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Molecular Characterization of Dissolved Organic Matter (DOM) in Seawater

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Moleculaire karakterisering van opgelost organisch materiaal (DOM) in zeewater

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor
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Aan mijn ouders

CONTENTS

ABBREVIATIONS

ASW	Artificial Seawater
BSA	Albumin Bovine Serum
CTD	Conductivity, Temperature, Dissolved Oxygen
CuPy	Curie-Point Pyrolysis
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
DON	Dissolved Organic Nitrogen
FID	Flame Ionization Detector
GC	Gas Chromatography
HRMS	High-Resolution Mass Spectrometry
HTCO	High Temperature Catalytic Oxidation
IR	Infra Red Spectroscopy
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
POM	Particulate Organic Matter
Py	Pyrolysis
TIC	Total Ion Current
TMAH	Tetramethylammonium Hydroxide
UDOM	Ultrafiltered Dissolved Organic Matter

SUMMARY

Dissolved organic matter (DOM) plays an important role in the global carbon cycle (chapter 1). However, not much is known about the molecular composition and the origin of DOM. The study described in this thesis was conducted to gain more knowledge on the molecular composition and the origin of DOM.

The molecular characterization of three DOM samples from the Pacific Ocean is described in chapter 2. These samples were obtained from and analyzed by Dr. Ronald Benner. They offered the opportunity to compare results and to evaluate our analysis methods. The samples were isolated using tangential-flow ultrafiltration. These DOM samples represent the size fraction of DOM between 0.2 μm and 1 nm (1000 Dalton), in this thesis referred to as ultrafiltered dissolved organic matter (UDOM). Samples from three different depths, representing a surface water sample (10 m), an oxygen-minimum layer water sample (765 m) and a deep ocean water sample (4000 m) were analyzed using analytical pyrolysis. Specific pyrolysis products of proteins, lignins and resistant aliphatic biomacromolecules were absent from all samples. A significant amount of nitrogen compounds was encountered in the 10 m-sample. The origin of these nitrogen compounds is currently unknown, but they may be partially derived from aminopolysaccharides originating from bacterial lipopolysaccharides and peptidoglycan. Pyrolyzates of all three samples contained significant amounts of alkylphenols of non-lignin origin.

The molecular characterization of UDOM from North Sea water during a *Phaeocystis* algal bloom using analytical pyrolysis is described in chapter 3. Some differences were observed between UDOM from the bloom area and the non-bloom area. The main difference was the presence of relatively large amounts of unaltered polysaccharides in the algal bloom UDOM sample and the absence of these compounds from the non-bloom UDOM samples.

In order to study the contribution of terrestrial or riverine DOM to seawater DOM, a pilot study of UDOM from the Ems-Dollart was conducted (chapter 4). Four different UDOM samples were collected from water masses with salinities varying from 0.4 to 20‰. The samples were analyzed using analytical pyrolysis, ^{13}C-NMR and stable and radiocarbon isotopes. All UDOM samples throughout the estuary revealed similar pyrolyzates and ^{14}C activities. Only the ^{13}C-NMR spectrum of the UDOM sample from the water with the highest salinity showed slight differences with those of other samples. The stable carbon isotope values suggest mixing of river DOM with seawater DOM of comparable ages. Also in the Ems-Dollart UDOM, pyrolysis

products of unaltered polysaccharides, lignins, proteins and lipids were not detected. This further suggests that UDOM consists of a large fraction of refractory organic matter and a very small fraction of fresh organic matter.

In order to test the hypothesis that polyphenolic constituents, present in UDOM and particulate organic matter (POM) are of algal origin, a number of macroalgae were studied using analytical pyrolysis (chapters 5 and 6). The algae were subjected to a number of chemical degradation treatments in order to test the chemical resistance of the polyphenolic moieties present in the algae. It has been shown that brown algae as well as green and red macroalgae contain chemically and possibly biologically very resistant macromolecules, which produce a number of alkylphenols upon pyrolysis. One of the conclusions of these chapters was that these algal polyphenolic macromolecules contribute significantly to DOM and POM.

