temperature changes in sedimentary environments (Wang et al., 2012), are small in all the organic fractions. Levoglucosan, formed during the pyrolysis of hexoses, was detected in the HA and FA isolates. It was absent from HU-top and present at very small concentrations in HU-bottom, suggesting a smaller contribution of saccharides to these recalcitrant fractions. The presence of thiophene in all isolates indicates the inclusion of marine OM in the refractory components of the estuarine OC pool. Pristane was present in the HUs and the HA isolates and is regarded as a marine biomarker. However, as this compound is derived from chlorophyll *a* it is also possible that it has terrestrial plant origin.

The major compositions of the HUs are illustrated in Figure 8.8(A, B) and these allow comparisons of how the HU compositions differ with time. It is evident that the HU-bottom has a higher concentration of aliphatic components, furans, lignin derivatives, and phenols, all of which suggest major contributions from higher plants to this recalcitrant organic fraction. Additionally, the HU-bottom has a small quantity of levoglucosan, indicating the presence of terrestrial biomass. HU-bottom also has a higher concentration of nitrogen compounds, compared to HU-top, suggesting that microbial necromass is an important contributor to C pools and that protein components are selectively preserved. Thiophene and pristine, both marine biomarkers, are present in slightly higher concentrations in the HU-top.

Polymethylene chains show a unimodal distribution maximising at C_{13} (Figure 8.8C). The HU-bottom shows a bimodal distribution which maximises at C_{13} and at C_{27}. As expected the hydrophobic HUs are abundant in the aliphatic polymethylene compounds, especially the HU-bottom. The FAs have low concentrations of all hydrocarbons, with the exception of C_{25} to C_{31}. *n*-Alkanes and *n*-alkenes (C_8-C_{31}) are known to derive from algaenan (Largeau et al., 1984), cutan, and suberan (Tegelaar et al., 1989; Deshmukh et al., 2001), and hydrocarbons of these chain lengths were identified in the pyGC/MS pyrograms. Chikaraishi and

Naraoka (2003), report that fresh water plants have an *n*-alkane maximum at C_{23} or C_{25} and seaweeds range from C_{13} to C_{17}. Both C_{13} and C_{25} chains are present at high concentrations, indicating inputs into the OM from the riverine, lacustrine and marine sources. Marine algae are dominated by *n*-alkanes in the range of C_{15}, C_{17}, C_{19}, whereas terrestrial plants are dominated by long chain C_{23} to C_{35} *n*-alkanes (C_{27}, C_{29} and C_{31}) are most common (Bianchi and Canuel, 2011). Such long chain alkanes are associated with the waxy protective coatings on leaf cuticles. Bacteria are generally composed of C_{10} to C_{30} hydrocarbons and these are often even-numbered homologues and branched (Bianchi and Canuel, 2011). The high concentration of long chain aliphatic hydrocarbons (Figure 8.8C), particularly in both HUs and in the FA (alkaline extract), is due to the contribution of the waxes of vascular plants (Matsumoto et al., 1992). The FAs have very low concentrations of the shorter chain hydrocarbons, suggesting a low input of aliphatic hydrocarbons from micro-organisms and algae.

Thermochemolysis with an *in-situ* methylating agent gives rise to volatile methylated compounds from substrates that otherwise would char or not be resolvable in the pyrograms. Fatty acid methyl esters (FAMEs) are generated via transesterification of triglycerides and other lipids when a methylating reagent, tetramethylammonium hydroxide (TMAH), is added (Challinor, 1991; Chefetz et al., 2002). There is a strong even over odd predominance in the concentration of FAMEs (Figure 8.8D). FAs have low concentrations of FAMEs, and odd FAMEs were not detected. Long chain fatty acids (C_{24} or longer) are assigned to higher plant sources (Rashid, 1985; Pulchan et al., 2003), and these are present at high concentrations in all the organic fractions. C_{16} and C_{18} FAMEs were identified in the FAs, along with C_{14}, C_{24} and C_{28} components in lower concentrations, suggesting higher plant contributions. HU and HAs had FAMEs in the range of C_{12} to C_{28}. C_{16} and C_{18} FAMEs in high concentrations were common to all fractions; these are the dominant aliphatic monomers in cutan (unsubstituted, ω-hydroxy, α, ω dicarboxylic, mid-chain functionalised monomers, Nawrath, 2006; Pollard et al., 2008).

Volatile aromatic compounds were identified in the organic fractions. Toluene was common to all samples and was present in the highest concentration. In contrast, benzene was a minor component. Both of these observations suggest that much of the aromatic rings are substituted. The xylenes were present in lower

concentrations and with no strong evidence for a longer alkyl aromatic series $>C_3$. The majority of the aromatic compounds produced during pyrolysis are oxygenated or substituted. Kaal et al. (2008a) suggest that benzene and toluene can be generated from the pyrolysis of fatty acids, lignin, tannins and proteins.

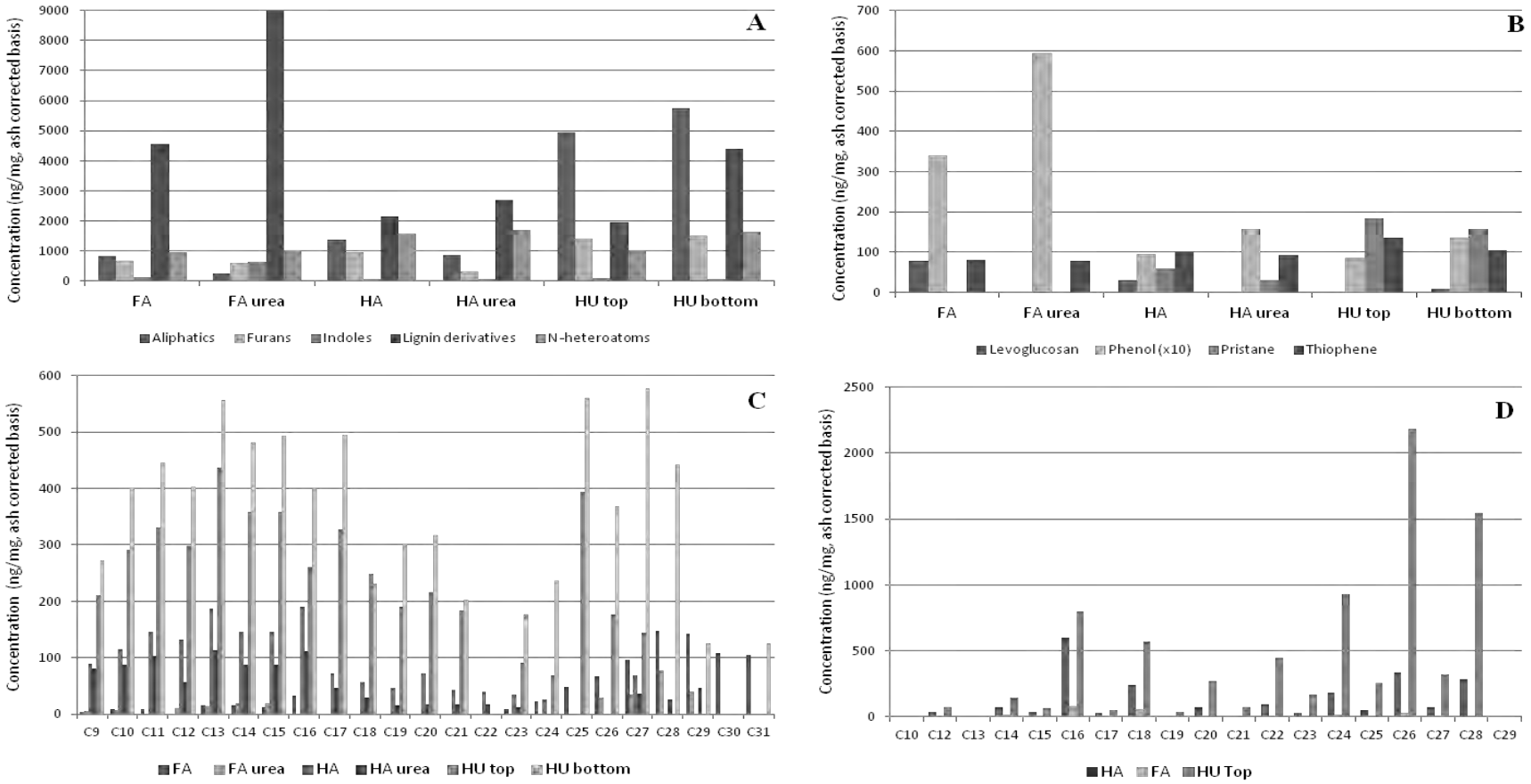

Figure 8.8. Analyses of the organic fractions in the isolates from the Galway Bay Core 1 sediment, as identified by flash pyrolysis Gas Chromatography Mass Spectrometry (pyGC/MS, single 720 °C pulse, ash and internal standard corrected); (A), major compound classes identified; (B), biomarkers; (C), distributions of aliphatic hydrocarbons; (D), distributions of the Fatty Acid Methyl Esters (FAMEs). HA= humic acids, FA = fulvic acids, HA urea and FA urea refer to the HAs and FAs isolated in 0.1M NaOH + 6 M urea, HU = humin from the top and base of the core. TMAH (tetramethyl ammonium hydroxide) was added to the isolates in D prior to pyrolysis.

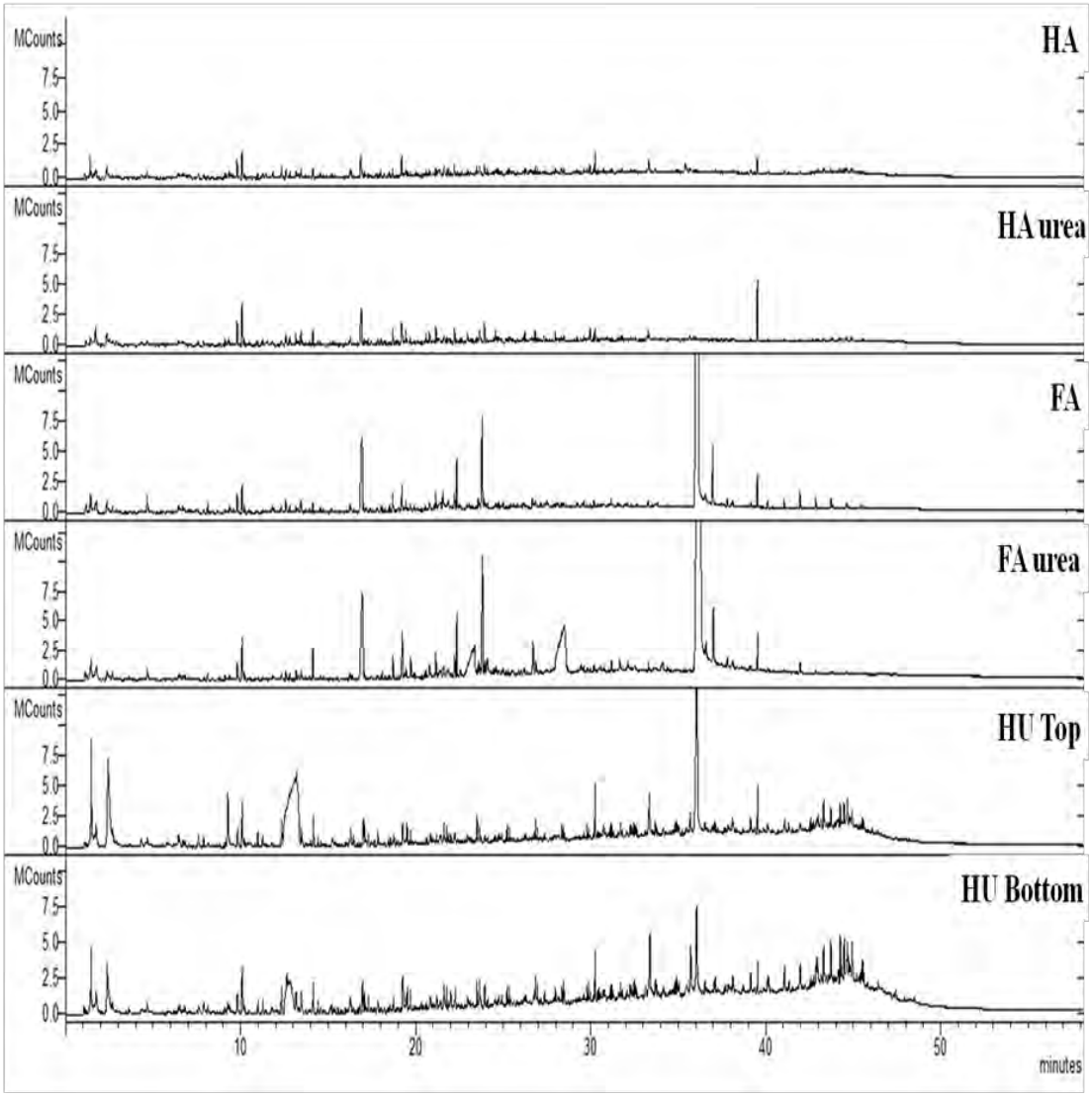

Figure 8.9. Pyrograms of the organic fractions isolated from sediments in Galway Bay (no TMAH (tetramethyl ammonium hydroxide) added, single 720 °C pulse). HA = humic acids, FA = fulvic acids, HA urea and FA urea refer to the HAs and FAs isolated in 0.1M NaOH + 6 M urea, HU = humin.

8.4. Discussion

FAs, the most polar isolates, can have considerable quantities of biologically labile components, such as protein and carbohydrate materials that are readily soluble in aqueous media. Acidic functional behaviour is provided by phenol-OH groups from lignin/cutan structures and CO_2H from fatty acids and acidic amino acids. The HAs are composed of both recalcitrant species (e.g. aliphatic methylene) and some labile components. Both HUs are predominantly composed of aliphatic methylene, with small amounts of carbohydrate and protein species entrapped in the aliphatic methylene components. Because of their hydrophobic nature such materials are not

extractable in aqueous media. The solvent systems used in this study were effective for the isolation of different OM fractions from sediments. In agreement with findings by Hayes et al. (2012), the alkaline isolates are shown to be compositionally similar to the alkaline-urea isolates. It is evident, from the spectra acquired and the ash determinations, that the organic extracts have strong interactions between the organic and inorganic components. The DMSO-acid solvent yielded isolates with lower ash contents which suggests that the solvents were more efficient at breaking bonds. It is possible that these strong organo-mineral interactions that were disrupted help the stability and preservation of OC in the environment. HU-bottom is shown to have a number of labile components that have been protected from degradation over a long period of time. It is important to note that HAs and FAs were not retained at this depth which suggests that these materials will eventually become re-mineralised with time. HU represents stable organic materials that will be transformed to kerogen/fossilised carbon rather than become re-mineralised.

The differences between the compositions of the HU samples, top and bottom, were evident in the NMR spectra (Fig 8.4B) and the data from the pyGC/MS (Figure 8.8, 8.9). It is evident that HU-bottom has a greater concentration of aliphatic hydrocarbons than HU-top, suggesting that these components are highly resistant to degradation. Surprisingly, the HU-bottom has a higher concentration of lignin derivatives, furans and phenols, with terrestrial plants being the most likely source. The HU-bottom also has a higher concentration of C_{16} and C_{18} fatty acids that can be indicative of cutan. Thiophene and pristine are at slightly higher concentrations in the HU-top, suggesting a greater marine influence. The HU-bottom has a higher atomic N concentration and a higher abundance of N- compounds, suggesting that proteins/microbial necromass are selectively preserved by the aliphatic components. The N composition of the HUs and the presence of compounds such as peptidoglycan, ergosterol, and hopanes, all suggest that micro-organisms provide considerable contributions to the OC sink. It is unlikely that the resonance at ca. 70 ppm in the HUs is from carbohydrate unless it is due to the presence of microbial sugars. Levoglucosan is absent from the HU-top and is present at very low concentrations in the HU-bottom. Therefore, the resonance in the NMR at 70 ppm is more likely to be from alkyl-OH from cutan.

8.4.1 Chemical Characterisations

The HUs had the highest C and H values as these are predominantly composed of aliphatic hydrocarbons (Table 8.1). The elemental N values correspond with the quantity of N-containing compounds/structures indicated by the pyGC/MS, and by the FTIR and ^{13}C NMR spectroscopy data. The atomic ratios assess structural changes in the organic materials (He et al., 2008). The C/N values for the HAs are in the marine range (2-10) and the HUs and the FA urea are approaching the value for higher plants (ca. 20; He et al., 2008; Giovanela et al., 2010). The influence from the higher plants on the C/N may be decreased due to the abundance of N-compounds in the humic fractions. The δ ^{13}C signatures from the isotope data indicate that the HAs and FAs are closer to marine signatures (-22 to -23‰; Nissenbaum and Kaplan, 1972) but the HUs signature is slightly more depleted in ^{13}C, which would indicate inputs from terrestrial C_3 plants.

The ^{15}N isotope ratio data for the organic fractions (Table 8.1) are in the range for seawater nitrate (5-7‰, Hedges et al., 1997) and soil N (0-8‰ Xue et al., 2009). FA has a value of 19‰ which is significantly different from the other values. This range of ^{15}N enrichment can be assigned to nitrate from manure and sewage. Volatilisation of ammonia, in sewage and manure, and subsequent oxidation to nitrate enriches the ^{15}N-value (Xue et al., 2009).

Neutral sugars can give some indications about the origins of the (poly)saccharide components of OM (Hayes et al., 2008). For example, arabinose (ARA) and xylose (XYL) are largely plant sugars, whereas rhamnose (RHA), mannose (MAN), and galactose (GAL) are believed to be predominantly of microbial or animal origin (Cheshire and Hayes, 1990). If the ratio [MAN + GAL)/ (ARA + XYL)] is < 0.5 it suggests that the origins of the OM are predominantly from higher plants, and if the values are ca. 2 this indicates that the origins of the OM are microbial (Oades, 1984). The HA from the top of the core has a value (Table 8.1) indicating compositions dominated by microbial sugars. During diagenesis, the OM is degraded and transformed; the plant carbohydrates are utilised and microbial sugars appear to be preserved.

8.4.2 Spectroscopic Characterisation

8.4.2.1 Major Compositions of the Organic Fractions

The FTIR spectra gave useful indications about the impact of the isolation procedure on the gross chemical/structural compositions of the fractions. Inorganic material present in the samples (reflected in the high ash contents) had a negative effect on the resolution of the spectra. ^{13}C NMR is now regarded as the best instrument for determinations of the complex structures and interactions in natural OM (Simpson et al., 2011), and 2D NMR helps to confirm assignments of resonances in the 1D spectrum. The NMR spectroscopy data highlight that the compositions of the FAs are clearly different from those of the HAs and of the HU. There are some differences between the two FA isolates in terms of their components and their relative amounts. The FAs are the most heterogeneous of the fractions, and have numerous oxygen functional groups present (also evident in the FTIR spectra).

Protein is an important biomolecule present in HSs (Kelleher and Simpson, 2006). The majority of the N detected in soil OM is from amide N (Smernik and Baldock, 2005) rather than from inorganic sources. N-containing heteroatoms and indoles were identified in the pyGC/MS spectra, and there are peptide and amide resonances at 50-60 and 170-180 ppm in the ^{13}C NMR spectra, as well as amide and N-H bonding identified in the FTIR spectra. Peptidoglycan resonances are also present in the HSQC (Figs. 8.5 and 8.6). This protein originates mainly from the cell walls of microorganisms and of plants (Kelleher and Simpson, 2006). Isolation of peptides is enhanced in the alkaline-urea medium.

In general, polymethylene straight chain aliphatic hydrocarbons dominate the fragments released on pyrolysis, especially in the case of the HU fraction. *n*-Alkanes and *n*-alkenes are shown to be the most abundant classes of compounds in the HAs. This tendency is biased, and is typical of the pyGC/MS technique, because these components are most likely to break free during pyrolysis, whereas oxy-aromatics, and carbohydrates are more likely to form chars. Aliphatic hydrocarbons are distributed throughout the pyrograms; however, the exact hydrocarbon compounds could not be identified and so these do not appear in Table 8.2. A number of peaks are evident in the aromatic region of the ^{13}C NMR spectra, especially in the FAs. The peaks in the aromatic region of ^{13}C NMR can be assigned as plant derived materials such as aromatic structures from cutan, tannins and lignin. The cutan

resonances at 160, 171, and 182 ppm are small but evident when the spectra are greatly expanded. There are phenolic resonances at 145-160 ppm in the ^{13}C NMR coupled with broad resonances at 107 ppm from -CH_2-O- in lignin or alkyl substituted aromatic C in cutan. Tannins are present at high concentrations in higher plants, and in less much lower concentrations in fungi, algae, mosses and grasses (Haslam, 1981; Kögel-Knabner, 2002).

Labile fatty acids can be retained in the sediments when these interact with stable compounds and mineral particles (Pulchan et al., 2003). Free fatty acids are present in the protective tissues of plants (cutin and suberin) and in free or combined states (waxes) on the outer surface of the leaves (Feng et al., 2010; Zocatelli et al., 2012). The chain lengths of fatty acids provide indications of their origins, i.e. all fractions have chain lengths indicative of cutin (e.g. C_{16} and C_{18}), especially the HU (Figure 8.8D). The HU has the highest concentration of odd FAMEs, indicative of bacterial inputs (Figure 8.8C, Chefetz et al., 2002); the contributions from micro-organisms to the isolates is evident with each analytical technique used. The preservation of peptide structures is consistent with aggregations of the microbial cells within the supramolecular associations of the HU. These aggregates exclude water and are consequently protected from microbial (Piccolo and Conte, 2000) and abiotic degradations.

A number of sterols were identified in the pyGC/MS using both flash pyrolysis (Table 8.2) and thermochemolysis. Cholesterol, an animal sterol found in human excreta (Eneroth et al., 1964), is believed to be a ubiquitous sterol in the environment (Matthews and Smith, 1968; Attaway and Parker, 1970; Gagosian, 1975; Hatcher et al., 1977). The presence of cholesterol and its derivatives (Table 8.2), along with the enrichment of $\delta^{15}N$ in the FA, indicates that sewage/agricultural wastes are contributing to the OM in Galway Bay. The identification of hopanes and ergosterol indicate that microbial components are retained in the isolates.

Furans, compounds derived from carbohydrates, are present in all samples. Free saccharides are removed from the HSs during fractionation on the XAD-8 resin, and therefore the remaining saccharides can be considered to be part of the humic structure. Levoglucosan, a pyrolysis product of cellulose and of hemicelluloses, had the highest concentrations in the HA and FA fractions. Hemicellulose is predominately found in plant cell walls, whereas cellulose is present in both plant

cell walls and in many types of algae. The carbohydrate signal was strongest in the FAs. The resonances at ca. 70 ppm in the HUs are most likely to be predominantly from alkyl-OH from cutan, and also from tannins, lignins, and with small contributions from plant and microbial saccharides. The small levoglucosan concentration and the high abundance of furans and furaldehydes in the organic fractions are most likely to originate from products of the microbial degradation of high molecular weight polysaccharides, or from microbial extracellular products (Nierop et al., 2001; Page et al., 2002; Kaal et al., 2007; Schellekens et al., 2009; Carr et al., 2010).

It is evident that lignin contributes to the compositions of FAs (Figure 8A, Table 8.2), and this is supported by lignin signals in the NMR spectra (Figure 8.4A, 8.5). Phenol, the biomarker present at the highest concentration in all organic isolates, indicates contributions from terrestrial vascular plants (phenol units are released when lignin is pyrolysed). Additionally, a number of other aromatic compounds were identified, namely; guaiacol, dimethoxylphenols, dimethoxybenzoic acid, methoxycinnamic acid, and dimethoxy cinnamaldehyde, that are indicators of lignin. The lignin contributions are confirmed by the presence of the methoxyl C resonances in the HSQC (Figs. 8.5, 8.6). A number of polyphenolic compounds were identified (Table 8.2). Phenols can also originate from tannins, cutans, or contaminants such as BPA. The HU-bottom has a number of lignin derivatives as illustrated in Figure 8.8A and Table 8.2; due to the terrestrial compositions of the HU, it is possible that in the past this was a terrestrial environment and that the organic inputs have been preserved over a very long time scale. Irish palaeogeographic maps indicate that substantial areas of land have been lost beneath the sea (Edwards and Brooks, 2008). The model suggests that around 8,000 years BP the Aran Islands were joined to the mainland, and that shallow embayments such as Galway Bay most likely contained considerable expanses of terrestrial and/or littoral environments (Edwards and Brooks, 2008).

8.4.2.2 Biomarkers

Both terrestrial and marine biomarkers were identified. Marine biomarkers include pristane, thiophene and methyl iodide. Tocopherols were identified in the pyGC/MS pyrograms (Table 8.2), but these are not used as biomarkers due to their widespread

occurrence in the biosphere, and are abundant in photosynthetic organisms such as higher plants and algae (Barakat and Rullkötter, 1997). However, tocopherols can be involved in the formation of pristane. Pristane was identified at low concentrations in the HAs and HU. It is absent from the FAs because pristane is an isoprenoid and is therefore concentrated in non-polar species. The highest concentration of pristane was in the HU, especially HU-top, and such isoprene species can form part of kerogen compositions (Tang and Stauffer, 1995). Thiophenes have been identified in all the organic fractions. Sediments may be a sink for sulfur, and sulfur linked macromolecular materials make a significant contribution to kerogen formation (Brassell et al., 1986). During early diagenesis, sulfur is incorporated into functionalised lipids, (Boussafir et al., 1995), and these lipids are protected against microbial mineralisation (Sinninghe-Damste et al., 1989). Thiophene concentrations are highest in the HU fractions, consistent with the enrichment of sulfur in kerogen. Methyl iodide is identified in the HSs (Table 8.2). The ocean is a main source for volatile halogenated organic compounds (from algae) resulting in the alkylation of halide ions during oxidation of the OM by an electron acceptor such as Fe (III) (Keppler et al., 2000). These marine biomarkers are present at much lower concentrations compared to the compounds which are indicators of terrestrial OM. Phenol, a terrestrial biomarker is present at high concentrations. This, coupled with the evidence for methoxyl-C in the ^{13}C NMR confirms the presence of terrestrial plant derived OM, in all the isolates. Other terrestrial indicators include cutan and tannins from higher plants, and aliphatic hydrocarbon chain lengths indicative of higher plants.

8.4.2.3 PAHs and Contaminants

Galway City has little heavy industry and few pollution sources (Dao et al., 2012). However, there is a waste water treatment plant on Mutton Island in Galway Bay (53°15'16N, 09°03'16W) and this may be the source of the majority of contaminants identified in the pyrograms. Contaminants include plastic derivatives, human steroids, personal care product compounds, as well as indications of manure/sewage (discussed in Sections 8.3.2 and 8.4.1). A number of plastic derivatives (BPA and phthalates) were identified in the organic fractions (Table 8.2). BPA was identified at high levels in both the FAs and in the HU-top (peak ca. 36.10 mins, Figure 8.9) but it

is almost absent in the HAs. It is possible that it is being sorbed by the hydrophobic species in the HUs and complexing with reactive functional groups in the FAs. pyGC/MS data from the HU-bottom (Figure 8.9) showed that the BPA and phthalate contamination is significantly lower when compared with that for the FA and HU-top. Plastic materials would not be expected in the HU associated with sediments from the base of the core, which are c.a. 9,700 BP. Therefore, the implication is that some plastic contamination arose in storage (core liners) and extraction procedures (plastic lab ware), but not to the extent present in the FAs and in the HU-top. Therefore, it must be assumed that there is a level of BPA contamination in Galway Bay, with the most likely route of entry to the sediments from the waste water treatment plant. BPA has been identified as a contaminant in the aquatic environment (Singh et al., 2010); it has been identified in river sediments (Kitada et al., 2008) and in the Changjiang River Estuary, and in the East China Sea (Haiyan et al., 2010). Kitada et al. (2008) found that the highest values were recorded in urban locations suggesting that the BPA originates from domestic wastewater whereas values in farmland sediments from an agricultural region were considerably lower. All fractions show similar levels of phthalate contamination (peak at ca. 39.5 mins), with the lowest concentrations in the HA (alkaline extract) and the HU-bottom. Phthalates are ubiquitous in the environment (Larsson et al., 1986) due to the global demand and use of plastic products. This is an environmental and health concern because both phthalates and BPA are endocrine-disrupting compounds (Kitada et al., 2008; USCPSC, 2010; Zia et al., 2013). It must be noted that the resonances at ca. 165 ppm (especially in the FAs) in the solid-state ^{13}C NMR could be C=O attributable to phthalates (Henrichs et al., 1988). Additionally, the aromatic resonances at ca. 130 ppm and OH-Ar resonances at ca. 152 ppm may have contributions from bisphenol A (BPA; Xie et al., 1999). Plastic derivatives were identified in the pyGC/MS pyrograms of the organic fractions, especially in the FAs (Table 8.2).

Other anthropogenic contaminants include sun-block derivatives (2-ethylhexyl 4-methoxycinnamate and oxybenzone) and animal steroid (cholesterol) derivatives (Table 8.2). Steroids and personal care products (e.g. sunblock) have been regarded as aquatic environmental contaminants during the past decade (Ying et al., 2002; Bound and Voulvoulis, 2004; Schwarzenbach et al., 2006; Kummerer,

2009), with the most important route of entry involving the release from waste water treatment plants (Lindqvist et al., 2005; Singh et al., 2010). There is no good evidence for polycyclic aromatic hydrocarbons (PAHs) suggesting a low concentration of organic pollutants (from hydrocarbon fuels) in Galway Bay. PAH contamination, should it occur, would be expected in the HU fraction because anthropogenic organic chemicals and PAHs can bind quickly and irreversibly to HUs (Rice, 2001). Picoline, a pyridine derivative, was identified only in the HAs isolates (HA 340, HA 720, and HA urea, Table 8.2). The presence of such pyridine compounds in the environment is attributable to the refining of fuel from coal and oil shale, the extraction and applications of creosote, and the extraction of chemicals from coal tar (Ronen and Bollag 1995; Ronen et al., 1998).

8.4.2.4 Comparisons of Fractions Isolated in the Sequential Solvent Systems

Overall, it is evident that the HAs, FAs and HUs have many compositional similarities, but it is clear that the HU is not a precursor or transformation product of the HSs. The HUs, especially HU-bottom, are dominated by aliphatic components which contribute to the hydrophobic nature of these organic materials. FAs are composed of less recalcitrant components, and have strong indications that suggest that terrestrial and microbial necromass including their excretion products are the major contributors to their compositions. The HAs are possibly the most marine influenced fraction, as these show lower concentrations of lignin derivatives, and do not have major concentrations of aliphatic hydrocarbons consistent with plant materials, and they have $\delta^{13}C$ signatures consistent with marine humic matter. The pyrolysis products, as well as the spectroscopic characterisations, of the alkaline-isolates, and the alkaline-urea isolates show that these were remarkably similar, irrespective of the solvent used. The initial exhaustive alkaline extraction was continued until the extracts were effectively colourless, and the subsequent exhaustive alkaline-urea extraction removed the more tightly held materials in the sediment matrix that meet the criteria for FAs and the HAs. The data presented illustrate the advantages of the addition of urea to the alkaline solvent to remove the remaining HA and FA materials. The isolates from the alkaline-urea extraction have additional protein and lignin compositions. This is due to the ability of the urea to solvate proteins and components that are strongly H- bonded. There are less

carbohydrate components in the alkaline-urea isolates because these are easily solvated in the alkaline solvent. The remaining carbohydrate may be more ordered in structure, making it more difficult for the solvent to interact with it. Contaminants were concentrated in the FA isolates with large BPA related peaks at 36.138 and 36.970 min, and a phthalate peak at 39.526 min. FA-urea has a number of additional peaks (not present in the FA), identified as styrene (14.142 min), phenylacetic acid (23.197 min), 4-ethyl-2-methoxyphenol (23.362 min), and a large peak at ca. 28 min which contains a mixture of compounds which could not be identified (Figure 8.9). The HA and HA-urea are very similar, with the most notable differences being a larger phenol peak (16.895 min), and a bis(2-ethylhexyl) phthalate peak (39.524 min) in the HA-urea (Figure 8.9). Guanidine is present only in the alkaline-urea isolates as it is possibly a pyrolysis product formed during the heating as the result of the presence of residual urea. The DMSO-acid solvent has the ability to solvate additional, highly recalcitrant components not solvated by the alkaline or alkaline-urea media. As the HUs are compositionally very similar, despite the age difference, with the major difference being that the HU-bottom has a higher concentration of aliphatic hydrocarbons and higher concentrations of plant derivatives, such as lignin compounds, furans and phenols. The HUs are dominated by aliphatic hydrocarbon species; however, 'labile' carbohydrate and protein components remain in the HUs. The HU-bottom was isolated from significantly older sediments; therefore these 'labile' components must have protection from re-mineralisation. The amorphous carbohydrate, the protein compositions, and the lignin may be encapsulated in hydrophobic domains, or have strong interactions with the clay minerals, preventing their re-mineralisation. The HU-bottom has a strong OCH_3 resonance from lignin in the HSQC, suggesting terrestrial inputs to the sediments from 9,700 BP. It is interesting that guaiacol, 4-ethenyl-2-methoxyphenol, and 2,6-dimethoxy-4-(1-propenyl)phenol (from lignin) were identified in the HU-bottom, but these are not identified in the HU-top. It is important to note the contributions from microbial biomass, which are evident from the protein and peptidoglycan resonances in all samples, along with the identification of microbial-type steroids (hopanes).

8.5 Conclusions

The sequential solvent systems are shown to be efficient for the extraction of organic components, with the urea adduct enhancing the HSs yields and solvating additional peptide and lignin (possible cutan/suberan) compounds. FAs have significant amounts of aromatic compounds with polar species, and low sterol and polymethylene contents. The HUs are composed predominately of aliphatic hydrocarbon components that prove too difficult and energetically unfavourable for degradations by microorganisms. The HAs were not dominated by any one compound class, though aliphatic hydrocarbons, lignin-derived compounds, furans, and N-compounds all made significant contributions. Overall, it is clear that marine OM has some input into the sequestered carbon in the form of HSs, as these isolates had compounds suggestive of marine origins, such as thiophenes, and hydrocarbons with chain lengths indicative of algae/seaweed. However, the analyses show that terrestrial components are the main contributors to the HUs, suggesting terrestrial OM is more recalcitrant. The HUs, especially from the bottom, are shown to have a significant terrestrial inputs because the FAMEs identified are consistent with those in higher plants, along with the evidence for some components from lignin and cutan. Additionally, the HUs had $\delta\ ^{13}C$ signatures similar to terrestrial humic matter and C_3 plants. HSs were not retained in the sediments over time; therefore, these must become re-mineralised and are not as stable as the HUs. The less recalcitrant components, in all samples, are retained through preservation mechanisms, such as sorption on the mineral components and inclusions in the resistant aliphatic hydrocarbon components.

HAs are predominantly marine from autochthonous productivity in the coastal environment. FAs are composed of a mixture of both marine and terrestrial OM. HUs, from both top and bottom, are dominated by terrestrial derived compounds. HU is not a precursor or a transformation product of HAs and/or FAs but a separate organic entity that does not conform to the operational definitions of a humic substance. It is most probable that, prior to the sea level rise, the Galway Bay region was a terrestrial environment, especially with the strong terrestrial compositions of the HU-bottom. Alternatively, terrestrial OM may be selectively preserved, whereas the aquatic OM may be consumed by aquatic organisms in the water column and re-mineralised. However, the HU may also encompass recalcitrant

marine components such as algaenan and encapsulate marine OM in its hydrophobic domains.

The organic materials studied represent a stable, long term sink for C in the environment. The retained OM in the estuarine sediments also has considerable inputs from microbial necromass and their by-products. The protein composition, coupled with the identification of peptidoglycan, and microbial sterols (hopanes and ergosterol) all indicate strong microbial inputs.

Table 8.2 List of pyrolysis fragments of biopolymers identified in the organic fractions, pyrolysed at 720 °C, with no TMAH (tetramethyl ammonium hydroxide) added. HU = humin (HU (b) = humin bottom; HU (t) = humin top), HA = humic acids, FA = fulvic acids, HAu = humic acid urea isolate, FAu = fulvic acid urea isolate (* indicates that the compound was detected in the sample). C= carbohydrate, S = sulfur compound, HC = hydrocarbon, P = protein (Phe = phenylalanine; Pro = proline; Trp = tryptophan), Ar = aromatic compound, PPC = polyphenol compound, L = lignin, AS = aminosugar, Pl = plastic derivatives, FAd = fatty acid, SS = sunscreen derivative, and S/T = sterol/terpene (Bruchet, 1985; Leenheer and Croué, 2003).

Compound	HU (b)	HU (t)	HA	FA	HAu	FAu	HA^{340}	HA^{720}	FA^{340}	FA^{720}	Class
Furan				*		*		*		*	C
Methyl iodide			*	*	*	*				*	
Guanidine					*	*					
2-Methylfuran	*	*	*	*		*		*		*	C
Benzene	*	*	*	*	*	*		*		*	Ar
Acetone				*		*				*	
Thiophene	*	*	*	*	*	*		*		*	S
1-Heptene	*	*	*		*			*			HC
Pyrrolidine	*	*	*		*			*			P
2-Ethylfuran			*		*			*			C
2,5-Dimethylfuran	*	*	*	*	*	*		*		*	C
2(5H)-Furanone			*		*			*	*	*	C
Dimethyl disulfide	*	*	*		*			*			S
Pyridine	*	?		*		*				*	P/AS
Pyrrole	*	*	*	*	*	*		*		*	P (Pro)
Toluene	*	*	*	*	*	*		*		*	P (Phe)
2-Methylthiophene (or 3-)			*	*	*	*		*		*	S
2- (or 3-) Furfural			*	*	*	*	*		*		C

Compound	HU (b)	HU (t)	HA	FA	HAu	FAu	HA^{340}	HA^{720}	FA^{340}	FA^{720}	Class
Cyclopentenone				*		*		*		*	C
3-Methylpyrrole (or 2- isomer)			*	*	*	*	*	*	*	*	P
2-Methylpyrrole (or 3- isomer)			*	*	*	*		*		*	P
Furfuryl alcohol							*				C
Ethylbenzene			*	*	*	*		*		*	P (Phe)
Picoline (3- or 4-)			*		*		*	*			
Xylene (isomer unknown)	*	*	*	*	*	*	*	*	*	*	Ar
Styrene			*	*	*	*	*			*	P (Phe)
2-Methyl-2-cyclopenten-1-one			*	*	*	*		*			C
5-Methylfuran-2-carboxaldehyde (or isomer)	*	*	*	*	*	*	*	*	*	*	C
Propylbenzene	*	*									Ar
3-Methyl-2-cyclopenten-1-one			*		*			*			C
Dimethyl trisulfide	*	*									S
Phenol			*	*	*	*	*	*	*	*	PPC/P/L
2-Methylcyclohex-2-enone (or isomer)				*		*				*	
1,2,3-Trimethylbenzene			*		*			*			Ar
2-(or 3-) Furoic acid					*		*				C
Pyrrole-2-(or 3-) carboxaldehyde					*		*				P
2-Hydroxy-3-methylcyclopent-2-ene-1-one			*		*		*	*			C
Undecene				*		*			*		HC
Indene	*	*		*	*	*					Ar
2-Methylphenol (or isomers)	*	*	*	*	*	*		*	*	*	PPC
2-Acetylpyrrole			*	*		*	*	*	*	*	P

Compound	HU (b)	HU (t)	HA	FA	HAu	FAu	HA^{340}	HA^{720}	FA^{340}	FA^{720}	Class
2-Acetyl-5-methylpyrrole			*	*	*	*	*	*	*	*	P
4- (or 3-) Methylphenol	*	*	*	*	*	*	*	*		*	PPC
Guaiacol (2-methoxyphenol, or 4- isomer)	*			*	*	*			*	*	L
2-Methylbenzo[b]furan				*		*			*	*	C
Maltol			*					*			
Levoglucosenone			*	*		*	*		*	*	C
Ethylphenol(3 isomers)/dimethoxylphenol(6 isomers)	*	*			*						PPC/L
Benzyl cyanide	*	*	*		*		*	*			
Xylenols (dimethylphenols, 6 isomers)	*	*		*					*	*	PPC
1-,2-, or 3- Methylindene		*									P
Tetramethylbenzene (3 isomers)	*	*									Ar
3-Methylphenol				*		*				*	PPC
Benzoic acid	*	*		*		*	*		*	*	
2-Methoxy-4-methylphenol				*		*			*	*	L
Catechol (1,2- dihydroxybenzene)				*	*	*	*		*	*	
2,3-Dihydrobenzo[b]furan				*		*	*	*	*		C
4-(1-Methylethyl)phenol (or isomers)				*		*			*		PPC
2-Hydroxyphenylacetic acid			*		*					*	AS/C
1-Methylethyl phenol (or isomer)				*		*					PPC
3-Phenylpropanenitrile			*		*			*			
Indole			*	*	*	*		*	*	*	P (Trp)
Phenylacetic acid				*		*				*	AS/C
4-Ethyl-2-methoxyphenol				*	*	*		*		*	L

Compound	HU (b)	HU (t)	HA	FA	HAu	FAu	HA^{340}	HA^{720}	FA^{340}	FA^{720}	Class
4-Ethenyl-2-methoxyphenol	*		*	*	*	*	*	*	*	*	L
2-(Hydroxymethyl)pyrrole	*	*									P
4-(1-Methylethenyl)phenol				*		*			*	*	PPC
4- (or 3-) Methoxyacetophenone				*					*		Ar
Phthalic anhydride				*		*				*	Pl
Dimethylbenzoic acid(s)				*						*	
2,6-Dimethoxyphenol			*	*	*	*	*	*		*	L
Vanillin							*				L
2,3-Dihydroxy-X, Y-dimethylbenzo[b]furan				*		*			*	*	C
Ethylbenzenediol isomer				*						*	
2- or 3- Methylindole				*		*			*	*	P (Trp)
4-Hydroxyacetophenone			*	*	*		*	*	*		
Phthalimide				*		*			*	*	Pl
Oxyindole			*	*	*	*	*	*			P
4-(1-Methylethyl)-2-methoxyphenol			*		*		*		*	*	L
2-Methoxy-4-(1-methylethyl)phenol	*	*		*				*		*	L
4-Ethyl-2,6-dimethoxyphenol	*	*	*		*			*			L
4-Ethenyl-2,6-dimethoxyphenol	*	*			*		*				L
2,6-Dimethoxy-4-(2-propenyl)phenol (or isomer)	*		*	*	*	*	*	*	*	*	L
Pyroccol			*	*	*	*		*	*	*	P
Tetradecanoic acid	*	*		*		*			*	*	FAd
Diphenyl carbonate				*		*			*		Pl
4-Hydroxy-3,5-dimethoxybenzoic acid				*		*			*	*	L

Compound	HU (b)	HU (t)	HA	FA	HAu	FAu	HA^{340}	HA^{720}	FA^{340}	FA^{720}	Class
Myristic acid (Pentadecanoic acid)				*						*	FA
Palmitate ester (hexadecanoic acid)	*	*		*		*	*	*		*	HC
Phthalate ester				*		*					Pl
Oxybenzone				*		*				*	SS
Oleic acid (Octadecenoic acid)				*							FAd
4-Hydroxybenzoic acid				*							
4-Methoxycinnamic acid				*							L
Bisphenol A	*	*	*	*	*	*	*	*	*	*	Pl
2-(4-Hydroxyphenyl)-2-(4-methoxyphenyl) propane				*					*	*	Pl
Monoacetate of Bisphenol A	*	*		*					*		Pl
Diacetate of Bisphenol A				*		*			*		Pl
2-Ethylhexyl 4-methoxycinnamate			*		*			*	*		SS
2-Ethylhexyl 4-methoxyphenylprop-2-enoic acid				*			*		*	*	SS
Benzoate ester (perhaps undecyl)				*						*	HC
Bis(2-ethylhexyl) phthalate	*	*	*	*	*	*	*	*	*	*	Pl
Stearate ester (perhaps hexyl ester)				*						*	HC
Bis(2-ethylhexyl) sebacate (decanedioate)				*		*			*		Pl
Squalene				*						*	S/T
Bis(2-ethylhexyl) sebacate	*	*								*	Pl
2-Cholestene (2, 3, 4,or 5-)		*									S/T
Cholesta-3,5-diene				*						*	S/T
4-hydroxy-3,5-dimethoxycinnamaldehyde				*						*	L
Myristic acid ester				*							HC

Compound	HU (b)	HU (t)	HA	FA	HAu	FAu	HA^{340}	HA^{720}	FA^{340}	FA^{720}	Class
Hopane	*	*	*	*	*						S/T
Tetradecyl myristate				*						*	HC
β-Tocopherol	*	*	*	*	*	*	*				
22,23-Dihydrostigma-3,5-diene	*	*					*				S/T
Cholesterol	*	*	*	*	*	*	*			*	S/T
Palmitoleic ester ((*Z*)-9-hexadecenoic acid)				*						*	HC
Myristate ester				*						*	FAd
Palmitate ester (hexadecenoic acid)				*						*	HC

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Chapter 9 Insights into the Compositions of Recalcitrant Organic Carbon using NMR Spectroscopy

9.1 Introduction

Microbial degradation of organic matter (OM) releases energy-rich labile (carbohydrates, peptides, lipids) molecules for microbial metabolism and essential elements (C, H, N, O, P, S) required for the proliferation of both microorganisms and plants. However, some biomolecules have a degree of resistance to degradation, and highly resistant molecules can be retained in the environment over long time scales. This chapter seeks to identify such resistant, so-called humin (HU) structures that are part of the sedimentary carbon (C) sink. These resistant molecules represent a link in the C sink between the active C pool and the generation of fossil C.

The use of mixtures of dimethyl sulfoxide (DMSO) and concentrated sulfuric acid (H_2SO_4) was proposed by Hayes (2006) as a solvent for HU. Insoluble materials remaining in the clay matrix following DMSO extractions are considered to be highly recalcitrant organic materials. The high concentration of C in the HUs allow comprehensive NMR experiments to be carried out and acceptable signal-to-noise (S/N) ratio to be achieved in a reasonable amount of time.

The NMR studies on these organic fractions provide unique insights into the compositions of recalcitrant organic carbon (OC), and into components that are preserved for extremely long periods of time. The HU fraction is the major focus, as this fraction accounts for a large portion of the recalcitrant organic materials, and therefore represents a long term C sink. Solid-state and comprehensive multiphase (CMP) NMR experiments were carried out on dried and swollen samples (soluble and insoluble HU) to obtain comparative information about physical and chemical properties of the whole and the fractionated samples. Additionally, samples that have not undergone any extraction processes were investigated. The total OM associated with the unextracted clay-sized fraction from the sediments provided a standard that would allow differences between the fractions to be observed. Comparing the extracted samples with the unextracted samples will help to ascertain if the extracting solvents affect the C compositions. The total OM samples provide insights into the organic C in its natural state, as well as indicating the types of molecules that are degraded or altered in the humification processes. The molecules in the DMSO-

soluble HU and in DMSO-insoluble HU must have a mechanism in place that prevents their degradation. These could include physical protection mechanisms (e.g. material protected inside dormant/dead microbes, or associations with minerals; Plaza et al., 2013), or the chemical recalcitrance of the materials themselves (e.g. plant cuticles are synthesised by plants for protection and remain recalcitrant in the soils and sediments; Feng et al., 2008). Finally, HUs isolated from two different estuarine sediment cores, at different depths will be compared in order to ascertain whether HU differs with location (due to different organic inputs) and with age (different depths in the sediments). The study has used standard and novel NMR procedures in order to gain a more comprehensive awareness of the compositions of sedimentary OM, and illustrates the level of information that can be gained when the full range of liquid, gel and solid components are studied in fractionated and intact samples using a CMP NMR probe. Two-dimensional (2D) NMR aids in the assignment of resonances from molecules that are masked in the traditional one-dimensional (1D) NMR experiments. The NMR data obtained have helped increase awareness of the molecular structures that become part of the long term C sink in sediments.

9.1.1. NMR Spectroscopy

NMR spectroscopy has been shown to provide a plethora of molecular information (reviewed in Ch. 5, 5.3). This study combines solid-state 1D NMR with 1D and 2D CMP NMR experiments to investigate sedimentary OM. Three different 2D NMR experiments were run on a HU sample to provide optimum characterisation. These included one heteronuclear (Heteronuclear Single Quantum Coherence, HSQC) experiment; and two homonuclear (Nuclear Overhauser Effect SpectroscopY, NOESY; and TOtal Correlation SpectroscopY, TOCSY) experiments. HSQC reduces spectral overlap due to the additional dispersion provided by the ^{13}C dimension. This allows identification of signals that are unresolved in the 1D (^{1}H, ^{13}C) spectrum due to overlap. NOESY is a through-space correlation experiment that enables the study of structures and conformations of HSs; cross-peaks in the 2D spectrum result from two ^{1}H nuclei that are in close spatial proximity, or from two nuclei undergoing exchange (Simpson, 2001). NOESY was used by Simpson et al. (2001) to study intramolecular interactions in species, including those between

polysaccharides, polypeptides, various aliphatics, and lignin aromatics. TOCSY can identify a range of compounds as it allows the identification of through bond interaction within a spin-system. CMP-NMR technology is a novel NMR approach (first reported in studies by Courtier-Murias et al., 2012). Samples were studied both in the dried (solid-state NMR) and swollen-state using DMSO-d_6. For the swollen-state, by combining various CMP-NMR techniques, it is possible to differentiate various components based on their approximate physical state. These include: (a), the most soluble molecules that show unrestricted diffusion; (b), molecules with restricted diffusion (including swollen biopolymers and gel-like materials); (c), 'swellable'-solids (for example rigid gels); and (d), intact rigid solids that do not interact with the solvent. As CMP-NMR can be used for solution, gel (HR-MAS) and solids, the experiments are carried out directly on the intact sample and do not require extraction. DMSO-d_6 is an excellent swelling solvent as it can break hydrogen bonds and penetrating into both polar and hydrophobic domains (Simpson et al., 2001). As such it swells the majority of organic components increasing their mobility and providing higher resolution ^{1}H NMR data.

9.2 Materials and Methods

9.2.1 Regional Setting

The sampling location is described in detail in Ch. 6 (6.1). This study focuses primarily on the near shore Core 1, and on a selection of samples from Core 4 (Figure 6.1). Core 1 was taken close to the mouth of the river (53° 15'22.21'N, 09° 02'14.4'W), and Core 4 was taken further out to sea (53° 14'15.44'N, 09° 05'21.54' W). Cumulative samples were taken from the top 100 cm of sediment from Core 1 (representing ca. 2,260 cal years before present, BP), the bottom 100 cm of Core 1 (representing ca. 9,770 years BP), the top 100 cm of Core 4 (representing ca. 9,200 years BP), and the bottom 100 cm of Core 4 (representing ca. 9450 years BP). Detailed results of radiocarbon dates are outlined in Dalton et al. (2010).

9.2.2 Sample Preparation

Four samples were prepared from the bulk sediment: a whole (unextracted) clay-sized fraction sample (clay); a demineralised (unextracted) clay-sized fraction (DCF); a DMSO-soluble humin (SHU); and a DMSO-insoluble humin (IHU). Examination of sedimentary OM in the clay-sized fraction used two different

approaches: (1), whole, non-extracted samples; and (2), chemically isolated samples. The OM in the non-extracted sample (clay) would be expected to be in its natural state. A subsample of the clay-sized fraction was demineralised (DCF) in order to concentrate the C and remove inorganic species to achieve better NMR sensitivity and higher spectral resolution. The clay-sized fraction and the DCF samples contain the total OM associated with the sediments (HAs, FAs, HUs and non-humic substances). The chemically isolated samples include SHU and IHU that were isolated from the clay-sized fraction. Prior to HU isolation, HAs and FAs were extracted.

9.2.2.1 Whole Clay-Sized Fraction Sample (Clay)

The clay-sized fraction was isolated from the bulk sediment by wet sieving (mesh size 63 μm) and sedimentation, following the method in Ch. 6, 6.3.4.1.

9.2.2.2 Demineralised Clay-Sized Fraction (DCF)

A subsample was taken from the whole clay-sized fraction sample. The clay-sized fraction was reacted with 10% HF to remove the inorganic components (repeated 10 times), following the method outlined in Ch. 6, 6.3.4.2.

9.2.2.3 Soluble Humin (SHU)

Humin was isolated from the clay-sized fraction (following HS extraction) using the DMSO + H_2SO_4 (94:6 *v/v*), following the method outlined in Ch.6, 6.6.3. The extraction was repeated a number of times in order to remove all the DMSO soluble HU (SHU) from the clay-sized fraction. Inorganic matter was removed using 0.3 M HF/0.1 M HCl.

9.2.2.4 Insoluble Humin (IHU)

Material remaining after the DMSO-H_2SO_4 extraction was demineralised using 10% HF following the method outlined in Ch. 6, 6.3.4.3.

9.2.3 NMR Analyses

All organic samples were ground, and dried thoroughly in a desiccator under vacuum, using P_2O_5 as the desiccant. Additional sample preparation was then based

on the NMR probe used. The parameters for the acquisition of NMR spectra are outlined in Ch. 6, 6.14. All NMR measurements were carried out at room temperature on a 500 MHz Bruker Avance III (Bruker BioSpin). Briefly, for solid-state NMR spectroscopy the samples (ca. 50 mg for SHU and IHU, 70-120 mg for DCF and the clay-sized fraction; the variability in weights reflect the quantity of the various materials that could be packed into the NMR rotors) were placed directly in the 4 mm zirconia rotors and sealed with Kel-F caps. For the swollen-state NMR spectroscopy the sample was placed in the 4 mm zirconia rotor and filled with DMSO-d_6 (75µL) both to swell the sample and to act as a lock solvent. Note the solid-state NMR analysis could also have been carried out using the CMP-NMR probe. However, a separate solid-state probe was employed as two spectrometers were available and simultaneous acquisition using two different spectrometers made optimal use and permitted the project to be completed during my visit to the Environmental NMR Center, University of Toronto.

9.3 Results

9.3.1 Proton NMR

^{1}H NMR spectroscopy can provide a wealth of compositional and structural information on natural OM. Three different ^{1}H spectra are illustrated in Figure 9.1, showing: (a), the conventional ^{1}H NMR spectrum; (b), inverse diffusion edited (IDE) NMR; and (c), diffusion edited (DE) NMR experiments. When applied to a sample, ^{1}H NMR detects both mobile (e.g. dissolved species) and gel-like components (e.g. swollen biopolymers). However, due to strong ^{1}H-^{1}H dipoles that form in the solid-state, ^{1}H NMR (at least as employed here) will not detect true solids. The detection of solid components requires different NMR experiments that are discussed in 9.3.3. The ^{1}H NMR therefore provides an overview of the swellable and soluble materials in the samples. The IDE is a sub-spectrum showing the most mobile molecules that exhibit unrestricted self-diffusion, i.e. molecules in solution. The DE provides a sub-spectrum of molecules that are not free to diffuse (i.e. gel-like materials and swollen biopolymers). From this point on, IDE will refer to the small, mobile molecules, and DE will refer to the gel-like materials.

The conventional ^{1}H spectrum (Figure 9.1a) shows that the main signals from the SHU arose from aliphatic methylene $(CH_2)_n$. There is also a disproportionately

large contribution from CH_3, some of which arises from the hydrocarbons themselves, with the remainder arising from terminal methyl groups in amino acid side chains, a phenomenon commonly reported in soils (see Simpson et al., 2007a, and there will be more detailed discussion of peptide/protein in Sections 9.4.2 and 9.4.5). Other signals are difficult to resolve from the baseline due to the dominance of the large aliphatic peaks in the conventional total ^1H spectrum. The small, mobile molecules (Figure 9.1b) indicate that there is a fraction of $(CH_2)_n$ that is mobile and able to diffuse, whereas the CH_3 groups in part arise from macromolecular or gel-like structures as these are greatly attenuated in the spectrum. The DMSO peak (ca. 2.5 ppm) is strongest in the spectrum of the small, mobile molecules spectrum due to the inherent mobility of the solvent. The gel-like material and macromolecules are emphasised in Figure 9.1c, and these mobile molecules are also dominated by $(CH_2)_n$ components. In addition to the aliphatic signals, resonances characteristic of carbohydrates and peptide/protein are also apparent. These resonances are more difficult to discern in the total ^1H spectrum but are emphasised by DE. The spectra (Figure 9.1a, b, c) suggest that the aliphatic molecules in the SHU consist of both small and mobile molecules, as well as large, rigid molecules. When the DE spectrum is further expanded a profile from protein/peptide is quite clear.

In Figure 9.2 the DE ^1H spectrum of the SHU sample is stacked with the ^1H spectrum for albumin, a well studied globular protein. It is clearly evident that the ^1H NMR spectrum of the HU is remarkably similar to the ^1H NMR spectrum of the albumin. The resonances in the aromatic amino acid region are especially similar for the SHU and albumin, confirming the presence of the aromatic amino acids, phenylalanine and tyrosine. The large CH_3 peak in the albumin confirms that proteins contribute to a large proportion of the CH_3 peaks identified in the conventional ^1H spectra (Figure 9.1a) of the SHU. A large contribution of protein has also been identified in soil OM and it has been shown to be result from living/dormant/and dead microbial biomass (Simpson et al., 2007a). A more in-depth investigation of the SHU can be achieved by looking at the ^1H-^1H and ^1H-^{13}C connectivities using 2D NMR experiments.

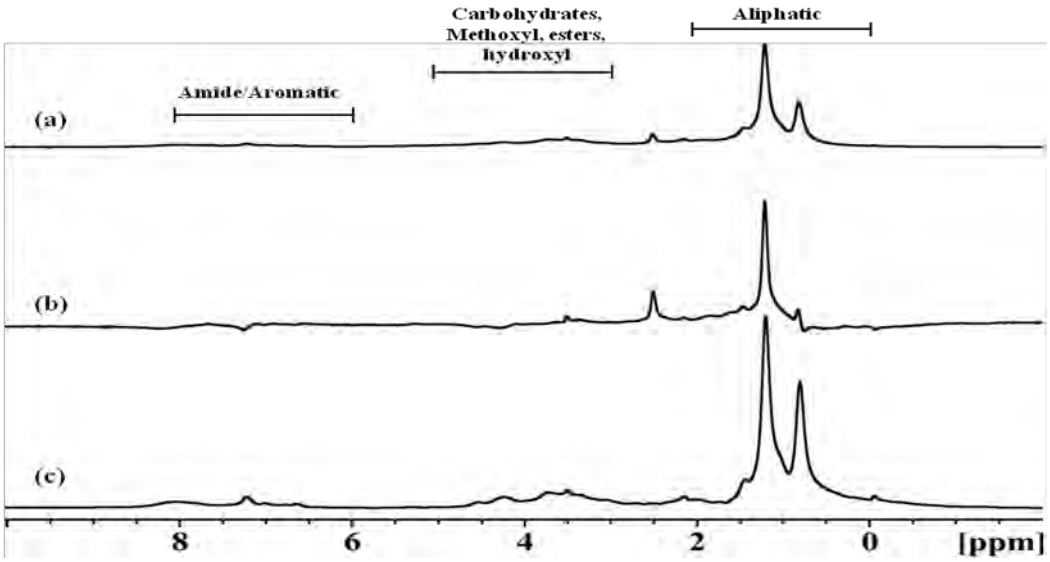

Figure 9.1. ^{1}H NMR spectroscopy of soluble humin (SHU) from Core 1 top. (a), is the conventional ^{1}H NMR spectrum showing all ^{1}H resonances; (b), is the inverse diffusion edited (IDE) spectrum showing ^{1}H resonances from the mobile/fast diffusing molecules: and (c), is the diffusion edited (DE) spectrum showing ^{1}H resonances from molecules with restricted diffusion (swollen materials and gels).

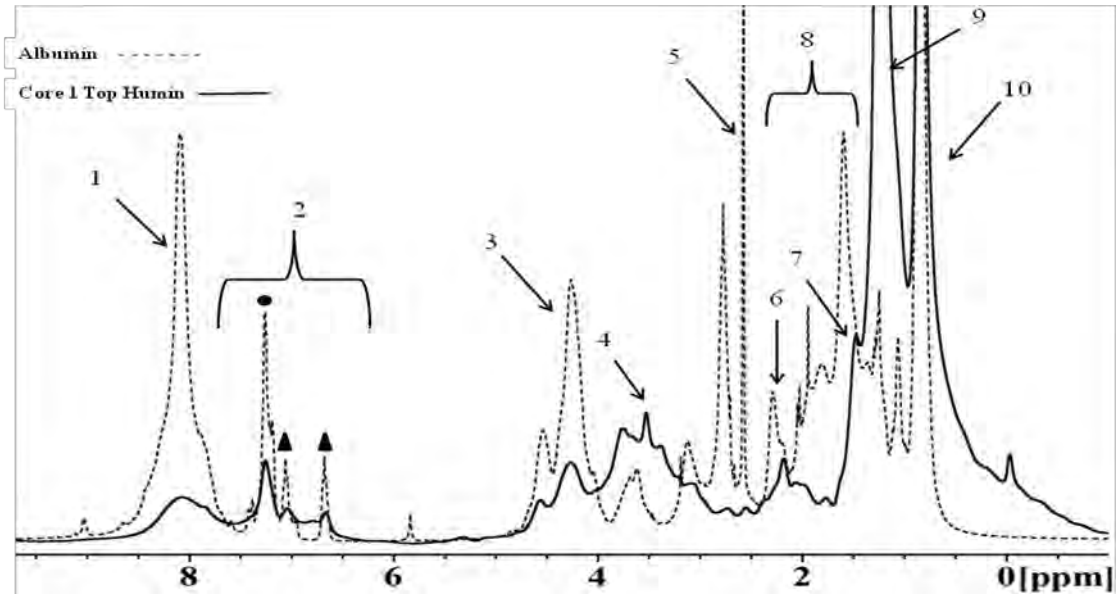

Figure 9.2. ^{1}H NMR spectrum of albumin, and diffusion edited (DE) ^{1}H NMR spectrum of DMSO soluble humin (SHU) from Core 1 top. Assignments are: (1), amide in peptides; (2), aromatic amino acids (● denotes phenylalanine, ▲ denotes tyrosine); (3), α-proton (peptides); (4), O-Aromatics (methoxyl signal in humin); (5), DMSO (solvent); (6), methylene adjacent to a carbonyl (R_2-OCO-CH_2-R_1, some appears to be in the form of lipoprotein, thus R_2 would be a protein); (7), aliphatic methylene units γ to an acid or ester; (8), amino acid side chains; (9), aliphatic methylene $(CH_2)_n$; and (10), CH_3, (Simpson et al., 2011).

9.3.2 2D NMR

9.3.2.1 HSQC

This 2D dispersion of the ^{1}H-^{13}C connectivities allows overlapping signals to be better resolved, providing more reliable assignment of resonances in the SHU. Previous detailed assignments from 2D NMR have been provided in the literature: see Kelleher and Simpson (2006); Simpson et al. (2007b); Simpson et al. (2011), for further details. As with the 1D data, aliphatic hydrocarbon resonances are also seen to dominate the HSQC (Figure 9.3, region 1). The presence of protein, observable in the 1D ^{1}H NMR spectrum Figure 9.1c, and clearly evident in Figure 9.3, is confirmed in the HSQC by the identification of amino acids and α- protons from peptides (Figure 9.3, regions 4 and 7). Carbohydrates are identified by the presence of signals from CH, CH_2 and anomeric protons (regions 2, 3 and 5 in the 2D spectrum, Figure 9.3). The HSQC confirms that the 'hump' in the 1D ^{1}H NMR between 3-4 ppm arises predominately from carbohydrate signals (Figure 9.1c) with the α-protons from protein/peptide superimposed on the left hand side with an apex from ca. 4-4.7 ppm. There are resonances in the HSQC from double bonds that indicate the presence of unsaturated aliphatic compounds. Such compounds are present in both microbial lipids, components derived from plant cuticles (Simpson et al., 2003; Simpson et al., 2007a) and algaenan (Blokker et al., 1998). As evident in Figure 9.3A, the aliphatic region of the HSQC is complex, with many overlapping resonances. When the spectrum is expanded it is possible to discern a range of additional aliphatic resonances. Contour levels are intense for the aliphatic CH_2 due to the significant contribution of methylene to the HU composition. Contour levels are also well defined for aromatic amino acids, especially Phe that has a methylene group, confirming that a significant portion of the SHU is from microbial origins. It is postulated that there are peptidoglycan signals in the HSQC (3 in Figure 9.3B); and while a distinct resonance is more discernible at lower plotting thresholds, clear identification is difficult due to the overlapping of signals. It is difficult to clearly see lignin resonances in the HSQC, although a relatively weak O-CH_3 resonance is apparent, but overlap makes it difficult to discern. α-Protons in amino acids and Ar-OCH_3 resonate in very close proximity; therefore, it is useful to look at NOESY spectra for more definitive identification of amino acids and lignin structures.

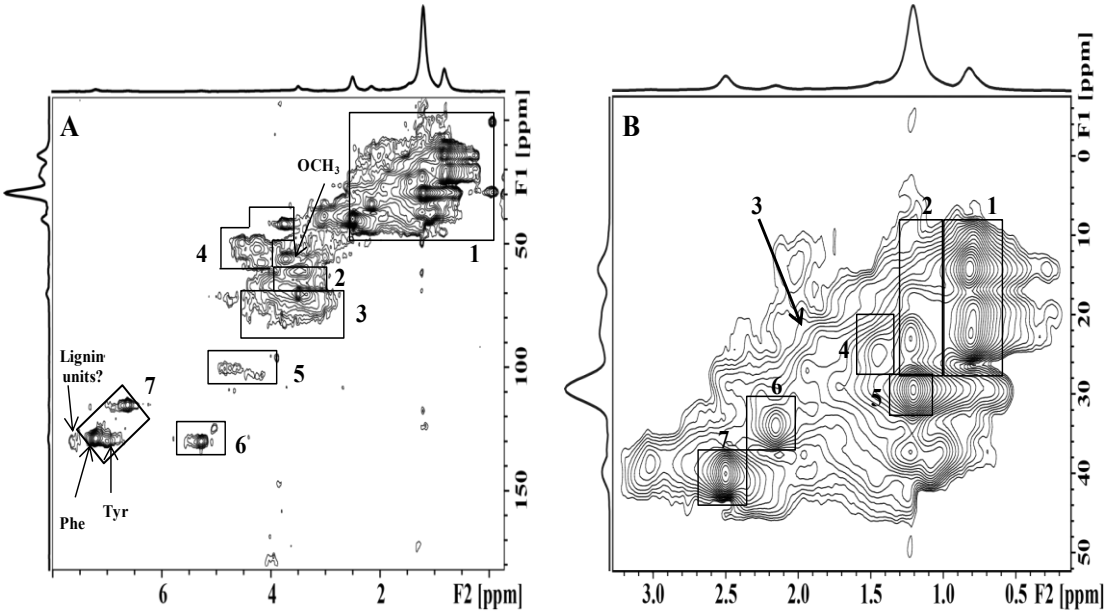

Figure 9.3. Heteronuclear Single Quantum Coherence (HSQC) 2D NMR spectra of Core 1 top Soluble Humin (SHU). A, All the resonances are from the SHU. Assignments: (1), Aliphatic CH_2 and CH_3, including signals from various hydrocarbons, and protein side-chains; (2), CH_2 from carbohydrate; (3), CH in carbohydrate; (4), α- protons in peptides; (5), Anomeric protons in carbohydrates; and (6), HC=CH; (7), amino acids (Phe and Tyr). B, Zoom of aliphatic region. Assignments: (1), CH_3 from amino acid side chains and terminal hydrocarbon groups; (2), aliphatic methylene to an acid or ester; (3), Peptidoglycan; (4), methylene unit β to an acid or ester; (5), $(CH_2)_n$; (6), methylene unit adjacent to carbonyl in lipids; and (7), DMSO.

9.3.2.2 NOESY

The NOESY spectrum (Figure 9.4) shows through space ^1H-^1H connectivities. The NOESY experiment was acquired to provide additional information in relation to peptide and lignin contributions to the SHU. Resonances attributable to amino acids are clearly visible in NOESY with strong resonances between 1-5 ppm, and 7-8 ppm. This region arises from correlations between amide (8-8.2 ppm), the α-protons in peptides (4-4.7 ppm), and various amino acid side chains (1.5-2 ppm). There are also many other protein signals in regions 4 and 5 of the spectrum but these are more challenging to differentiate due to overlap. There are also small but key lignin resonances in region 2 of the spectrum; these result from the spatial correlation between the methoxyl group on lignin and the aromatic protons (Simpson, 2001). The overlap of signals in regions 4 and 5 make additional structural interpretation from the NOESY difficult. Therefore TOCSY NMR experiments which highlight coupling through bonds tend to be less crowded and provide additional useful information from these spectral regions.

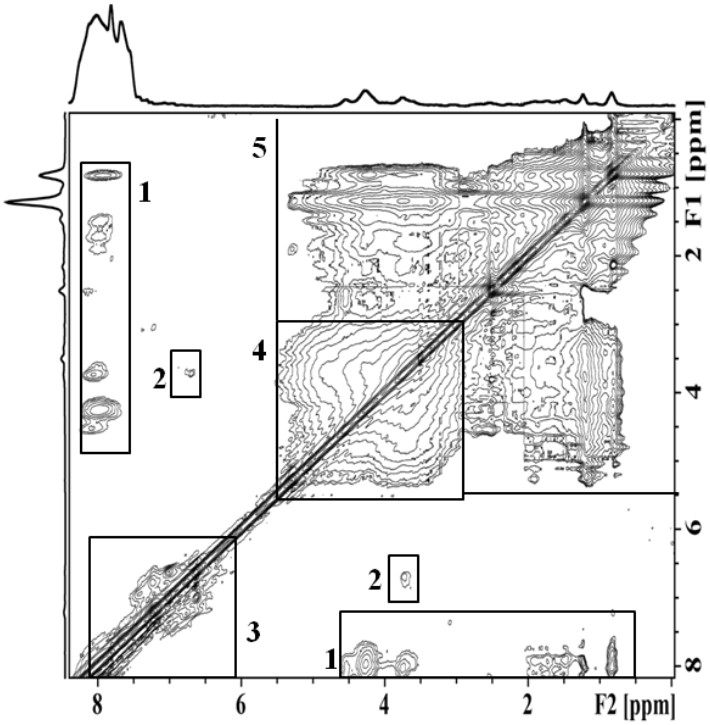

Figure 9.4. Nuclear Overhauser Effect Spectroscopy (NOESY) of Core 1 top soluble humin (SHU). Assignments: (1), amino acids; (2), lignin-aromatics (methoxyl-aromatic interactions); (3), lignin aromatic structures (interactions between aromatic protons); (4), methylene and methyl units (bonded to amino acids, and hydrocarbon structures); and (5), mixture of long chain aliphatics and protein signals (Simpson et al. 2002; 2011).

9.3.2.3 TOCSY

Consistent with all other NMR experiments on (Core 1 top) SHU, the aromatic resonances in TOCSY are relatively weak (region 1, Figure 9.5A), indicative of small contributions from lignin structures, and aromatic amino acids. Region 2 in the spectrum has overlapping correlations from protons associated with sugars, and amino acids that are complicated and difficult to interpret, but it is obvious that there are significant resonances in this region; amino acids are the most likely source as SHU would be expected to have very small sugar compositions. Region 3 contains mainly resonances from correlations with amino acids side chains. These strong resonances reinforce the concept that protein is making significant contributions to the recalcitrant OM. Regions 4 and 5 contain considerable overlap from a range of aliphatic residues, including hydrocarbons and amino acid side chains. It is difficult to confidently assign structures because of the proximity of the resonances. However, by taking a slice through the main chain $(CH_2)_n$ resonance aliphatic region, a more in-depth observation of the hydrocarbon composition is obtained (Figure

9.5B). This slice shows all the functionalities primarily associated with long chain hydrocarbons in the SHU. Resonances shown in the slice are consistent with resonances identified in cutin/cutan by Deshmukh et al. (2005) and/or biological lipids (Simpson et al., 2007a). Terminal methyl groups and main chain methylenes are represented by the large broad peaks between 0 and 1.8 ppm. Identification of such aliphatic structures in that resonance region shows that there may be inputs from waxy components from terrestrial higher plants to the organic materials in the recalcitrant SHU associated with the sediments. However, it is difficult to differentiate between microbial lipids and cuticular species, and this cannot be stated with certainty.

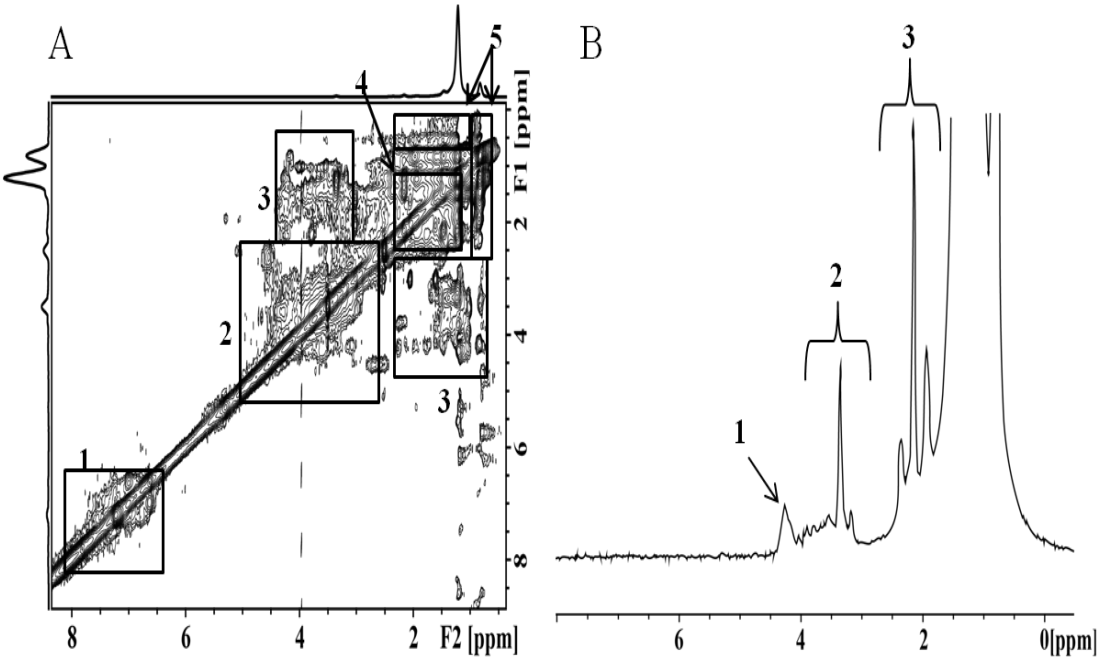

Figure 9.5. A: Total Correlation Spectroscopy (TOCSY) of Core 1 top Soluble Humin (SHU). Assignments: (1), aromatics; (2), sugars, methine units bridging lignin aromatics, amino acids (α-β couplings); (3), methylene units adjacent to ethers, esters, and hydroxyls in aliphatic chains, amino acids (α-β-γ couplings); (4), methylene in aliphatic chains; and (5), CH_3 units in amino acids and aliphatic chains, (Simpson et al. 2002). B: ^{1}H slice through the aliphatic region of the TOCSY spectrum. Assignments: (1), H attached to the O side of an ester; (2), methylene α to H in primary alcohols; and (3), methylene bonded to (a), free fatty acids, and (b), C=O side of an ester (Deshmukh et al. 2005).

9.3.3 Solid-State and Swollen-state ^{13}C NMR

^{13}C CPMAS NMR experiments on the organic samples were carried out both in the dry state and after swelling with DMSO-d_6 (Figure 9.6). Cross polarisation magnetises the protons and then passes it to the carbons in proximity via a dipole network. Strong dipole networks arise only in true solids and rigid-gels. Thus the swollen-state experiment selectively observes the solid/rigid gels components while suppressing mobile components (Courtier-Murias et al., 2012). Therefore, CPMAS of the dry sample will give an overview of the entire C in the sample, whereas CPMAS in the swollen-state will emphasise the domains that cannot be accessed by the solvent. The clay-sized fraction sample (non-HF treated) was run only in the solid dried state (Figure 9.6A). Although the S/N is very low, despite running a large number of scans (80 K), the resolution is sufficient to show that the profile of the C distribution in the clay-sized fraction broadly matches those of the organic isolates, and of the DCF sample. The clay-sized fraction was not run on the CMP probe because it was predicted that useful S/N could not be obtained in a reasonable amount of time (Masoom et al., 2013). The resonances in the spectrum of the clay-sized fraction are significantly less defined than those in the isolated organic fractions. The lack of definition is likely to be attributable to the lower concentration of organic C (note: HF dissolves minerals thus concentrating the organic components on a mass basis). The clay-sized fraction from the top of the core had an ash content of 80% (See Appendix 1-4) and an elemental C value of 26 % compared to the SHU that has an ash content of < 1% and elemental C value of 53%. The high C value and low ash value results in a good S/N ratio for the SHU; however, all the major resonances are evident for the clay-sized fraction sample despite the low S/N. The ash content is decreased considerably in the IHU (41%) and DCF (32 %), due to the prolonged HF treatments that these were subjected to. The improvement in the S/N ratio in the clay-sized fraction after HF treatment, compared to the clay-sized fraction, is evident. The presence of paramagnetic species also has a negative impact on the acquisition of spectra. The clay-sized fraction sample has a Fe content of 13%, and this value increases to 19% in the DCF sample due to the increase in the concentration of all the other inorganic species following the removal of silicate (< 1% following HF treatment). The clay-sized fraction sample has a significant carbohydrate peak, as identified in the ^{13}C solid-state spectrum (O-alkyl region,

Figure 9.6A). Indeed that would be consistent with the large carbohydrate signal in the DCF. The spectrum for the DCF sample has large peaks for lipid, carbohydrate, and protein (in the solid-state, Figure 9.6A). The carbohydrate peak is smaller in the HUs (SHU and IHU, Figure 9.6A) compared to the whole samples (clay and DCF) because labile sugars (carbohydrates) are readily degraded in the environment as they are preferentially utilised by microbes (Volk et al., 1997; Amon et al., 2001; Kalbitz et al., 2003). Therefore, these would not form part of the recalcitrant organic fractions unless some protection mechanism prevented their degradation. That could include, for example, entrapment within living, dead, or dormant cells. The peaks at 30 ppm and at 33 ppm are assigned as amorphous and highly-ordered $(CH_2)_n$, respectively, the main components of waxes, lipids, including lipoprotein, or cutins, suberans, algaenans, and fatty acids/esters (Song et al., 2008). The highly-ordered $(CH_2)_n$ resonance in IHU in Figure 9.6A is greater than that for the amorphous species. That explains why this fraction is insoluble in DMSO as the crystallinity impedes access to the extraction solvent. This can be shown definitively by comparing the spectra after swelling in DMSO (Figure 9.6B). The CPMAS spectrum in the swollen-state of the IHU shows components that are not swollen by the solvent and remain solid. The highly-ordered $(CH_2)_n$ also dominates in the SHU (over amorphous $(CH_2)_n$), indicating that portions of this isolate do not swell in DMSO. It also highlights how the H_2SO_4 enhances the solvation ability of the DMSO because components are not soluble in the DMSO for NMR analysis but were extractable in the laboratory with the H_2SO_4 adduct. The components identified in the CMP NMR are examples of how the chemical arrangement of the C species plays an important role for its sequestration and accumulation in the environment. The amorphous $(CH_2)_n$ still dominates in the DCF, but of this fraction will ultimately be degraded in the environment whereas the highly-ordered $(CH_2)_n$ are selectively preserved and concentrated.

Carboxylates and aromatic components are attenuated in the swollen-state spectrum as these can be swollen (Figure 9.6B) and gain mobility. The carboxyl region that remains, at least in part, may arise from long-chain lipids/fatty acids and could be associated with the highly-ordered aliphatic fraction. The DCF retains its strong carbohydrate signal in the swollen-state NMR. The rigid carbohydrate in the swollen-state spectra suggests the presence of highly-ordered cellulose, or large

cellulose (carbohydrate) domains that cannot be completely penetrated by the solvent. After swelling, spectra are better resolved, mainly due to decreased overlapping resonances. The CH_3 peak is better defined, whereas it is seen as a shoulder on the CH_2 peak in the dry state. There is a large hump in the 50-60 ppm region of the solid-state (dry) spectrum due to overlapping of signals from peptides and methoxyl, possibly from lignin units. Two peaks are evident in the 50-65 ppm resonance in the samples in Figure 9.6B. These could represent peptide and highly-ordered cellulose (65 ppm) structures. There is evidence in each of the spectra for carbohydrate structures confirmed by the anomeric C resonances. Carboxyl/ester resonance is most evident in the IHU and DCF spectra (Figure 9.6B); fatty acids and esters are a likely source.

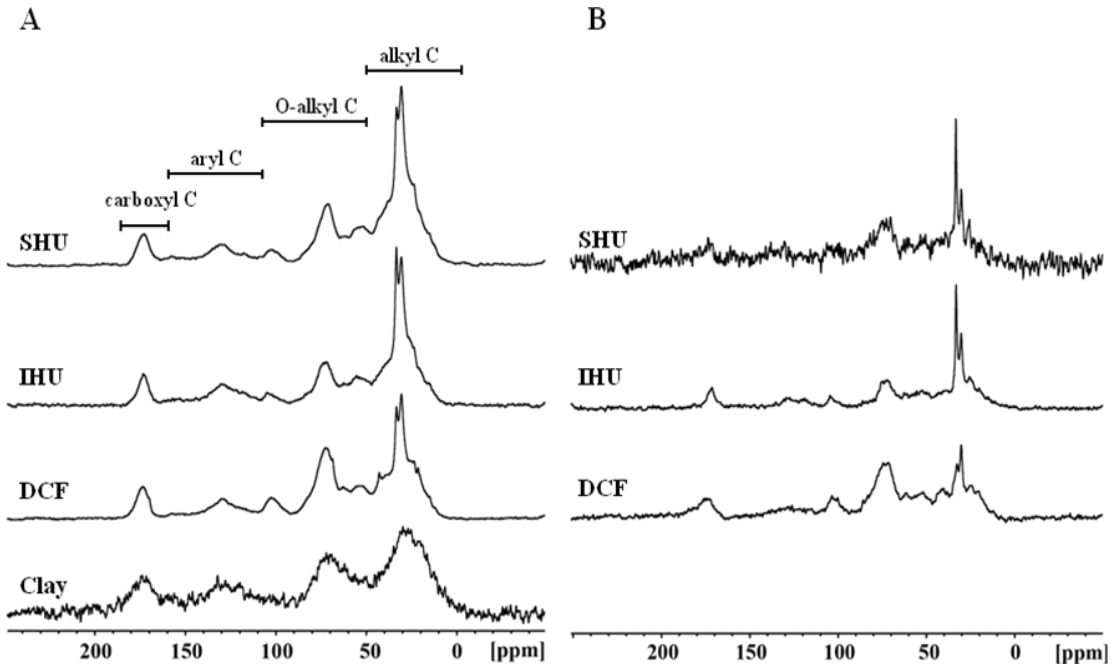

Figure 9.6. A: ^{13}C Cross Polarisation Magic Angle Spinning (CPMAS) solid-state NMR spectroscopy of Core 1 top soluble humin (SHU), insoluble humin (IHU), demineralised clay-sized fraction (DCF), and whole clay-sized fraction sample (clay), (number of scans; SHU and IHU = 4K, DCF = 16, clay = 80K). B: Swollen-state ^{13}C NMR spectroscopy of Core 1 top SHU, IHU and DCF, (32K scans were acquired for all samples).

The solid-state (dry) spectra of the samples from the base of the core show similar C distributions (Figure 9.7A). The spectrum for clay-sized fraction has very poor S/N, and thus it is difficult to make any meaningful interpretations. The clay-sized fraction has a very high ash content (90%) which is 12.5% higher than the ash content of the clay-sized fraction from the top. The SHU and IHU are dominated by

aliphatic methylene. SHU has stronger contributions from amorphous methylene, whereas the IHU has almost equal contributions from both highly-ordered and amorphous species. The DCF spectrum is dominated by aliphatic methylene, but it also has significant contributions from carbohydrate, confirmed by the anomeric C resonance at 105 ppm. The carbohydrate resonances are smaller in the HUs. There are strong resonances between 50-60 ppm indicating the presence of both peptide and lignin structures. The lignin contributions are confirmed as there are signals at 56 ppm, especially in both SHU and IHU. There are also aromatic resonances suggesting inputs from higher plants. These resonances are larger for the HUs from the base compared to the HUs from the top, suggesting greater plant inputs to the OM retained in the sediments deposited ca. 9,450 years BP. The aromatic resonances are strongest and the methoxyl sharpest in the IHU, suggesting that plant materials form part of the chemically unextractable and highly recalcitrant OC in that environment. The DCF has a sharp resonance at 42 ppm indicative of secondary methine carbons, possibly from chlorophyll-like/terpene-like structures (Skjemstad et al., 1983; Conte et al., 2011). These are also present in the DCF from the top. All three samples also have a strong resonance in the carboxyl/amide/ester region suggesting significant contributions from fatty acids, protein, and cutan structures.

The resolutions of the resonances in the swollen-state spectra (Figure 9.7B) are poor compared to those for the solid-state spectra, and to the swollen spectra of the sample from the top of the core (Figure 9.6B). The strongest resonances are in the aliphatic hydrocarbon region, most notably in the SHU. The SHU has well defined resonances for highly-ordered and amorphous methylene. The highly-ordered resonance dominates; the same trend is evident in the SHU from the top of the core. The other major resonance at ca. 70 ppm are most likely from alkyl-OH, as the anomeric C is not evident above the noise of the spectrum. There are resonances at 19 and 40 ppm indicative of methyl groups (Wilson, 1987; Chefetz et al., 1998; Conte et al., 2010; 2011), and a resonance at 38 ppm indicative of terpene-like structures (Skjemstad et al., 1983; Conte et al., 2011). These resonances were less defined in the solid-state due to overlapping signals. The aliphatic hydrocarbon resonances are broader in the IHU, and highly-ordered and amorphous methylene are present in almost equal proportions. Similar to the SHU, there are resonances at ca. 70 ppm, but there is no evidence for anomeric C. The DCF sample has very poor

S/N and differentiation between true resonances and noise is difficult. The poor S/N can be attributed to the high ash content of the sample (73%) despite the removal of silicates. A small amorphous methylene is present, along with a smaller, poorly defined highly-ordered resonance. There are resonances in the 70 ppm region assigned as carbohydrate, substantiated by the presence of an anomeric resonance at 105 ppm. Additionally, the DCF has small resonances at 65 and 89 ppm indicating the presence of highly-ordered cellulose. All the samples have weak resonances for carboxyl/amide/ester; the most definition in this region is observed in the SHU. There is evidence for microbial sugars in the SHU and IHU (resonances at 25-26 ppm, Chefetz et al., 1998).

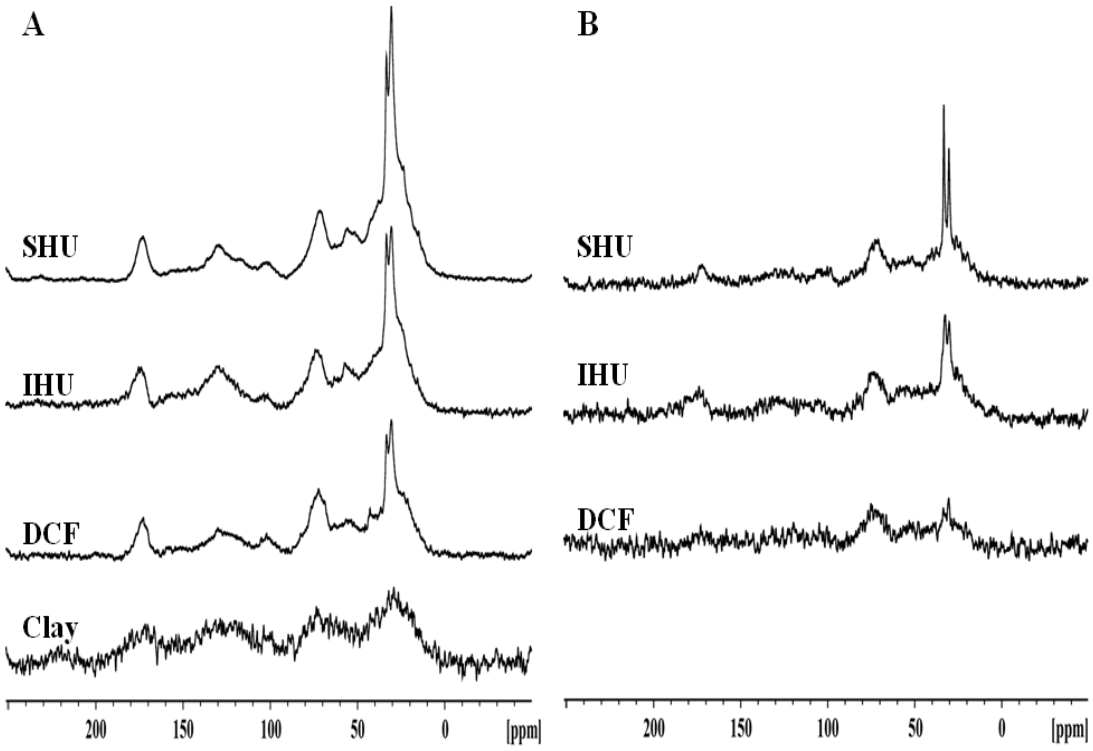

Figure 9.7. A: ^{13}C Cross Polarisation Magic Angle Spinning (CPMAS) solid-state NMR spectroscopy of Core 1 bottom Soluble Humin (SHU), Insoluble Humin (IHU), Demineralised Clay-sized Fraction (DCF), and whole clay-sized fraction sample (clay), (number of scans; SHU and IHU = 4K, DCF = 16, clay = 80K). B: Comprehensive Multiphase (CMP) ^{13}C NMR spectroscopy of Core 1 bottom Soluble Humin (SHU), Insoluble Humin (IHU) and Demineralised Clay-sized Fraction (DCF). 32K scans were acquired for all samples.

9.3.4 Comparisons of Humins using 1D ^{1}H and ^{13}C NMR

Both the SHU and IHU from Core 1 and Core 4 were studied using both solid-state ^{13}C NMR and ^{1}H NMR spectroscopy. The HUs were isolated from the top and bottom of Cores 1 and 4. It is assumed that the HUs from the base of the sediment cores are older organic materials (because the sediments they are associated with are older than those from the top of the core) and would therefore be composed of highly recalcitrant organic materials that might originate from different organic precursor materials. The base of the cores are about ca. 9,450-9,770 years old and the environment in Galway Bay could have been very different at that time. Subsequent changes include sea level rise, deforestation of the landscape, urbanisation, and recent industrialisation. It is also postulated that HUs from Core 1 would differ from HUs from Core 4, because Core 1 has a strong terrestrial influence (attributable to the proximity of the coring position to the mouth of the river Corrib) whereas Core 4 should have a greater marine influence. There are a number of notable differences between the HUs in the two cores. It is evident that Core 1 (top) has more highly-ordered $(CH_2)_n$ in the IHU (Figure 9.8B) than for the SHU (Figure 9.8A). There is evidence for highly-ordered $(CH_2)_n$ in all samples suggesting some level of ordered structure in the aliphatic hydrocarbon species in SHU and IHU. This structured $(CH_2)_n$ may originate from algal cell walls, from the protective layers in higher terrestrial plants (i.e. cutin/cutan; Hu et al., 1999), or from microbial lipids (Simpson et al., 2007a). The highly-ordered $(CH_2)_n$ species contributes to the insoluble nature of these recalcitrant organic materials. The SHU from Core 4, top and bottom, have well defined aliphatic hydrocarbon resonances, and the amorphous $(CH_2)_n$ resonances dominate. Resonances centered ca. 70 ppm are likely to represent carbohydrate structures and this is strengthened by the weak anomeric C resonances (105 ppm). However, this resonance (70 ppm, Core 4) could also be from OH structures, such as those found in cutan, as well as from carbohydrate. In the case of the IHU spectra, Core 1 top contains more highly-ordered than amorphous $(CH_2)_n$, suggesting that the amorphous species have been solubilised to an extent in the DMSO-acid extraction media. Core 1 IHU (top and bottom) have clear resonances in the carbohydrate and anomeric C regions, and these have strong broad aromatic resonances centered at 125 ppm (also identified in cutan by Deshmukh et al., 2005). The evidence for carbohydrate is less convincing for Core 4 IHU (top and bottom) as

the anomeric C at 105 ppm is not defined. The resonance at 50-60 ppm, which is clearly evident in the spectra for Core 1 (top and bottom), is more strongly in evidence for Core 4 bottom than for the top sample. This resonance is likely to be contributed by peptide structures. However, it could arise from lignin as there are strong lignin resonances in Core 1 bottom SHU, as evidenced from pyGC/MS analysis (Ch. 8, 8.3.4).

The aromatic resonances are most prominent in the Core 4 IHUs; resonances between 120-150 ppm suggest contributions from cutins, cutans, peptides and lignins, and resonance at 140-150 ppm, coupled with a resonance at 56 ppm are suggestive of lignin (Figure 9.8B), indicating strong inputs from terrestrial sources to this fraction.

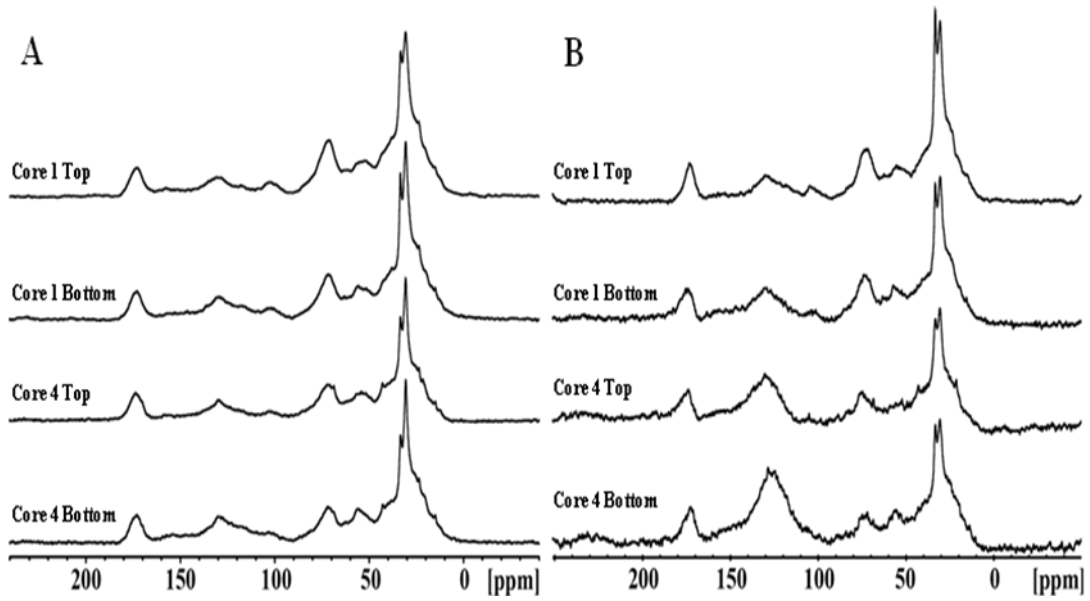

Figure 9.8. ^{13}C Cross Polarisation Magic Angle Spinning (CPMAS) NMR spectroscopy of humins (HUs) isolated from different locations. HU was isolated from the top and base of two cores which have different geographical locations in Galway Bay (Core 1 and Core 4). A: soluble HUs (SHU) from Core 1 and Core 4; B: insoluble HUs (IHUs) from Core 1 and Core 4.

The conventional ^{1}H NMR spectra (total signals, Figure 9.9A.) and the DE spectra (rigid macromolecules, Figure 9.9B) for the SHU's from Core 1 and Core 4 are shown in Figure 9.9. Both $(CH_2)_n$ and CH_3 resonances are large in the SHU's due to their aliphatic compositions (already evidenced in Ch. 8 for the core 1 top SHU sample using 1 and 2D NMR, as well as in the pyGC/MS data). The aliphatic resonances are quite broad for Core 1 SHUs due to the mixture of highly-ordered and

amorphous methylene. The Core 4 SHU's, as shown in the ^{13}C NMR, are predominately amorphous $(CH_2)_n$ resulting in a sharper and more symmetrical resonances when compared to that observed in Core 1 SHU (Figure 9.9A). However, the Core 1 SHU's contain almost equal amounts of highly-ordered and amorphous $(CH_2)_n$, and this mixture of hydrocarbons results in broadening of the proton resonance. The CH_3 peak in Core 1 bottom is large compared to the $(CH_2)_n$ peak suggesting a large amount of methyl groups indicative of a high protein input. These protein peaks are somewhat masked in the total proton spectrum (Figure 9.9A), and this highlights the benefits of the DE experiment. Strong protein signals are evident in the Core 1 bottom SHU (Figure 9.9B). Amino acids regularly have a terminal methyl group; therefore, the large CH_3 peak in the Core 1 bottom SHU corresponds to the amino acid resonances in the spectrum. Indeed when compared with the DE spectrum Core 1 bottom SHU is very similar to that obtained from cultured microbes (Simpson et al., 2007a) and as demonstrated in Figure 9.10. There are strong similarities in the amide, aromatic amino acids, amino acids side chain, α- protons from peptides, lipoprotein, $(CH_2)_n$, and CH_3 chemical shift regions of both spectra. That suggests that the sample is dominated by microbial residues, and the strong similarity between the spectral profiles suggests the material is preserved in a relatively intact state. Core 4 SHUs also have strong protein compositions as evidenced by a broad hump for α-protons from peptides and strong resonances for aromatic amino acids (Figure 9.9B). The total ^{1}H spectra (Figure 9.9A) show signals that could be from lipoprotein peaks (at 2.25ppm), and peptidoglycan peaks (seen as a shoulder on the large aliphatic peak at ca. 1.5 ppm), in all four samples (especially in Core 4 SHU's). The fact that these peaks are absent from the rigid, macromolecular spectrum suggests that these molecules have some molecular mobility. Such biological molecules are found in microbial cell walls. Resonances for carbohydrates are greatest for Core 1 top. This may suggest that with time carbohydrates are slowly degraded and the peptides are selectively preserved. Otherwise, the abundance may be due to a different organic input to the sediments.

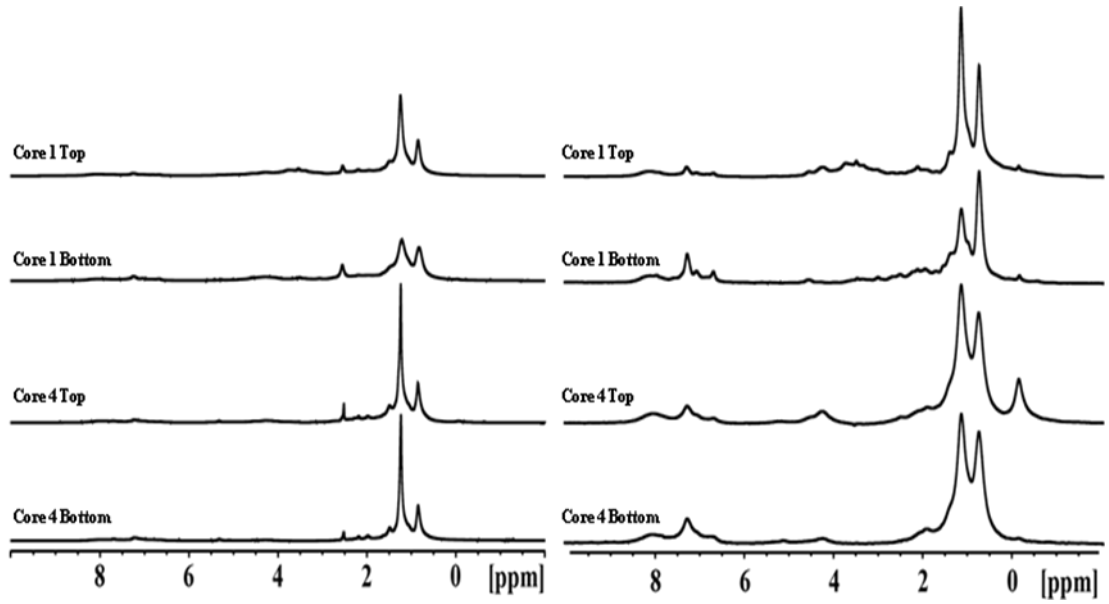

Figure 9.9. ^{1}H NMR spectra of soluble humins (SHUs) from Core 1 and Core 4. A: the total ^{1}H signals; B: ^{1}H signals from rigid macromolecules (diffusion edited spectra).

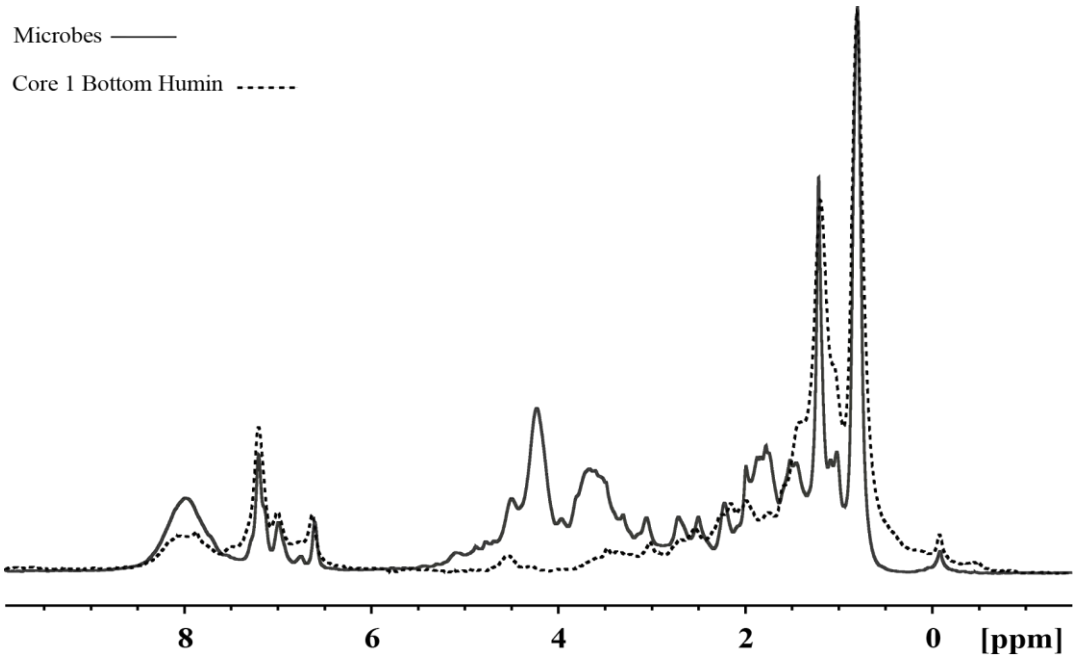

Figure 9.10. ^{1}H diffusion edited (DE) spectra of microbes and Core 1 bottom soluble humin (SHU).

9.4 Discussion

NMR spectroscopy has been used extensively in this research in order to understand the compositions of the NOM (both labile and recalcitrant components) in sediments from an estuarine environment. Wilson (1987) stated that the NMR spectra for small molecules are simple, but macromolecules produce a complex spectrum of overlapping peaks producing a spectrum showing average structural entities. The spectra shown in this study are complex, and with overlapping signals. Therefore a number of techniques were employed (DE, 2D NMR, CMP-NMR) to obtain additional information about the samples. Structures with major aliphatic hydrocarbon compositions and proteins/peptides have been shown to be major contributors to the recalcitrant OM sink, and carbohydrates have also been shown to exhibit a degree of recalcitrance.

9.4.1 Aliphatic Hydrocarbons

As shown in previous research, the HU fraction is composed mainly of functionalities with major aliphatic hydrocarbon structural components, and its compositions contain considerably less aromatic structures compared to the HAs (Almendros et al., 1991, 1996; Nierop et al., 1999; Salloum et al., 2001; Kang et al., 2003; Guignard et al., 2005; Wang and Xing, 2005b; Keeler et al., 2006; Simpson and Johnson, 2006; Bonin and Simpson, 2007; Simpson et al., 2011). Our study complements these findings, as is evident from the strong signals from $(CH_2)_n$ and CH_3 in the ^1H NMR spectra (Figs. 9.1a,b,c), in the ^{13}C NMR spectra (Figs. 9.6, 9.8), and as well in the 2D NMR spectra (Figs. 9.3, 9.4, 9.5). A slice taken from the TOCSY spectrum (Figure 9.5B) shows proton resonances similar to those identified by Deshmukh et al. (2005) who investigated the cuticular components found in higher plants. Cutan is a highly aliphatic biopolymer mainly composed of highly-ordered and amorphous methylene (Deshmukh et al., 2005). In addition, there are resonances identified in the ^{13}C NMR for alkyl-OH (possibly from cutan) in the IHU and SHU. Core 4 top and bottom SHU and IHU has a split peak in the carbohydrate region with the peak at 74 ppm that is assigned as alkyl-OH (Figure 9.8A, B). Deshmukh et al. (2005) identified resonances at 74 ppm as methines attached to ester-linked mid-chain hydroxyls. Cuticular lipids are highly resistant to degradation in the environment and subsequently form part of the long

term C sink. Suberin/suberan may also contribute to the alkyl-OH resonances. Cutans, suberins, and algaenan are insoluble, non-hydrolysable aliphatic biopolymers found in algae, higher plant cell walls, and in the fossil remnants. The recalcitrance of these compounds is due to their highly-ordered structure (Deshmukh et al., 2005), and impedes their degradation by microorganisms. A study by Hatcher and Clifford (1997), using ^{13}C NMR and pyGC/MS, found that the structure of cutan includes a cellulosic backbone to which long-chain aliphatic hydrocarbon structures are linked through ether bonds. This cutan-cellulose linkage may explain the persistence of the carbohydrate signal in the SHU and IHU. The highly aliphatic nature of structures in algae is long established (Hatcher and Clifford, 1997). Algaenan, an aliphatic biopolymer, will resonate between 0-50 ppm, along with other methylene structures. Therefore, algaenan could not be identified directly from the ^{13}C NMR spectra. Additionally, the aliphatics can arise from biological lipids in microbial cells, as shown in Figure 9.10. Algaenans are not soluble in any organic solvent (Nguyen et al., 2003), which makes these difficult to study. Their persistence in the environment, possibly in the SHU and IHU fractions, can be attributed to their inherent insolubility; these biomolecules, along with cutan and suberan, may provide hydrophobic domains wherein labile components are trapped and protected from re-mineralisation.

9.4.2 Protein and Carbohydrate

Carbohydrate and protein signals are shown to have rigid, macromolecules domains (Figure 9.1c, Figure 9.9B). Their presence in the HUs (SHU and IHU) indicates that these biomolecules are preserved long term in the sedimentary organic C pool. There is abundant evidence for carbohydrates in the solid-state ^{13}C NMR, and also in the HSQC of Core 1 top SHU. Carbohydrates account for a large portion of the DCF sample (Figure 9.6A, B). The majority of carbohydrates are labile and are quickly re-mineralised in the water column, or in the soil. Carbohydrates may form part of the HU through the formation of covalent bonds between lipid components and carbohydrates in plants, from tannins, from highly-ordered cellulose, or they from encapsulation in hydrophobic domains. Such labile biomolecules would not survive without a protective mechanism preventing their decomposition. Clay minerals can also provide protection for labile OM (discussed in Ch. 2, 2.2.3). There is much

evidence in the ^{13}C NMR to suggest the presence of cellulose in the IHU and SHU (Figure 9.6). Peaks identified at 63 and 84 ppm are assigned to amorphous cellulose, and peaks at 65 and 89 ppm are assigned to highly-ordered cellulose (Albrecht et al., 2008; Conte et al., 2010). The SHU and IHU from Core 1 and Core 4 (Figure 9.8) all show a peak at 25 ppm assigned as microbial deoxy sugars (Chefetz et al., 1998). This peak is stronger in the Core 1 top SHUs. It is postulated that microbial biomass makes a significant contribution to the compositions of the organic materials, and so it is not surprising to identify microbial sugars in the NMR spectra. Microbes re-mineralise nitrogen in decomposing plants. However, some nitrogen is retained and forms part of the sedimentary OM (Hsu and Hatcher, 2005). Additional sources of nitrogen containing compounds would include microbial and marine biomass (living and necromass). When the ^{1}H spectrum of rigid, macromolecules in the SHU (Core 1 top) is compared to albumin, the presence of protein in the recalcitrant OM is confirmed. 2D NOESY also confirmed the presence of amino acid side chains, and identified α- protons in peptides. Marine biomass also has significant nitrogen contents, with up to 30% protein in the composition of phytoplankton (Romankevitch, 1984; Rullkötter, 2006). ^{13}C NMR spectroscopy by Dickens et al. (2006) showed plankton to have a strong peak between 50-60 ppm, suggesting it has a significant amount of protein in its composition. It is proposed that cellular debris makes a large contribution to the HU composition (Lichtfouse et al., 1998; Simpson et al., 2002; Kelleher and Simpson, 2006; Lorenz et al., 2007; Simpson et al., 2007a, b; Song et al., 2011). The contributions from microbial cellular debris to the HUs in this study are confirmed by the identifications of peptidoglycan and of lipoproteins (Figure 9.9A), and by the similarity of the HU spectrum (Core 1 bottom) with that of fresh microbes (Figure 9.10). The presence of cellular debris is also supported by the weak resonances for peptidoglycan in the HSQC (ca. 23 ppm, 1.8 ppm), also identified in soil HU by Song et al. (2011). These resonances are masked by overlapping in the spectra for HU in this study, but they were also identified in the HSs from Core 1 top (spectra shown in Ch. 8, Figs. 8.5, 8.6).

9.4.3 Aromatics/Lignin

All the NMR experiments have shown that the sedimentary organic C samples are mainly aliphatic materials, with only small amounts of aromatic functionalities. The majority of the aromatic signals are likely to arise from aromatic amino acids and to a lesser extent contributions from lignin units. Aromatic amino acids give strong resonances in the ^{1}H DE NMR spectra, and these assignments are confirmed by comparing these spectra with albumin and cultured microbes. Lignin contributions cannot be overlooked due to the concentration of lignin derivatives identified in the pyGC/MS and Core 1 SHU's (Ch. 8, Figure 8.8A), as well as the evidence for lignin in the Core 1 bottom samples (Figure 9.7). In all experiments, resonances indicating lignin are small; however, methoxyl is evident in the HSQC spectrum and the spatial interaction between methoxyl protons and aromatic protons are weak but evident in the NOESY spectrum, of Core 1 SHU. The solid-state ^{13}C NMR has overlapping signals in the 50-60 ppm region, and so the presence of an OCH_3 peak is masked (Figure 9.6, 9.8). However, using the CMP probe it was possible to see evidence for methoxyl in the IHU of Core 4 bottom (Figure 9.8B). There are small guaiacyl (147 ppm) and syringyl (153 ppm) peaks identified in Core 1 bottom IHU and in Core 4 bottom IHU (Figure 9.8B). The broad peak centred at 105 ppm in Core 1 top IHU (Figure 9.8B) could be a mixture of anomeric C from carbohydrate and quaternary C from tannins (Nierop et al., 1999; Roscoe et al., 2004). The strong aromatic resonances in Core 4 IHUs could be attributable to plant materials such as lignin, tannins, cutin/cutan, and suberin/suberan. It can be said with confidence, based on the NOESY experiment, coupled with the information provided by ^{13}C NMR (CMP), the HSQC and the TOCSY, that there is lignin derived material in the HUs in the estuarine sediments. However, the lignin contributions are very small. Early studies on HSs suggested lignin to be a major component of the humified OM (Waksman, 1932) and lignin compounds were believed to contribute to the recalcitrance of HSs. This study shows lignin to be a minor organic input to the sedimentary organic C sink.

9.4.4 ^{13}C CPMAS in the Dry versus Swollen-State

There are broadly similar C distributions in all the organic fractions (Figure 9.6A, 9.7A) in the samples from the top and base of Core 1. The C distribution in the clay-sized fraction matches that of the HUs and the DCF suggesting little chemical alteration is introduced by the extraction and/or sample pre-treatment. This implies that solvation and fractionation of the sediment components is useful in order to obtain high resolution molecular level details about organic environmental samples. By comparing the clay-sized fraction and DCF with the HU samples, changes observed in the OM compositions can be related to protection. Carbohydrate materials are greatly decreased in the HUs when compared with those in the DCF. The carbohydrate signal decreases as labile sugars are utilised as an energy source by microbes in the environment. Highly-ordered methylene is shown to be a major component of the sedimentary OM, especially in the IHU. It is more evident in the spectra acquired using the CMP probe (Figure 9.6B). This is attributable to the highly-ordered aliphatics remaining immobile despite their being swollen in DMSO. Generally, when materials are swollen in DMSO their non-rigid molecules will gain molecular mobility and swell or dissolve. The highly-ordered nature of the components may be the reason for the recalcitrance of this fraction (IHU) and for its contribution to the hydrophobicity of the HUs. Hu et al. (1999) explains that highly-ordered domains are challenging for chemicals and enzymes to penetrate, and thus are more difficult to degrade than their amorphous counterparts. Conversely, the SHU from the bottom of the core is shown to have more highly-ordered methylene than the IHU. Another factor contributing to the insolubility of the HUs is the smaller composition of carboxyl groups. The acidic functional groups are less prominent in the HUs compared to the DCF. Resonances at 160-180 ppm in the HUs may be from ester and amide functionalities. The ^{13}C NMR spectra for the DCF (Figs. 9.6A, B, Figure 9.7 A) have good resolution, and that makes it possible to study HSs in their natural state. For studies of the DCF in using the CMP probe, it is necessary to have low ash contents in order to achieve good S/N. There has been no fractionation of the DCF samples; therefore, there is overlapping of resonances and the sample may contain 'non-humic substances' which would traditionally be removed using an XAD-8 column during HS fractionation. It is highly beneficial to acquire ^{13}C spectra in both the dry and swollen-states (Figs. 9.6, 9.7A and 9.6, 9.7

B). The dry state provides an overview of all the functional groups present. Only molecules which are not swollen or solubilised by DMSO-d_6 are shown in the CP-MAS of the DMSO swollen material (Figs. 9.6B, 9.7B). Amorphous aliphatics are attenuated to some extent, as some of this material would be accessible to the solvent and have molecular motion. The remaining amorphous $(CH_2)_n$ may have adapted some mechanism of protection from degradation in the environment. For example, the material could be highly dense and cross-linked making it difficult for microbes to access (Deshmukh et al., 2003). The amorphous and highly-ordered $(CH_2)_n$ species that remain solid even in the presence of a swelling solvent may be fundamental to the recalcitrant nature of the OM in the environment and result in its long term preservation in the sediments. If the components are not accessible to an aggressive solvent such as DMSO, it is logical that these will not be accessible for the microbial activities requiring water necessary for the biological transformations**.** The major resonances in the ^{13}C CPMAS solid-state and the swollen-state NMR spectra of the IHU from the top of the core were similar. However, it would appear that there were differences in the amounts of the functionalities representing the resonances. For example, the ratio of highly-ordered (33 ppm) to amorphous $(CH_2)_n$ (30 ppm) is greater in the swollen-state spectrum because the amorphous component is more mobile in DMSO. The IHU is likely to be more macromolecular because soluble, labile compounds have already been removed in the extracting solvent system. The IHU from the base of the core loses its strong aromatic resonances, and its sharp methoxyl resonance in the swollen-state spectrum. Carbohydrate, acidic functional groups, and aromatic resonances are decreased on swelling as their molecular mobility is increased thorough contact with DMSO-d_6, but these are unlikely to be completely solubilised. Some carbohydrate signals remain, possibly due to the presence of highly-ordered cellulose, or possibly from large fragments of cellulose, from algae and higher plants, that cannot be fully swollen. Additionally these resonances at 70 ppm may have contributions from alkyl-OH, especially in the HUs.

The DCF from the bottom of the core provided much less compositional information, due to low S/N, compared to the sample from the top. The ash content for the DCF was 73% which is significantly higher than the DCF from the top (32%). In order to gain meaningful results from swollen-state NMR it is paramount

to remove as much inorganic species as possible. However, harsh chemicals used to dissolve metals can cause structural changes to the organic materials.

9.4.5 Traditional Considerations of Organic Preservation in the Environment

As evident from the data, a comparison of the HUs with the DCF indicates that certain biomolecules are degraded quickly in the environment whereas some molecules are retained and concentrated in the long term C sink (Figure 9.6A). The $(CH_2)_n$ and CH_3 are inherently recalcitrant due to their structure and their hydrophobicity. We can assess the preservation of peptides by comparing Core 1 bottom SHU with the ^1H NMR spectrum of fresh microbes (Figure 9.10). Resonances in a number of chemical shift regions show similarities. The similarities of these spectra coupled with the significant protein contributions identified in Core 1 top SHU (confirmed in Figure 9.2) leads to the hypothesis that protein, originating mainly from microbial biomass, makes important contributions to the recalcitrant OC materials sequestered in the environment. The main difference between the two spectra in Figure 9.10 is that the region from 3.5-4 ppm is attenuated in the SHU spectrum. This attenuation arises from the additional pre-saturation that was required when acquiring the SHU spectrum in order to suppress the dominant water signal in the region (see Ch. 6, Section 6.14.3.). Protein components i.e. aromatic amino acids and amide structures in the SHU may exhibit a degree of recalcitrance. For example, the aromatic amino acids have a degree of hydrophobicity that could enable these to interact, and hence be protected by clay minerals or other hydrophobic components such as long chain aliphatic hydrocarbons. The similarities of the protein and microbial signatures suggest that much of the protein in the organic samples is from microbial sources. It is not clear whether it arises from living, dormant, or necromass sources. Microbes have been shown to survive in sediments for very long periods of time (Parkes et al., 2005).

A number of suggestions have been put forward for this selective preservation of biomolecules. The degradation of highly-ordered molecules is likely to be retarded because their structures inhibit access to degradative enzymes/microbes. However, it is evident that amorphous molecules can also be preserved. Physical protection, encapsulation, and the formation of covalent and non-covalent bonds are prominent methods accepted for biomolecule retention.

Peptides have been shown to become encapsulated within the OM in sedimentary environments (Knicker and Hatcher, 1997; Nguyen and Harvey, 2001; Hsu and Hatcher, 2005, 2006). Physical protection is provided by organo-minerals associations (Keil et al., 1994; Mayer, 1994; Hedges and Keil, 1995; Hsu and Hatcher, 2005), or by the entrapment of OM within minerals and aggregates (Sollins et al., 1996; Guggenberger and Kaiser, 2003; Hsu and Hatcher, 2005). One encapsulation mechanism is based on hydrophobic and non-covalent associations (Nguyen and Harvey, 1998, 2001; Hsu and Hatcher, 2005, 2006). Labelled ^{15}N peptides have been shown to have the ability to form strong covalent and non-covalent interactions with HAs, confirming that proteinaceous materials can be incorporated into HSs (Hsu and Hatcher, 2006), and it has long been known that proteins can be sorbed by clay minerals (McLaren, 1954). These protection methods could explain the retention of proteins and of carbohydrates in the HU fractions from the sediment cores. Alternatively, preservation/protection inside living or dead cells could also explain the abundance of these materials in the HU fractions.

9.5. Conclusions

Humins (HUs) are predominately composed of methylene compounds that contribute to the recalcitrance of organic materials in the environment. ^{1}H NMR spectroscopy shows that the aliphatic molecules in SHU are composed of both small, mobile molecules and rigid, macromolecules. ^{1}H DE NMR is useful to identify peptide and carbohydrate signals which can be dwarfed in the conventional ^{1}H spectrum due to the large aliphatic signals. It is shown that protein is a significant component of the HU as evidenced by the signals from aromatic amino acids, signals from α-protons from peptides and from methyl groups. It is possible that the protein in SHU is derived from cellular debris. This is supported by the similarity of the ^{1}H spectrum of Core 1 bottom SHU to that of microbes. Protein signals are masked in ^{13}C NMR but can be identified using 2D NMR. The ^{13}C NMR shows that SHU differs from the total organic fraction (DCF) as it is less aromatic and has less carbohydrate and COOH groups. Lignin contributions to the SHU from the top are detectable but very small. Stronger lignin/plant contributions are evident in the samples from the base of the core.

Extraction and fractionation has been shown to be beneficial because it does

not give rise to chemical alterations of the OM, but improves the NMR sensitivity and gives greater resolution, with fewer scans. Unextracted demineralised clay-sized fraction (DCF) provides good indications of the total organic composition of the sediments in their natural state. However, NMR spectra of unfractionated samples have overlapping signals that make the identification of the components difficult. The quality of the spectra can be compromised by high ash compositions, as evidenced in the sample from the bottom. When the silicates are dissolved, other elements become concentrated in the sample. Improvements to the spectra could be achieved by using solutions of HF/HCl, and acquiring additional scans. Therefore, this approach is promising for studying OM in its natural state. The CMP probe provided details of the truly recalcitrant species in the organic samples. This probe could be exploited further to provide additional levels of compositional and structural detail. The use of the CMP NMR probe (coupled with traditional NMR approaches) will encourage future studies of environmental organic samples in multiphase media.

NMR spectroscopy data alone do not definitively indicate how C is sequestered in sediments. However, the spectra show that HU is comprised of recognisable biomolecules. Improved methods are required for the isolation and study of the highly resistant biomolecules such as algaenan, suberan, and cutan. The recalcitrant organic C in the sediments is mainly composed of aliphatic hydrocarbon materials that may be unavailable to or protected from degradation by microbial activity. Microbial peptides and carbohydrates are also shown to be important contributors, and these may be from living or preserved necromass. Lignin residues form only a small part of the OM in this estuarine environment. Lignin is more evident in the base of the core and appears to make strong contributions to the highly recalcitrant IHU fraction. Direct comparisons with the OM in non-extracted clay-sized fractions (clay and DCF) shows that the isolation procedures used did not chemically alter the compositions of the isolates. The highly-ordered components in HU resist swelling by DMSO and represent the most rigid materials in HU, and play a major role in C sequestration.

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Chapter 10 Characterisation of Organic and Inorganic Components Associated with Core 4 Sediments

10.1 Introduction

Quantifying and structurally elucidating the uncharacterised organic matter (OM) pool is vital for understanding the mechanisms that regulate OM preservation over geologic time. Despite the volume of relevant research that has been carried out, this still remains one of the greatest challenges in marine organic geochemistry (Gélinas et al., 2001). This chapter focuses on Core 4, taken furthest from the terrestrial environment, and it would be expected that this core would have the greatest marine influences. The most noticeable difference between marine and terrestrial OM is the absence of lignin in marine OM (Baldock et al., 2004) and enrichment in nitrogen and aliphatic species (Hedges and Oades, 1997; Baldock et al., 2004).

To attain a complete overview of all the organic materials retained in the sediments the total organic fraction, including un-humified OM, humic substances (HSs), and recalcitrant components (humins, HUs) are studied. Organic fractions that are insoluble in aqueous media represent the most recalcitrant materials, and these form an integral part of the long term carbon (C) sink. Soluble humin (SHU) and insoluble humin (IHU) were isolated from the clay-sized fraction of the sediments using an acidified organic solvent and hydrofluoric acid (HF), respectively. The study of these HU fractions is more challenging as they are highly insoluble due to their hydrophobic domains and their associations with clay minerals. OM is concentrated in the clay/silt fractions (Clapp and Hayes, 1999; Cheshire et al., 2000) due to ligand exchange, polyvalent cation bridges, and weak interactions (van der Waal forces, hydrophobic interactions and hydrogen bonding) between the clay and the OM (Theng, 1979; Oades, 1989). Therefore, the clay-sized fraction of the sediments was isolated for: (a), extraction of SHU (following HS extraction); and (b), the concentration of the organic materials for NMR spectroscopy (no prior extractions). The clay-sized fraction (no pre-treatment) was studied using solid-state ^{13}C NMR to determine the main compositions of the OM in its natural state. A sub-sample of this clay-sized fraction was pre-treated using hydrofluoric (HF) acid in order to increase the sensitivity and resolution of the NMR spectra through the removal of silicates which concentrate the OC in the sample.

In general CPMAS is not quantitative but it is an excellent technique for looking at relative changes in C distributions across a series of samples, and that is the aim of this study. A major disadvantage of ^{13}C NMR in the study of natural OM is the inherent insensitivity of the ^{13}C nuclei. This is problematic in samples with low C concentrations such as the clay-sized fraction, resulting in lower sensitivity and lengthy instrument time being required. Multiphase (swollen-state) NMR provides details about the mobile and rigid composition of the sample when spectral editing is utilised.

Paramagnetic species (e.g. Fe-bearing minerals) are undesirable in the samples being studied by NMR spectroscopy as they decrease sensitivity of specific functional groups and can affect the whole range of C species through broadening of the resonances (Gélinas et al., 2001). HF has been the most successful chemical for the concentration of OC by the dissolution of silica (Gélinas et al., 2001). However, concentrated HF/HCl solutions can cause chemical alterations to the OM, coupled with OC loss through hydrolysis and the solubilisation of labile biomolecules (Gélinas et al., 2001). The HSs and HU samples in this study were pre-treated with 0.1 M/0.3 M HCl/HF solution to decrease interference from paramagnetics and to concentrate the OC. This low molarity HCl/HF solution was chosen as the OC in the HS and HU had been concentrated through the extraction process but some inorganic components (clay colloids) may be co-extracted. To isolate IHU from the clay-sized fraction, and to demineralise the unextracted clay-sized fraction sample, a 10% HF (*v/v*) solution was used to dissolve the silicates.

The total OM provides an opportunity for spectroscopic assessments to be made in relation to components which degrade quickly in the environment, whereas the HSs and HU will show which materials are retained as part of the OC sink. Differences in the organic materials from the top and the base of the sediment core are also accessed to determine if organic materials differ with depth and age. Finally, the inorganic species in the clay-sized fraction and demineralised clay-sized fraction are studied using scanning electron microscopy energy dispersive X-ray (SEM-EDX) spectroscopy in order to determine the inorganic compositions of the sediments and to evaluate the suitability of the HF pre-treatment. Clay minerals have a substantial effect on the sequestration of OM in the environment.

10.2 Methodology

10.2.1 Regional Setting

Core 4 (53 ^{o} 14'15.44N, 09 ^{o} 05'21.54W, Figure 6.1) was taken using a vibrocorer. The sea floor at the sampling point was under 13.5 m of water, and the core is 500 cm in length. Details of the location and collection of the core are outlined in Ch. 6, 6.1. Sediments from the top 100 cm of the core represent ca. 9,200 cal years before present (BP), and sediments from the base of the core represent ca. 9,450 years BP. Detailed results of radiocarbon dates are outlined in Dalton et al. (2010).

10.2.2 Extractable Organic Components

The aqueous extraction and fractionation of HSs is outlined in Ch. 6, 6.3.2 and 6.4.4, respectively. The extraction of HU using an organic solvent is outlined in Ch.6, 6.3.3.

10.2.3 Preparation of Humic Substances and Humin for NMR Spectroscopy

All extracts were mixed with 0.1 M/0.3 M HCl/HF to remove fine clay particles and paramagnetic minerals. The procedure was repeated twice. The HCl/HF was removed from the HAs and HUs by centrifugation and the samples were dialysed against distilled water, (the method is outlined in Ch. 6, 6.4.6). As the FAs remain in solution, they were adsorbed on an XAD-8 resin, washed thoroughly with distilled water and desorbed using 0.1 M NaOH. The FAs were H^{+} -exchanged by passing down through an IR-120 resin.

10.2.4 Preparation of the Clay-Sized Fraction, Demineralised Clay-Sized Fraction, and Insoluble Humin for NMR Spectroscopy

The clay-sized fraction (clay) was isolated from bulk sediments that had no prior chemical extraction; the method is outlined in Ch.6, 6.3.4.1. A sub-sample of the clay-sized fraction was reacted with 10% HF and the remaining solid material is labelled as the demineralised clay-sized fraction (DCF); the method used is outlined in Ch. 6, 6.3.4.2. To recover the insoluble humin samples (IHU), the clay-sized fraction following SHU extraction was reacted with 10% HF to dissolve the silicates. The remaining solid material is labelled IHU; the method is outlined in Ch. 6, 6.3.4.3.

10.2.5 NMR Spectroscopy

Prior to NMR spectroscopy all samples (HAs, FAs, HUs, IHUs, DCFs, and clays) were freeze dried and then stored in a desiccator over P_2O_5 to remove moisture. NMR tubes and rotors were also stored in the desiccator in an effort to decrease H_2O interference in the spectra.

10.2.5.1 Solution-State and 2D NMR Spectroscopy

HAs and FAs (ca. 20 mg) were dissolved in 800 μL of DMSO-d_6 (deuterated dimethyl sulfoxide). Solution-state NMR parameters are outlined in Ch. 6, 6.14.3. The 2D NMR parameters are outlined in Ch. 6, 6.14.4.

10.2.5.2 Solid-State ^{13}C CPMAS NMR Spectroscopy

Samples: HA (ca. 53mg), FA (ca. 20 mg) HU (ca. 39 mg) IHU (ca. 87 mg) DCF (ca. 100 mg) clay (ca. 112 mg) were placed directly in the 4 mm zirconia rotors and sealed with Kel-F caps. The variability in weights reflects the amount of sample that could be packed into the NMR rotor, and the limited quantity of the FA sample that was available for analyses. The NMR parameters are outlined in Ch. 6, 6.14.2.

10.2.5.3 Swollen-State ^{13}C NMR

HUs and DCF (ca. 25 mg) were swollen in ca. 75 μL of DMSO-d_6 The NMR parameters are outlined in Ch. 6, 6.14.5.

10.2.6 Elemental Analyses

Elemental analyses were carried out at the University of Limerick; the method is outlined in Ch. 6, 6.9.2.

10.2.7 SEM-EDX

Only the clay-sized fraction (clay) and DCF samples were analysed with this process as these samples have high inorganic contents. The method is outlined in Ch. 6, 6.16.

10.3 Results

10.3.1 Humic Substances

10.3.1.1 ^{13}C CPMAS NMR spectroscopy

The main compositions of the HSs include alkyl C (0-45 ppm), amine and methoxyl C (45-65 ppm), O-alkyl C (65-90 ppm), di-O-alkyl C (90-110 ppm), aromatic and unsaturated C (110-145 ppm), phenolic C (145-165 ppm) and carbonyl/amide C/ester C (165-190 ppm) (assignments from Gélinas et al., 2001). NMR assignments are based on resonances reported in the literature for OM from a variety of environments as this is the first time complete assessments of the organic fractions from an estuarine environment have been characterised extensively using NMR spectroscopy. The ^{13}C NMR spectra (Figure 10.1) show that the HAs and FAs have a number of compositional differences. The HAs are more aliphatic (with a large $(CH_2)_n$ peak centred at 30 ppm), a feature that decreases their solubility (due to hydrophobic domains) in aqueous media, compared to the FAs. Interestingly, the FA top isolate has a peak at 33 ppm, for highly-ordered $(CH_2)_n$, that may arise from aliphatic hydrocarbon species such as cutan, suberan, or algaenan. Both the HAs and FAs from the top of the core have small resonances at 39 and 42 ppm which can be assigned as secondary methine carbons (=CH-), possibly generated by chlorophyll/terpene-like systems (Skjemstad et al., 1983; Conte et al., 2010), but these resonances are absent from the bottom samples. Both HAs and FAs (top and bottom) have a large peak at ca. 72 ppm. This chemical shift region is generally assigned to carbohydrate materials (C_2, C_3, and C_5 in cellulose, Taylor et al., 2008; Conte et al., 2010). Carbohydrate contributions are tentative in the isolates as the anomeric C resonances (105 ppm) are very weak. Therefore, alkyl-OH groups may be contributing to the signals at ca. 74 ppm, especially in relation to both of the HAs from the base of the core which have a split peak at 71 and 74 ppm. The HAs have resonances at 63 and 68 ppm that are indicative of C_6 in amorphous and highly-ordered cellulose, respectively (Conte et al., 2010). All samples have resonances between 17-28 ppm and these can be assigned as microbial deoxy sugars and methyl C (Chefetz et al., 1998; Conte et al., 2010; 2011).

The region between 50-60 ppm is assigned to nitrogenated species (Conte et al., 2010) and OCH_3 from lignin. The OCH_3 group resonates at 56 ppm, with a characteristic sharp peak. Assignments of methoxyl in the HAs (top and bottom) are

tentative as the resonance at 56 ppm lacks definition, as well as no definitive lignin resonances in the other chemical shifts. There is more convincing evidence for lignin in the FA-top, FA-bottom and especially in the HA urea bottom isolate as it has a sharp resonance centred at 56 ppm. Lignin derived units resonate at 128 (*p*-hydroxyphenol derivative structures), 147 (guaiacyl like units), and 152 (syringyl like units) (Roscoe et al., 2004; Conte et al., 2011). HA-urea has a sharp peak at 56 ppm but it is large compared to the other lignin signals in its spectrum; therefore, it is possible that the peak is a mixture of OCH_3 and of protein. Urea, a proton acceptor, has the ability to solubilise cellular proteins. Lignin/protein signals are smaller in the HA compared to the HA-urea. This highlights that the urea has the ability to bring more hydrogen bound materials into solution. The FAs from the base of the core have a large resonance centred at 130 ppm, coupled with a resonance at 56 ppm for methoxyl, indicating considerable lignin contributions to this isolate. Resonances at 130 ppm can also be attributed to fused aromatic structures from black carbon-like compounds (Novotny et al., 2006; Song et al., 2008). Resonances centred at 173, in all the isolates, are assigned as carboxyl, amide (Chefetz et al., 2002) and ester C (Song et al., 2008). The FAs have strong resonances at ca. 170 ppm; these are most likely to be predominantly COOH groups which enhance the solubility of these fractions. Resonances in the ^{13}C NMR spectra can be confirmed when the data are coupled with the ^{1}H NMR resonances.

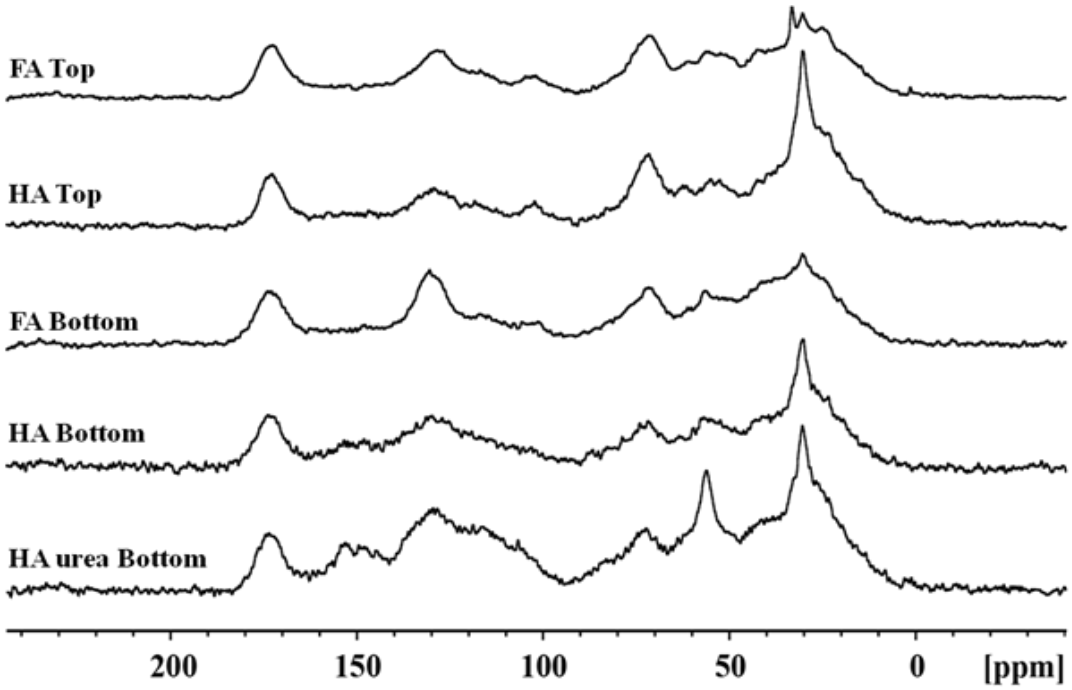

Figure 10.1. ^{13}C CPMAS NMR of humic substances (HSs) extracted from the top and bottom of the sediment core (HA = humic acid, FA = fulvic acid, HA urea refers to the HAs extracted in 0.1 M NaOH + 6 M urea).

10.3.1.2 ^{1}H NMR Spectroscopy of Humic Substances

A strong DMSO resonance (2.5 ppm) dominates all samples in the total ^{1}H spectra (Figure 10.2). All the samples have resonances for $(CH_2)_n$ and CH_3 at 1.25 and 0.8 ppm, respectively. The strong CH_3 (relative to the $(CH_2)_n$ peak) is due to strong contributions from proteins (Song et al., 2008). The FAs (top and bottom) have resonances visible in the carbohydrate/lignin chemical shift region, especially in the case of the FA bottom. The FA bottom has a strong methoxyl signal (3.5 ppm) indicating that lignin contributes to the compositions. The strong lignin signal in the FA bottom corresponds with lignin signals (56 and 130 ppm) identified in the ^{13}C NMR (Figure 10.1). Additionally, the FA bottom has a doublet at 7.6 ppm that was not present at any of the other samples. This resonance is a major component of the isolate as it is the largest resonance in the spectrum (excluding the solvent resonance). The unidentified doublet remains in the IDE spectrum (Figure 10.3) and that suggests it is a small, mobile molecule. The other signals that remain in all the IDE spectra of the HSs are $(CH_2)_n$ (especially in the HA urea bottom) and a carbohydrate signal at 3.5 ppm (FA bottom). The HAs are dominated by aliphatic hydrocarbon signals. The HA urea bottom and both FAs show small resonances at

1.5 and 2.2 ppm for peptidoglycan and lipoprotein, respectively. These resonances are indicative of components from microbial cell walls.

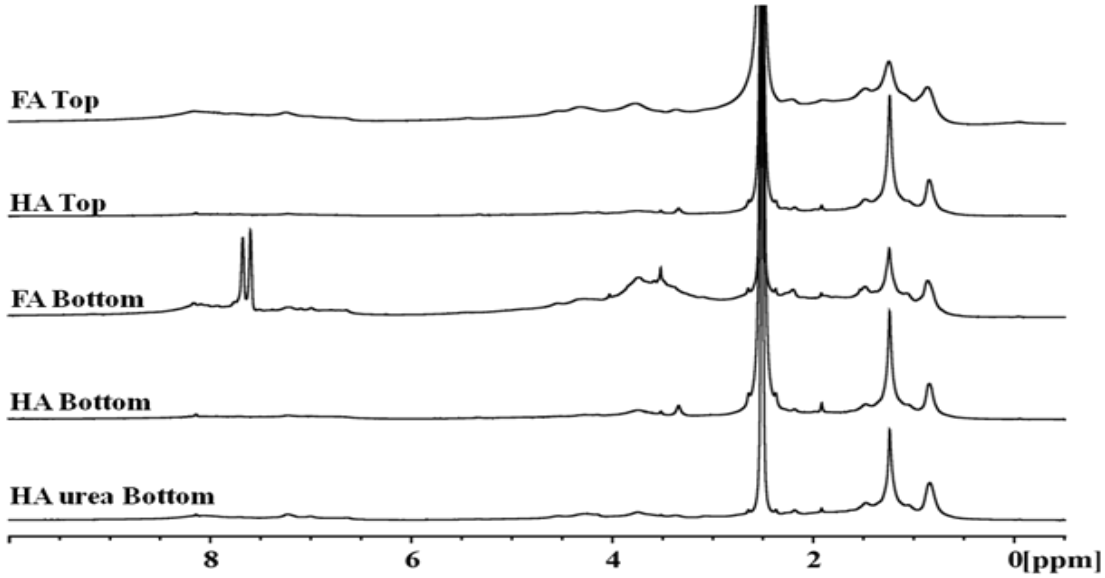

Figure 10.2. Conventional ^{1}H NMR of humic substances extracted from the top and bottom of the sediment core (HA = humic acid, FA = fulvic acid, HA urea refers to the HAs extracted in 0.1 M NaOH + 6 M urea).

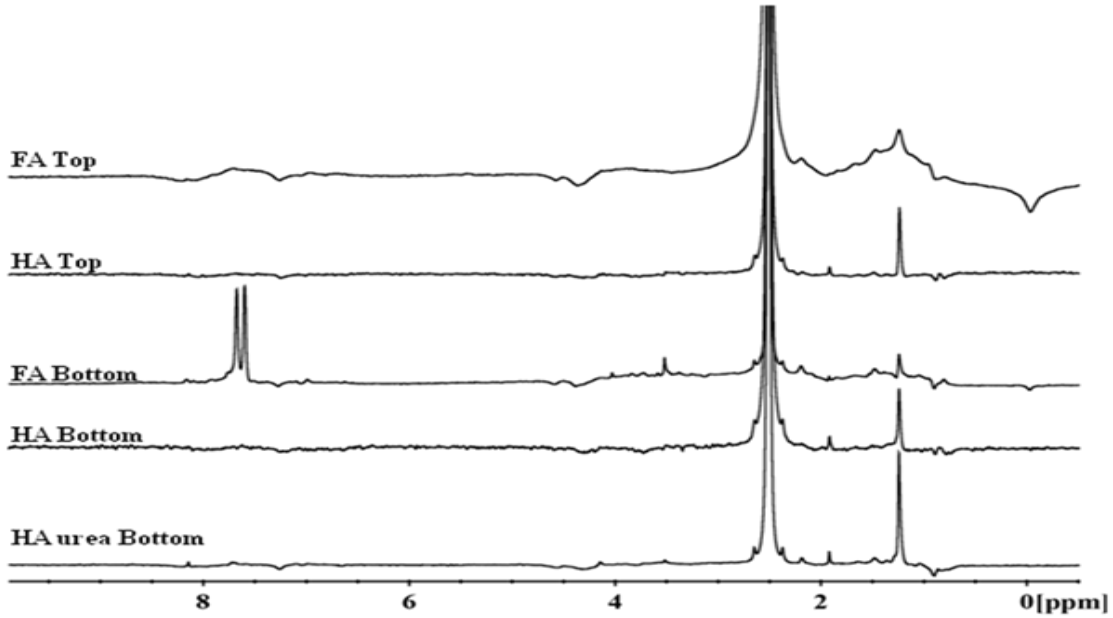

Figure 10.3. Inverse Diffusion edited (IDE) ^{1}H NMR of humic substances extracted from the top and bottom of the sediment core (HA = humic acid, FA = fulvic acid, HA urea refers to the HAs extracted in 0.1 M NaOH + 6 M urea).

The FAs (top and bottom) have the strongest responses in all chemical shift regions of the diffusion edited (DE) spectra. The DE spectra (Figure 10.4) provides additional compositional information compared to the conventional ^{1}H spectra

(Figure 10.2) as the mobile components (most importantly the DMSO signal) are removed from the spectrum. The resonances suggest that there are many rigid, macromolecular components in the HSs. The dominance of $(CH_2)_n$ and CH_3 remains in the DE spectra.

Apart from aliphatic hydrocarbons, there are contributions from peptides and lignin. The height of the CH_3 resonance relative to the $(CH_2)_n$ resonance suggests methyl groups from peptides. Peptide materials are identified by an amide broad resonance (7.8-8.3 ppm), a resonance from α- protons in peptides (centred at 4.3 ppm), aromatic amino acids; tyrosine and phenylalanine are identified (7.0 and 7.25 ppm) and amino acids side chains (1.77 and 1.9 ppm). These signals are stronger in the FAs than in the HAs, and especially for the FA isolate from the top. The HA-urea has a stronger response in all chemical shift regions compared to the HAs. This is due to the ability of urea to break H- bonds and to bring macromolecular components into solution. Aromatic amino acids were seen as a small bump in the baseline of the conventional ^1H spectra. However these are much better separated and defined in the DE spectra. Evidence for lignin is present in all the isolates (with the exception of the HA top) with a methoxyl signal ca. 3.7 ppm. Methoxyl lignin resonances are confirmed by the HSQC of the FA bottom and the HAs (Figs. 10.5, 10.6). The resonances at 3.7 ppm (Figure 10.4) and at 56 ppm (Figure 10.1) are particularly strong in the HSs from the base of the core, suggesting contributions from lignin.

Small carbohydrate signals are evident at 3.8-4.0 ppm in HA (top and bottom) and FA bottom. Anomeric protons in carbohydrate are not visible in the conventional ^1H spectra. Carbohydrate signals are not evident in the HA urea as this carbohydrate has been solubilised in the alkaline media, and carbohydrates do not form strong associations with the sediments. Based on the ^{13}C NMR spectra, the HSs from the top appear to have strong resonances for carbohydrate (ca. 70 ppm, Figure 10.1). However, carbohydrate resonances are relatively small in the ^1H spectra (Figs. 10.2, 10.4), which would suggest that the resonances at 70 ppm also have contributions from alkyl-OH groups. The HAs have similar spectra to the FAs with the exception of resonances at ca. 1.7 (amino acids side chains) in the FAs (Song et al., 2008). The HAs are shown to be predominantly composed of aliphatic hydrocarbons with much smaller contributions from peptides, lignins, and carbohydrates. The HA and HA-urea are shown to be compositionally similar in all

chemical shift regions, except for the lack of carbohydrate in the HA urea, and protein signals are better resolved in the HA urea sample. Based on resonances in the three ^{1}H spectra, protein, lipoprotein, and peptidoglycan resonances suggest strong microbial inputs to isolates from the top of the core and the lignin signals suggest a strong terrestrial input to the isolates from the base of the core.

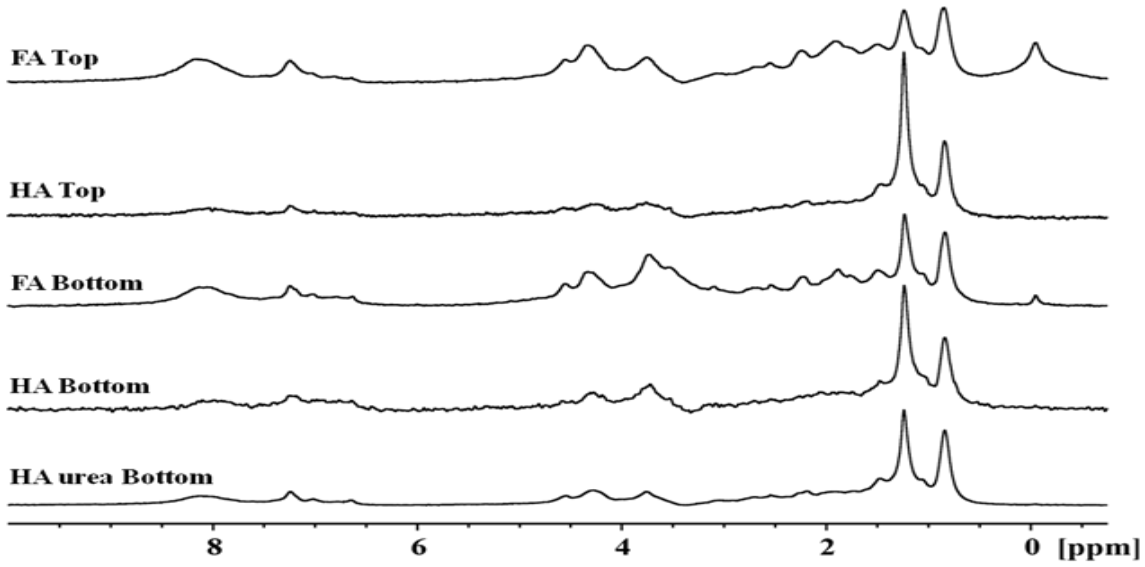

Figure 10.4. Diffusion edited (DE) ^{1}H NMR of humic substances extracted from the top and bottom of the sediment core (HA = humic acid, FA = fulvic acid, HA urea refers to the HAs extracted in 0.1 M NaOH + 6 M urea).

10.3.1.3 Heteronuclear 2D NMR

The HSQC NMR experiment (Figure 10.5) was carried out on the FA from the base of the core in an attempt to make an assignment for the doublet in the ^{1}H spectra (Figs. 10.2, 10.3), as well as confirming the interpretation of the resonances in the 1D ^{13}C and ^{1}H NMR spectra. There are no visible anomeric C resonances from carbohydrate, and the resonances from CH and CH_2 in carbohydrate (region 3) are weak. That would suggest small quantities of carbohydrate have been retained over time. Therefore, it is most likely that the resonances at 70 ppm in the 1D ^{13}C NMR are from alkyl-OH groups possibly from cutan/suberan. Peptide resonances are also weak, but there are strong resonances in the region of aromatic amino acids that correspond with resonances in the ^{1}H DE spectrum (Figure 10.4). The methoxyl group from lignin has a strong resonance (region 2), coupled with aromatic resonances (region 6), that suggest lignin units. Lignin contributions were speculative

in the 1D NMR but they are confirmed in the 2D spectrum as the result of the dispersion afforded in the second dimension. The peaks at 7.58, 130.9 and 7.66, 128.3 ppm (Figure 10.5 B) have not been previously identified in other isolates from the sediments in this study (Core 1 and Core 4). These are most likely to represent a small, mobile aromatic component of the FAs composition based on their chemical shift and on their resonances in the IDE spectrum. To view the 'unusual' resonances and to differentiate these from amino acids, the aromatic region was expanded (Figure 10.5 B). Wen et al. (2013a, b) suggest that these resonances (ca. 130, 7.5 ppm) represent C–H bonds in *p*-coumarate, a major component in lignin. An extensive NMR spectroscopy study of lignin at the Georgia Institute of Technology identified ^{1}H resonances at 7.53 ppm as aromatic protons in benzaldehyde units, and ^{13}C resonances at 128 ppm as C–α and C–β in φ–CH=CH–CH_2OH (http://www.ipst.gatech.edu/). But the absence of other ^{1}H and ^{13}C resonances rules these possibilities out as the sole contributors. Additionally, there is a resonance at 114.5, 6.70 ppm (Figure 10.5 B) that is identified as C-H in guaiacyl units (Wen et al., 2013a, b). The HSQC spectra, when greatly expanded, of HU from the base of Core 4 and from the top of Core 1 also show such lignin resonance at 131, 7.57 ppm.

The HSQC spectrum of the HA from the top and from the bottom are similar (Figure 10.6). The resonances are much stronger in the HA from the top at all chemical shifts, with the exception of the methoxyl C resonance at 56, 3.7 ppm. The strong methoxyl in the HA bottom corroborates with the strong lignin signals evident in the FA-bottom isolate. However, neither spectrum shows strong lignin resonances in the aromatic region. The HA top shows weak resonances for aromatic amino acids and α- protons in peptides. The HA bottom has weak resonances for α- protons in peptides, and shows a very weak resonance in the aromatic region (Figure 10.6B), but this is more visible when the contour levels are greatly increased. Both HAs, especially the HA top, have resonances for alkyl species from carbohydrate, but the anomeric C is not visible. The resonances for double bonds (130, 5.3 ppm) are very weak in the HA top (Figure 10.6A), suggesting low inputs from olefinic C. Similar to the 1D NMR the HSQC for both HAs show strong contributions from $(CH_2)_n$ and CH_3.

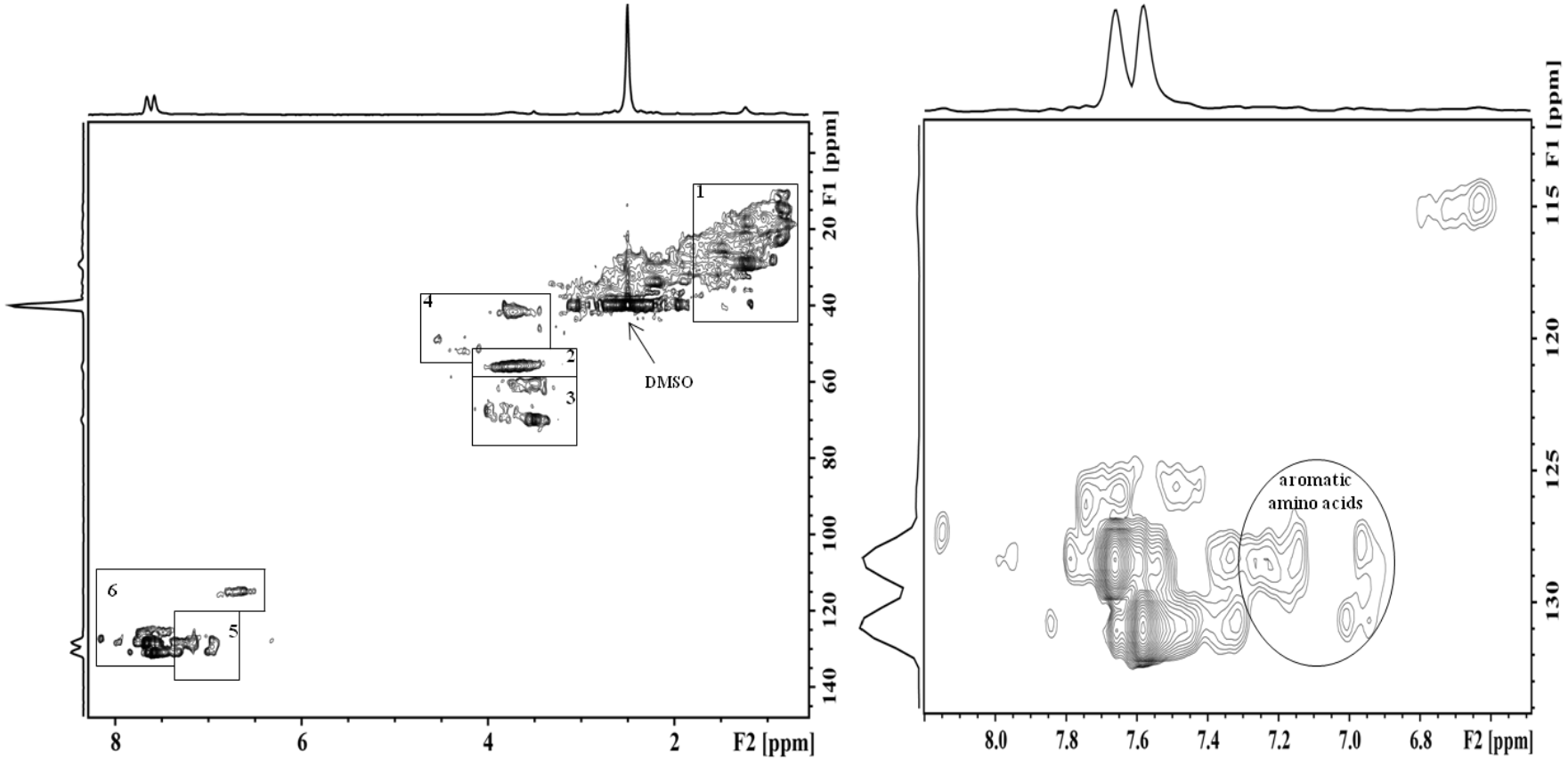

Figure 10.5. A: Heteronuclear Single Quantum Coherence (HSQC) NMR of FA bottom. 1= aliphatic CH_2 and CH_3, including signals from various hydrocarbons, and protein side-chains; 2 = methoxyl from lignin; 3 = CH and CH_2 from carbohydrate; 4 = α- protons in peptides; 5 = aromatic amino acids; 6 = aromatic units from lignin. B: Zoom of aromatic region.

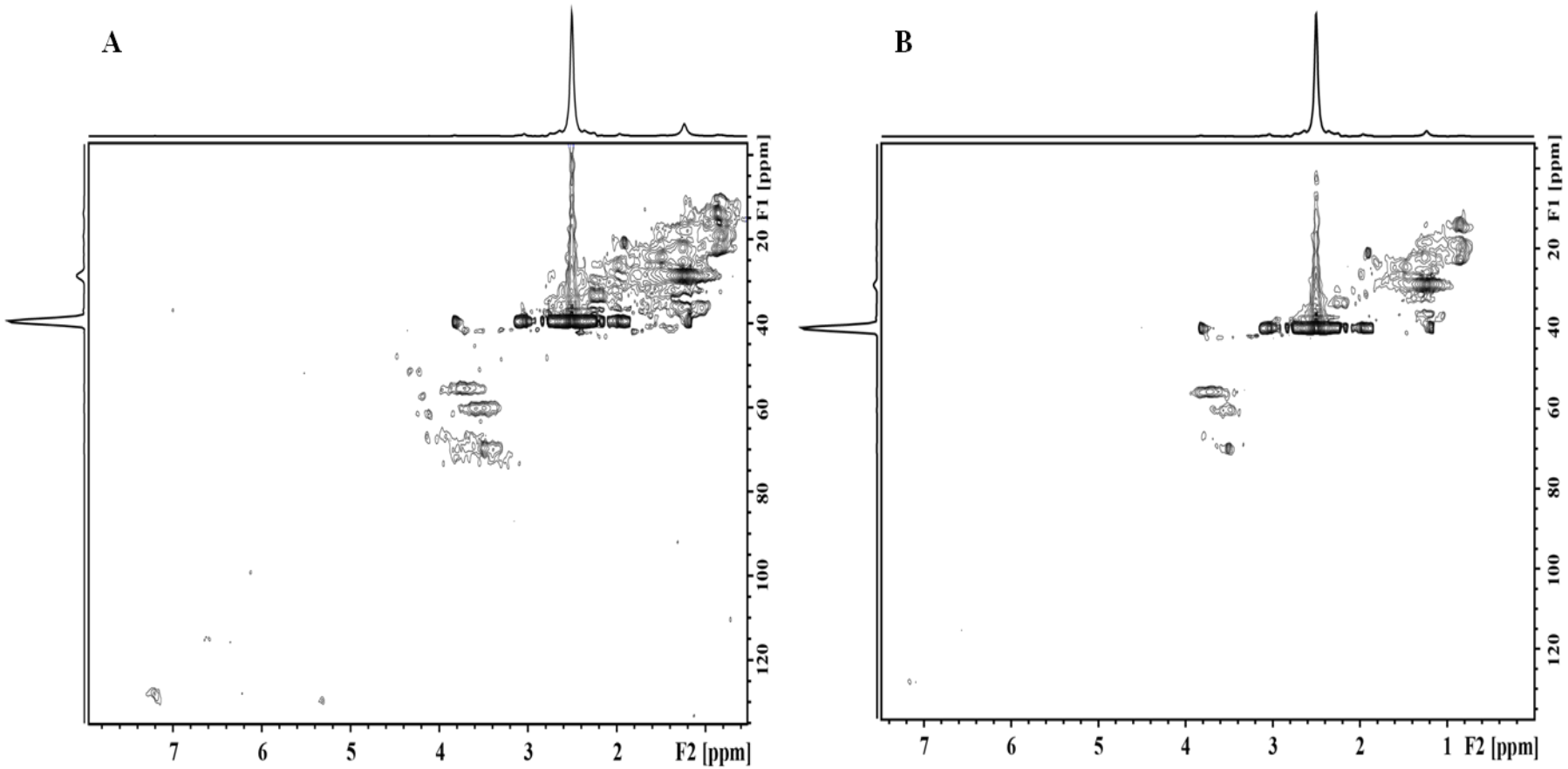

Figure 10.6. Heteronuclear Single Quantum Coherence (HSQC) NMR of A: HA top, and B: HA bottom

10.3.2 Organic Matter associated with the Sediments

10.3.2.1 ^{13}C NMR Spectroscopy

^{13}C NMR is useful to compare the composition of the organic fractions retained in the sediments. Following on from the HSs, the focus will now be on both the total OM, a mixture of both labile and recalcitrant components associated with the clay-sized fraction (clay and DCF) and also the most recalcitrant fractions (SHU and IHU). The ^{13}C NMR spectra were acquired using both a solid-state probe (Figs. 10.7A, 10.8A) and a CMP probe (Figs. 10.7B, 10.8B). The solid-state probe shows all the C functionalities in the sample, whereas spectra acquired using the CMP probe (swollen-state) shows only true solids, i.e. the species that did not dissolve/gain mobility in the DMSO-d_6 media. The compositions of the clay-sized fraction (clay) and demineralised clay-sized fraction (DCF) contain a mixture of HSs, HUs, and non-humic substances (OM that has not yet been re-mineralised or begun the diagenetic transformation process) whereas the HUs are predominantly composed of highly refractory C species.

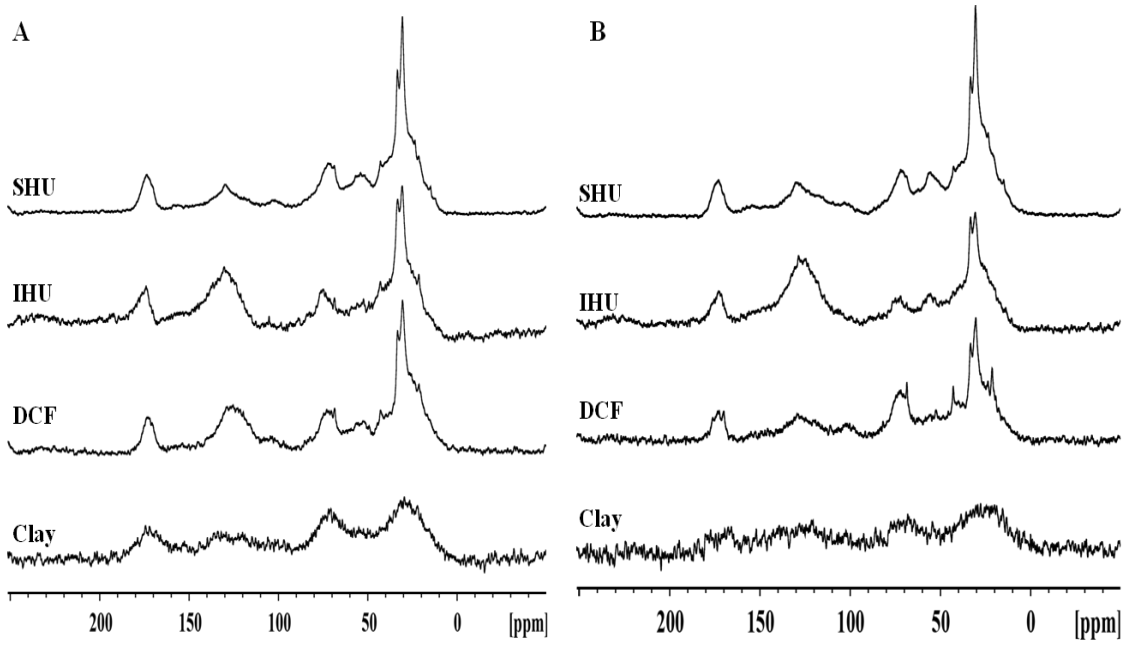

Figure 10.7. Solid-state ^{13}C CPMAS NMR of soluble humin (SHU), insoluble humin (IHU), demineralised clay-sized fraction (DCF), and of the clay-sized fraction (clay) (number of scans: SHU and IHU = 4K; DCF = 16; clay = 80K); A: fractions from the top of the core, B: fractions from the bottom of the core.

Overall, the spectra of all organic fractions from the top of the core have broadly similar profiles (Figure 10.7A). The clay-sized fraction has poor S/N but

resonances are evident in the aliphatic hydrocarbon region, the 'carbohydrate' region (65-85 ppm), and the carboxylic/ester/amide region (165-180 ppm). Aromatic resonances (C=C, 110 - 140 ppm) are only just distinguishable above the baseline in the clay-sized fraction. The other organic fractions (SHU, IHU and DCF) have resonances in the same chemical shift regions, but these have enhanced S/N that enables peaks to be assigned with more confidence. In the aliphatic hydrocarbon region (0-45 ppm) the clay-sized fraction has a broad peak, whereas this peak is split in the SHU, IHU and DCF showing the highly-ordered and the amorphous $(CH_2)_n$. Methyl C (21.5 ppm) is well defined in the SHU, IHU and DCF. There are resonances at ca. 39 ppm (IHU) and at 43 ppm (SHU, IHU and DCF) that can arise from terpene-type structures. These resonances are also in the HSs from the top of the core (Section 10.3.1.1). The protein/OCH_3 signals are difficult to assign in the clay-sized fraction due to the poor resolution of the spectrum. There is a distinct OCH_3 peak at 56 ppm in the IHU. There are broad resonances in the 50-60 ppm region of the SHU and DCF. It is probable that the majority of this signal arises from peptides, especially in the SHU because the aromatic resonances associated with lignin structures (between 125 and 155 ppm) are small. Lignin resonances were also less in the HSs from the top of the core. The IHU and DCF samples have resonances at 128, indicating the presence aromatic structures, possibly from lignin. The resonance ca. 70 ppm is generally assigned as carbohydrate; however, the anomeric C resonance at 105 ppm is small in the SHU. The anomeric C resonances are less evident in the IHU and the DCF samples. As both the clay-sized fraction and the DCF samples have not been fractionated on the XAD-8 resin, these contain both fresh and transformed OM. Therefore, it would be expected that there would be higher amounts of carbohydrate in these fractions compared to the HUs that have been extracted exhaustively removing all the HSs. The remaining carbohydrate in the SHU and IHU must have a degree of protection from degradation in the environment. All samples, except the clay-sized fraction, have a peak at 68 ppm indicating the presence of highly-ordered cellulose, and a peak at 21.5 ppm, which can be assigned as CH_3 in acetyl groups of hemicellulose (Rezende et al., 2011).

Core 4 bottom samples (Figure 10.7B) have resonances similar to those for samples from the top. However, the S/N in the clay-sized fraction (bottom) is much less, which makes meaningful interpretation difficult. The DCF (bottom) has strong

carbohydrate signals with resonances at 21.3 ppm, 68 ppm, 70-80 ppm, and 100-105 ppm. Resonances between 50-60 ppm in the DCF (bottom) are from a mixture of protein and lignin; the aromatic resonances are small, but there are resonances evident at 148 and 153 ppm indicative of lignin. There is a sharp peak at 43 ppm (terpene-like structures). The resonances in the carboxyl/ester/amide region are split with resonances at 170, 173, 176 ppm.

The IHU (bottom) has a strong highly-ordered $(CH_2)_n$ composition (resonance at 33 ppm almost equal in height to that at 30 ppm). It also has a defined highly-ordered cellulose resonance at 68 ppm. The carbohydrate resonances at ca. 70 ppm and 105 ppm are weak. There are strong aromatic resonances centred at 126 ppm, and small lignin resonances at 148 and 153 ppm. The resonances at ca. 170 ppm represent a mixture of carboxyl from fatty acids and lignin, ester from cutan, and amide from protein.

The SHU (bottom) has strong aliphatic hydrocarbon resonances. It also has a strong hump between 50 and 60 ppm that is most likely from both protein and lignin resonances. The carbohydrate contributions are indefinite and there is no anomeric C visible at 105 ppm. Thus the resonances at ca. 70 ppm are most likely from aliphatic alcohols.

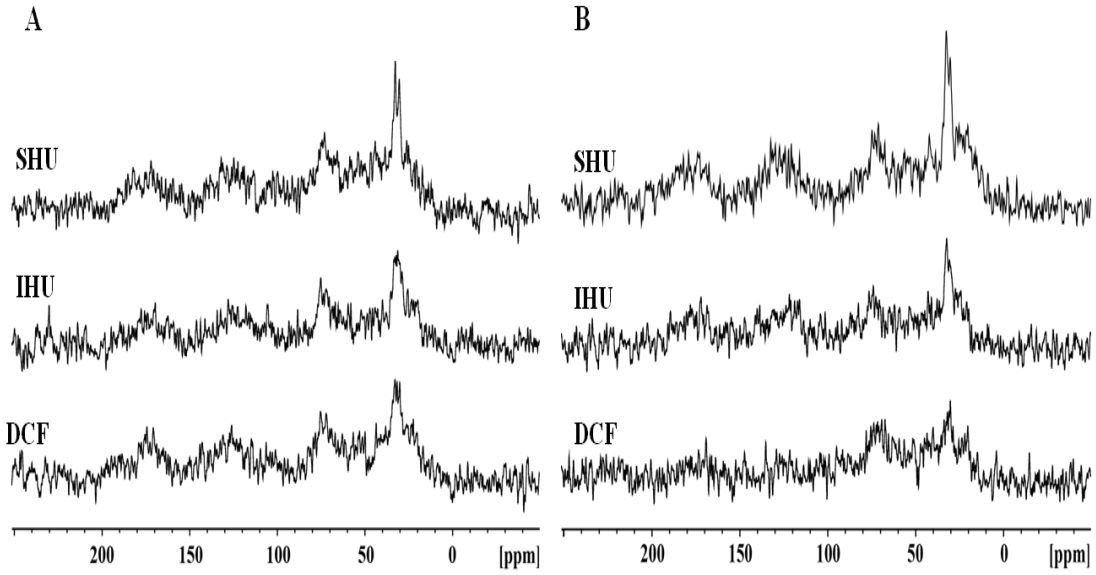

Figure 10.8. ^{13}C NMR of fractions swollen in DMSO. Fractions include: soluble humin (SHU), insoluble humin (IHU), demineralised clay-sized fraction (DCF); 32K scans were acquired for all samples. A: fractions from the top of the core; B: fractions from the bottom of the core.

^{13}C spectra for all the samples that swelled in DMSO have low S/N (Figure 10.8), which hinders the identifications of resonances. The clay-sized fraction samples were not swollen in DMSO as the S/N was low in the solid-state. Any component that swells and gains molecular motion is not detected; therefore only true solid domains are illustrated in these spectra (Figure 10.8). Aliphatic hydrocarbons are the dominant signal with highly-ordered $(CH_2)_n$ having stronger resonances in the SHUs and IHU bottom. Due to their ordered structure these species cannot be penetrated by the solvent. The S/N is very poor in the DCF from the bottom of the core; aliphatic $(CH_2)_n$ and resonances at ca. 70 ppm are scarcely discernible. IHU top (Figure 10.8A) has a sharp resonance at 105 ppm for anomeric C suggesting important contributions from carbohydrate to this fraction. IHU bottom also has a resonance at 105 ppm but it is difficult to differentiate it from the noise in the spectrum. It is also possible that there are resonances between 17-26 ppm for methyl C and deoxy sugars.

10.3.3 Chemical Characterisation

Extraction yields of HSs for the top of Core 4 (Figure 10.9) are similar to those from the top of Core 1 (Figure 8.2). However, there was no isolatable HSs in Core 1 bottom, but this was not the case for Core 4. Considerably lower HSs are extractable from the base of the core due to the lower OC content at depth, and perhaps also due to the re-mineralisation over time of the HSs. The urea adduct extracted additional HS components, but not to the same extent as in Core 1. The HU had lower tields in Core 4, top and bottom, compared to Core 1.

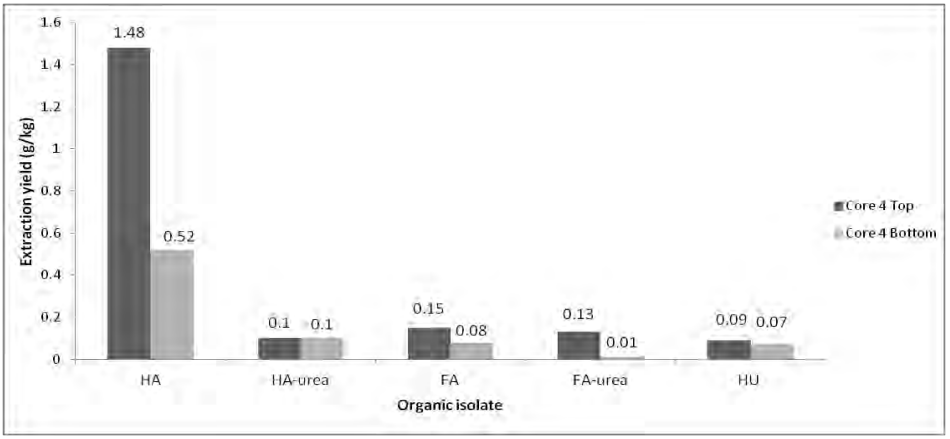

Figure 10.9. Extraction yields of the humic substances from the top and base of the core (HA = humic acid, FA = fulvic acid, HA urea and FA urea refers to the HAs and FAs extracted in 0.1 M NaOH + 6 M urea, HU = humin).

The sugar ratios suggest that the HAs and DCF samples are mainly composed of microbial sugars rather than sugars from higher plants (Table 10.1) (Oades, 1984). The HA bottom has the lowest ratio value ratio suggesting a greater plant input to the sugars in this isolate. This corresponds with strong lignin signals from the FA from the base of the core.

Table 10.1. Ratio of [MAN + GAL]/[XYL + ARA] from the humic acid (HA) and demineralised clay-sized fraction (DCF) from the top and the base of the core.

Sample	[MAN + GAL] / [XYL + ARA]
HA Top	3.15
HA Bottom	1.96
DCF Top	2.31
DCF Bottom	2.55

The C contents are low in the HAs from the alkaline extractions (Table 10.2). A similar trend was apparent in HAs from Core 1 top (Ch. 8, Table 8.1), attributable to high ash compositions. N composition is highest in the FA top isolate and that correlates with strong protein signals in the NMR spectra (Figs. 10.4, 10.5). The C/N values are highest in FA bottom indicating a strong plant contribution. This supports the strong lignin signals identified in the FA bottom isolate (Figs. 10.3, 10.4, 10.6). C/N values are higher in the isolates from the base of the core, suggesting greater terrestrial plant inputs to the sediments at this depth.

Table 10.2. Elemental composition and C/N ratio of the humic substances from the top and bottom of the core (FA = fulvic acid, HA = humic acid, HA urea = HAs isolated in the 0.1 M NaOH + 6 M urea media).

Sample	%N	%C	%H	%S	C/N
FA Top	04.10	46.62	03.26	02.52	11.37
HA Top	02.87	29.68	03.17	02.52	10.34
FA Bottom	03.23	48.34	03.20	03.06	14.97
HA Bottom	02.30	25.42	02.92	01.58	11.05
HA Urea Bottom	03.18	41.14	02.95	02.16	12.94

The ash contents are very high for the clay-sized fraction samples as these are predominantly inorganic materials. The clay-sized fraction was subjected to an extensive treatment with HF to dissolve the silicates (DCF), the dominant inorganic species (Table 10.3). The high ash contents of the DCF samples suggested that there were other inorganic species remaining following the silicate dissolution. Therefore, SEM-EDX analysis was carried out to determine the compositions of the inorganic species. It is evident in Table 10.3 that the HF treatment was effective in dissolving the silicates. As a consequence of Si-O removal, other inorganic species, such as Al and Mg, are concentrated. These remaining inorganic species have a negative effect on spectroscopic studies as they lower the quantity of OC in the sample being studied. A high concentration of Fe will affect the signal strength and the resolution of the NMR spectra acquired. Fe, Al (in the top) and Ca are present at high concentrations in the clay-sized fraction samples. These are important cations that mediate C sequestration in the environment (Li et al., 2002; Tipping, 2002; Wiseman and Püttmann, 2006; Setia et al., 2013). Other elements (e.g. Na, Ti) were also detected at lower levels and the relevant data are available in the appendices (1-2).

Table 10.3. Elemental and ash compositions of the clay (clay-sized fraction) and demineralised clay-sized fraction (DCF) from the top and bottom of the core.

Sample	% Ash	% Si	% Fe	% Al	% Mg	% S	% K	% Ca
Clay Top	84.83	39.52	11.31	16.08	02.13	01.16	06.83	21.84
DCF Top	68.82	01.11	11.44	28.68	16.82	17.80	07.74	04.38
Clay Bottom	90.33	51.48	07.56	20.65	02.31	00.64	07.76	08.95
DCF Bottom	75.46	0.19	01.73	23.70	07.53	02.11	20.00	41.45

10.4 Discussion

The low extraction yields (Figure 10.9) can be attributed to the high inorganic compositions of sediments, as well the low organic inputs to the sediments due to low productivity and extensive re-mineralisation in the water column. Additionally, the presence of inorganic species such as Fe and Al hydroxides tightly bind OM making it difficult for the solvent to remove the OM from the mineral matrix. These low yields impacted characterisation studies as there was not sufficient sample for a suite of experiments to be carried out.

10.4.1 NMR Spectroscopy of Humic Substances

The information acquired from the ^{1}H and ^{13}C NMR complement each other, and together help to assign resonances with more confidence, especially when samples are also studied using 2D NMR. The NMR spectra show the materials from the top and base of the cores to have some differences in their compositions. Therefore, HSs will vary depending on their OM inputs and their stage of diagenesis. The ^{1}H DE spectra show that HSs are predominantly composed of rigid components and the only mobile components include aliphatic $(CH_2)_n$, lipoprotein, peptidoglycan, and aromatic lignin units in the case of the FA bottom isolate.

The materials from the base of the core are slightly older and show enrichment in plant materials. The coring location in the past could have been more strongly influenced by terrestrial OM inputs and by terrestrial environmental conditions. The age gap between the sediments from the top and base of the core is small and overall the compositions from the top and bottom show strong inputs from plants and from peptide sources (similar to those from the base of Core 1, which represent a similar age). The HA and HA-urea (bottom) are compositionally similar, but the HA urea sample was included in the Figures to illustrate the enhanced solubilisation of lignin and protein structures, attributable to the ability of urea to solubilise H- bonded components. Urea is used routinely to denature proteins; therefore, it could be releasing additional protein components into solution that are bound to the mineral matrix.

The FAs (top and bottom) are shown to be less aliphatic and have more carbohydrate and aromatic components in their compositions compared to the HAs. The strong aromatic peak centred at 130 ppm coupled with the peak centred at 56 ppm suggests lignin inputs in the compositions of FA bottom (Figure 10.1). This strong aromatic resonance at 130 ppm required more detailed investigations using 2D NMR in order to identify the resonance (Figure 10.5).

Protein assignment is tentative in the ^{13}C NMR due to the overlapping with OCH_3. As lignin and protein resonate at different chemical shifts in ^{1}H NMR, it is useful to assign the peak between 50-60 ppm in the ^{13}C NMR. Therefore, ^{1}H NMR (Figs. 10.3, 10.4) and 2D HSQC (Figs. 10.5, 10.6) resolve resonances for the protein and lignin components. Protein is evident in the ^{1}H NMR especially in the aromatic and amide region. α- Protons in peptides have weak resonances in the HSQC (Figs.

10.5, 10.6). The methoxyl from lignin is clearly identifiable in the HSQC of all three isolates, especially the FA bottom. Additionally the C/N ratios suggest plant contributions, especially in the FA bottom which has the highest ratio value.

HSQC was also beneficial to confirm the contributions from carbohydrates, as these were tentative in the 1D experiments as the anomeric C was not clearly evident, especially in the isolates from the bottom of the core. Anomeric C is also not identifiable in the HSQC but weak resonances are present for CH_2 and CH from carbohydrate. The carbohydrate resonances are stronger in the HA top HSQC (Figure 10.6A), confirming greater carbohydrate compositions of the isolates from the top of the core. The HAs and FAs were fractionated using XAD-8 resin which removes any materials not bound to the humic matrix. Therefore, resonances in the NMR spectra are not arising from co-extraction products. The sugar analysis of the HAs show that the majority of the sugars have microbial origins. Additionally, there are resonances in the 1D ^{13}C NMR spectra for deoxy sugars from microorganisms. Labile plant sugars possibly have been degraded in the environment and not retained long-term in the sediments. The microbial sugars are consistent with the signals for protein, lipoprotein, and peptidoglycan resonances identified in the DE and IDE spectra.

10.4.2 Organic Fractions in Sediments

The ^{13}C NMR spectra of the fractions acquired that swelled in DMSO-d_6 are difficult to interpret due to the low S/N ratio. The HUs are composed of less labile components; for example, resonances in the carbohydrate region of the spectrum are smaller. There is evidence of highly-ordered carbohydrates present in both the solid-state and swollen-state spectra, especially in the IHU top. The carbohydrate compositions were also greater in the HSs from the top. The retention of carbohydrate in the refractory IHU suggests that strong protection mechanisms are responsible for its retention. Schmidt et al. (1997) and Gélinas et al. (2001) both reported loss of carbohydrate following HF treatment. This may have occurred to a small extent (smaller resonance in the IHU relative to the HU) but the resonances remain at ca. 70 ppm, and the resonances at 105 ppm are sharper.

The total OM contained materials in varying stages of diagenesis. It is difficult to differentiate noise from true signal in the clay-sized fraction, especially in

the clay-sized fraction from the base of the core. Therefore, assignments must be made with caution and should be coupled with resonances identified in the organic fractions. However, the broad resonances show that the overall compositions are similar and that the organic materials are not significantly changed through chemical pre-treatment or extraction.

The resolution of the spectra are greatly improved after the HF pre-treatment of the clay-sized fraction (despite the high ash content). The carboxyl/amide/ester, carbohydrate, terpene-like structures and aliphatic hydrocarbons are much better defined in the DCF (top and bottom). The DCF top also shows improved definition in the aromatic and protein/methoxyl chemical shift regions.

The SHU and IHU from top and bottom are broadly similar. The IHU have more highly-ordered $(CH_2)_n$ relative to the amorphous $(CH_2)_n$, owing to its insolubility and recalcitrance. The SHUs have more defined resonances at 70 ppm, but it is unlikely that this is from carbohydrate, as there is no evident anomeric C resonance, and it would be expected that the majority of carbohydrate materials would be quickly degraded in the environment, unless these are protected from degradation. The resonance at 70 ppm is more likely from alkyl-OH groups from plant OM. The highly-ordered hydrocarbons, which are abundant in the HUs, are responsible for the long term retention of OC in the environment as these are inherently recalcitrant due to their structure. Additionally, these species provide hydrophobic domains for the protection of labile carbohydrates and proteins. The retention of carbohydrate and the concentration of protein in the HUs suggest that these components also have a degree of recalcitrance, conferred by highly-ordered and semi-crystalline domains in cellulose and hemicellulose, as well as methyl groups and hydrophobic side chains in amino acids. Microbial sugars are evident in the total OM samples as well as in the HUs suggesting these carbohydrate components are more recalcitrant than plant sugars. These resonances corroborate with the [MAN + GAL]/[XYL + ARA] ratio which suggests that the sugars are predominantly from microbial sources.

10.4.3 Mineralogy of the Clay-Sized Fraction and the Demineralised Clay-Sized Fraction

It is evident from Table 10.3 that the 10% HF treatment is effective for Si removal, and it causes little or no alterations to the OM, as evidenced in the ^{13}C NMR spectra (Figs. 10.7, 10.8). The Si is decreased to < 1% following the HF treatment. As a consequence of the mass removal of Si-O, other elements become concentrated in the samples such as Mg and Al. The persistence of these inorganic species accounts for the high ash contents in the DCF samples. Fe was solubilised to a small extent in the HF pre-treatment, as this was not concentrated in the DCF samples. Fe species affect the NMR spectrum by quenching signals from functionalities in close proximity to the paramagnetic species, and also causes line broadening. Removal of these Fe-bearing minerals with mineral acid should increase the quality of the spectra acquired. Most of the other elements detected could also have been dissolved using mineral acid (HCl or H_2SO_4). Therefore, an improvement in the methods used in this study would be to dilute the HF in a weak acid solution rather than in distilled water. Minerals bearing Fe and Al are all associated with strong retention of OM. Studies by Song et al. (2011) and by Chang et al. (2014) found that soils high in Fe/Al hydroxides had low HU extraction yields in the acidified-DMSO media compared to soils that are predominantly composed of aluminosilicate clays. It is evident in Fig 10.9 the HU yields were low, and this is attributable to the low OC content of the sediments and the strong interactions of the OM with the clay minerals. Also, Ca^{2+} is regarded as a very important cation involved in the stabilisation of soil OM and organic aggregates through its role in the formation clay–polyvalent cation–OM complexes (Peele, 1936; Myers, 1937; Peterson, 1947; Muneer and Oades, 1989; Clough and Skjemstad, 2000; Six et al., 2004). These interactions with inorganic species, especially the Fe and Al hydroxides, are important for long term C sequestration.

10.5 Conclusion

The isolates have less indications of marine OM compared to HS isolates for the top of Core 1, which are significantly younger and closer to the terrestrial environment. This suggests that terrestrial inputs dominated ca. 9,000 years BP. Components from higher plants are evident in all the NMR spectra, and the C/N ratio shows influences from plant materials. By comparing organic materials from the top and base of the core, it is evident that the base of the core has more lignin components, especially the FA isolate. The presence of lignin suggests a terrestrial influence, suggesting that Galway Bay may have been a terrestrial environment in the past. Microbial inputs are also strong in the HSs, in the HUs, and in the total OM, as evidenced by signals from peptide structures in the NMR, the sugars released by hydrolysis are dominated by microbial saccharides, and a high N content.

The benefit of carrying out extraction and isolation methodologies is evident when these samples are compared to spectra of the total OM (clay-sized fraction and DCF samples). ^{13}C NMR spectra of clay-sized fraction (no pre-treatment) samples are difficult to interpret due to poor resolution of resonances despite a large number of scans. HF pre-treatment greatly improves the resolution of the spectra through dissolution of silicates, and good compositional information can be acquired using solid-state ^{13}C NMR spectroscopy. However, the high inorganic content affects the acquisition of swollen-state NMR as the samples showed poor S/N, but this may be improved by removing the remaining inorganic species evident in Table 10.3. This DCF and the HU samples are examples of multiphase samples specifically suited for studies by the CMP probe.

Similar to the isolates from Core 1 top, the HA and HA urea are very similar, with the exception of the higher concentration of protein and lignin components solvated in the alkaline/urea media. Components in the SHU and IHU represent stable C which most notably include highly-ordered $(CH_2)_n$ and cellulose that are difficult to degrade (chemically or biologically) due to their structure. Other stable C components include amorphous $(CH_2)_n$, protein, aromatic structures, and microbial sugars (perhaps from living microbial communities, or derived from preserved cellular materials). These components are protected from re-mineralisation through their associations with clay minerals and hydrophobic domains in the OM.

The HF pre-treatment is shown to be efficient in removing silicates without compromising the integrity of the sample. However, the addition of mineral acid may aid in the removal of other inorganic species which are concentrated in the DCF and can decrease spectral resolution. The clay-sized fraction have high proportions of Fe, Al and Ca which are known to strongly bind OM, thus protecting it from re-mineralisation but hindering the extraction process in the laboratory. Low SHU isolation can be attributed to interaction with these species. Therefore, it was necessary to isolate the remaining HU (IHU) using HF. The SHU and IHU are very similar with the major difference being related to the enhanced order of the compositions in the IHU.

NMR spectroscopy provides a complete characterisation of the components retained in the sediments of Core 4. The age gap between the sediments from the top and base of the core is small, suggesting it to be a low depositional environment. Despite its distance from the land, lignin signals are strong in the samples suggestion important contributions from terrestrial biomass. It is possible that the coring location was once part of the river Corrib estuary or even dry land prior to sea level rise.

10.6 References

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Chapter 11 Conclusions

11.1 Main Conclusions of the Study

The main research aims, to isolate and to characterise the organic components from surface sediment and sediment core materials from an estuarine environment, were achieved. It was clearly demonstrated that the sequential solvent extraction system developed for soils can also be successfully applied to aquatic sediments. The major adaptation was the omission of the extractions in aqueous alkaline media at pH 7 and at pH 10.5. These were not necessary as the pH of sea water is usually ca. 8, and therefore negligible yields are isolated below pH 12.6. Extraction yields were low for the sediments from Core 1 (inner estuary) and Core 4 (outer estuary). However, yields can be improved when high concentrations of urea are added to the alkaline medium. These isolates are very similar in composition to the alkaline isolates, except for the enhanced solvation that takes place of protein and lignin structures. Overall, the extraction yield of fulvic acids was very low. This low yield is attributed to the fact that FAs will remain in solution in the (mildly alkaline) aquatic environment and become re-mineralised rather than be sequestered. Extraction of the humic substances (HSs) from the older sediments at the base of Core 1 was not successful; this is attributed to re-mineralisation of the HSs in these sediments which are a significant age (ca. 9,500 years old). Humin (HU) can be successfully isolated and studied when both DMSO-H_2SO_4 and 10% HF are utilised. The DMSO-H_2SO_4 isolates the soluble HU components. These are shown to contain more amorphous aliphatic hydrocarbons that are easier for the solvent to penetrate. Additionally, they have more carbohydrate compositions relative to the IHU. Following extraction of the clay-sized fraction with the DMSO-H_2SO_4, the truly refractory components are concentrated by dissolving the clay silicates. These components of the IHU show strong resonances for highly ordered structures. These are the most difficult to degrade biologically or abiotically. The enhanced solvation power of the DMSO-H_2SO_4 is evident when the HU samples were studied in a DMSO-d_6 media for NMR spectroscopy experiments. Some species did not swell/solvate. However, these species were solubilised in the DMSO-H_2SO_4 medium.

Total organic matter (OM) was concentrated to facilitate characterisation by (1) isolating the clay-sized fraction from the sediments, and (2) taking a subsample

of the isolated clay-sized fraction and removing the silicates with HF (demineralised clay-sized fraction, DCF). The NMR spectroscopy revealed that it is very difficult to study the clay-sized fraction as the high inorganic composition and low organic composition resulted in a poor signal-to-noise ratio. This may be a more successful approach for the clay-sized fraction from a high organic matter soil. The DCF spectra had good signal-to-noise in the solid-state ^{13}C NMR spectra; however, additional experiments prove difficult due to the high ash content of the sample which provided poor signal and decreased spectral resolution. Comparing the spectra of the clay-sized fraction sample with the DCF sample, and with the chemically extracted isolates showed that the chemical extraction procedure does not alter the organic compositions.

FT-IR spectroscopy provided general compositional information about the samples. However, broad bands and the overlapping of signals hinders in-depth characterisation. Conversely, it did prove useful for identification of inorganic species co-extracted with the organic isolates. The pyGC/MS experiments provided an abundance of data in relation to the compositions of the samples. Protein, lignin, carbohydrate derivatives (furans), and lipids are the major compositions of the estuarine sediment samples. A number of unusual compounds were identified which suggest there is pollution in the bay from compounds such as plastics, personal care products and sewage. The protein compositions of the organic fractions were confirmed with the presence of pyrroles, and the influence of microbes indicated through the identification of microbial-type sterols such as ergosterol and hopanes.

A suite of NMR spectroscopy experiments were carried out to produce a wealth of information on the OM from the sediments. Proton NMR is a quick experiment that can provide a high level of information about the analyte, especially when spectral editing is used. The solubility issue can be overcome for less soluble samples (e.g. HU, DCF) by using the complete multiphase probe. The ^{13}C solid-state NMR provided an overview of all the major functionalities in the samples, but the broadness of the lines resulted in overlapping of some signals. Swollen-state ^{13}C NMR has narrower lines so interpretation of resonances is enhanced. This experiment was used to study the species that did not swell. These are the highly ordered structures which are responsible for the recalcitrance of OM in the environment. Additional compositional information can be attained when 2D NMR

is utilised. This was evident in the HSQC spectra where the additional dispersion separated out the resonances from peptide and methoxyl signals. It also helps identify lignin aromatics. The aliphatic components of the samples can be studied when using the TOCSY experiment, as this experiment detects protons in the same spin system. NOESY provides information on protons which interact through space. This experiment is especially useful for the identification of proteins, and also confirmed the presence of lignin.

Overall, the results show that the HSs are composed of numerous labile components and lack a large aliphatic hydrocarbon composition which offers long-term protection from re-mineralisation, especially in the case of the FAs from the top of Core 1. The FAs from Core 4 (top and bottom) showed stronger compositions of aliphatic hydrocarbon, and even the presence of some highly-ordered species. The presence of these species in higher abundance suggests that with time the aliphatic hydrocarbon species are preserved and these are necessary to prevent the FAs solubilising. The HUs are principally composed of aliphatic hydrocarbons with origins in plant biomass. A fraction of the aliphatic hydrocarbons is highly ordered and extremely recalcitrant. The inclusion of lignin and protein structures in the HUs is due to the encapsulation of these species thereby enabling these to be preserved for possibly thousands of years. The HUs are most strongly associated with the mineral matrix. Dissolution of silicates greatly concentrates the DMSO-insoluble HU, enabling characterisation. However, the ash composition remained high in the insoluble HU and DCF despite extensive treatments with 10% HF. SEM-EDX analysis revealed that the HF treatment was effective in removing the silicon from the sample; however, other elements remained and in some cases became concentrated. C/N values were generally 10 or greater suggesting a large influence from terrestrial plants. This may indicate that the marine OM is less recalcitrant and is re-mineralised in the water column and during early diagenesis, relative to the terrestrial OM.

11.2 Future Work/Recommendations

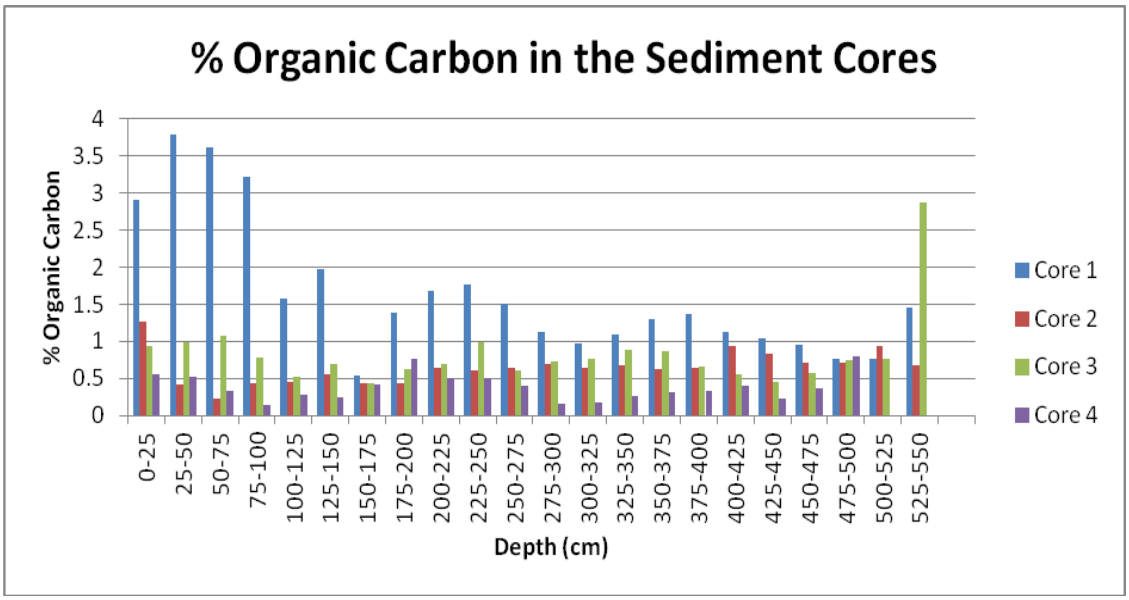
- In future studies of the organic components associated with aquatic sediments the use of larger quantities of sediment is recommended in order to isolate

sufficient quantities of the organic fractions for in-depth characterisation studies.

- pyGC/MS studies on the FAs from the older sediments at the base of Core 4 would be useful in order to access the lignin compositions, as unusually strong lignin resonances were evident in the NMR spectra of this isolate.
- Sugar analysis on all samples would give better indications of the saccharide compositions of the isolates, and it could be said with more confidence that microbial sugars dominate.
- Amino acid analysis of the isolates would potentially corroborate with the strong protein signals in the NMR spectra, thus enabling more confidence assignments of sources.
- A procedural blank would have been useful as the plastic contamination could either be assigned to the laboratory procedures used or to the Galway Bay environment.
- The addition of HCl or H_2SO_4 to the 10% HF solution could be effective in dissolving the elements that were concentrated/remained after the dissolution of Si.
- Additional radiocarbon dates would assist in the discussion of changes to OM in relation to time.

Appendices

Appendix 1-1 Organic Carbon Content of Core 1, Core 2, Core 3, and Core 4.



Appendix 1-2 SEM-EDX Data from Core 1 and Core 4 Clay-Sized Fraction (Clay) and Demineralised Clay-Sized Fractions (DCF).

	Core 1 Top Clay	Core 1 Top DCF	Core 1 Bottom Clay	Core 1 Bottom DCF	Core 4 Top Clay	Core 4 Top DCF	Core 4 Bottom Clay	Core 4 Bottom DCF
% Na	2.04	6.14	1.49	2.55	-	-	-	-
% Mg	2.03	5.36	2.37	6.65	2.13	16.82	2.31	7.53
% Al	20.35	11.61	22.37	22.09	16.08	28.68	20.65	23.7
% Si	45.36	0.59	49.24	0.46	39.52	1.11	51.48	0.19
% S	7.87	35.12	-	3.37	1.16	17.8	0.64	2.11
% K	8.23	14.65	8.87	20.09	6.83	7.74	7.76	20.0
% Ca	-	-	7.89	44.83	21.82	4.38	8.95	41.45
% Ti	1.33	7.94	0.73	-	1.17	12.04	0.68	3.41
% Fe	12.78	18.59	7.05	-	11.31	11.44	7.56	1.73

Appendix 1-3 Radiocarbon Dates for Core 1 and Core 4.

Core	Core Placement by section (cm)	Shell placement in the core (cm)	Marine Calibrated age ranges (cal BP)
Core 1	Section 1of 6 5-7	5-7	89-280
Core 1	Section 1 of 6 37-38	37-38	224-467
Core 1	Section 2 of 6 26-27	66-67	6099-6411
Core 1	Section 6 of 6 26-28	464-466	9562-9980
Core 4	Section 1 of 5 16.5-17	16.5-17	8889-9249
Core 4	Section 1 of 5 55-57	55-57	9127-9524
Core 4	Section 5 of 5 60-61	450-451	9296-9598

Appendix 1-4 Ash Contents of the Clay-Sized Fraction (Clay) and Demineralised Clay-Sized Fractions (DCF).

Sample	% Ash
Core 1 Top Clay	80.00
Core 1 Top DCF	31.81
Core 1 Bottom Clay	90.18
Core 1 Bottom DCF	72.78
Core 4 Top Clay	84.83
Core 4 Top DCF	68.82
Core 4 Bottom Clay	90.33
Core 4 Bottom DCF	75.46

Appendix 1-5 Ash Contents of the Insoluble Humin (IHU).

Sample	% Ash
Core 1 Top IHU	41.14
Core 1 Bottom IHU	54.14
Core 4 Top IHU	61.11
Core 4 Bottom IHU	67.97