However, it is further shown that alkylphenols present in pyrolyzates may have many different precursors (chapter 7). A number of samples containing organic matter were studied, *i.e.* UDOM, POM, sediments, polysaccharide/protein standards, algae, hydrolyzable tannins, lignins, lignites, coals, soils and insect cuticles. These samples were subjected to various chemical degradation treatments. To some extent, the distribution patterns of the alkylphenols in the pyrolyzates can be used to discriminate their precursors: lignins, proteins and transformed protein. From this study it was concluded that alkylphenols in pyrolyzates of UDOM, POM and sediments reflect polymers, which are formed by cross-linking of protein units containing tyrosine with polysaccharide units, forming non-amide bonds on hydrolysis of polysaccharide/protein material in the water column. Experiments with salts added to the polysaccharide/protein standards were also conducted in this study. It was demonstrated that the presence of a mineral matrix does not cause the production of any artifacts when analyzing these standards using analytical pyrolysis.

One hypothesis is that the nitrogen-containing compounds detected in the pyrolyzates of UDOM from the Pacific Ocean (chapter 2) are derived from chitin. To this end the decay of shrimps (chapter 8) was studied to test this hypothesis. Solid state ^{13}C-NMR and analytical pyrolysis was used to analyze the shrimps, the decayed shrimps and a shrimp chitin standard. Comparing the pyrolysis products detected in this study to those, encountered in the pyrolyzates of the Pacific Ocean UDOM (chapter 2) led to the conclusion that the nitrogen containing compounds are probably of another origin.

The molecular characterization of UDOM in North Sea water using analytical pyrolysis, ^{13}C-NMR and stable and radiocarbon isotopes is described in chapters 9 and 10. Overall, not much difference in composition of the UDOM from the North Sea was observed. It consisted mainly of refractory organic matter, composed of altered carbohydrate and proteinaceous moieties.

The characterization of the Ems-Dollart UDOM samples along with a number of other fluvial samples by thermochemolysis using tetramethylammonium hydroxide (TMAH) is described in chapter 11. This method is a powerful method to study and release lignin moieties from macromolecular organic matter. Only traces of lignin (2-4% w/w) were detected in the Ems-Dollart UDOM samples. As concluded before, "fresh" lignin may be excluded as a major contributor to UDOM.

SAMENVATTING

Opgelost organisch materiaal (DOM – dissolved organic matter) speelt een belangrijke rol in de koolstofkringloop (hoofdstuk 1). Er is echter niet veel bekend over de moleculaire samenstelling en de oorsprong van DOM. De studie beschreven in dit proefschrift, werd uitgevoerd om meer inzicht te verkrijgen in de moleculaire samenstelling en de oorsprong van DOM.

De moleculaire karakterisering van drie DOM-monsters uit de Grote Oceaan wordt beschreven in hoofdstuk 2. Deze monsters werden verkregen van en waren geanalyseerd door Dr. Ronald Benner. Ze boden de gelegenheid om resultaten te vergelijken en onze analysemethoden te evalueren. De monsters waren geïsoleerd d.m.v. tangentiële-stromingultrafiltratie. Deze monsters vertegenwoordigen de fractie van het DOM met een diameter tussen 0,2 μm en 1 nm (1000 Dalton), welke in dit proefschrift geültrafiltreerd opgelost organisch materiaal (UDOM – ultrafiltered dissolved organic matter) wordt genoemd. Monsters van drie verschillende diepten, die een oppervlaktewatermonster (10 m), een watermonster uit de zuurstofminimumzone (765 m) en een diepzeewatermonster (4000 m) vertegenwoordigden werden geanalyseerd m.b.v. analytische pyrolyse. Specifieke pyrolyseprodukten van eiwitten, lignines en resistente biomacromoleculen waren afwezig in al deze monsters. Een belangrijke hoeveelheid stikstofbevattende verbindingen zijn aangetroffen in het 10-m-monster. De oorsprong van deze stikstofbevattende verbindingen is tot nog toe onbekend, maar mogelijk zijn ze gedeeltelijk afgeleid van aminopolysacchariden, afkomstig van bakteriële lipopolysacchariden en peptidoglycan. De pyrolysaten van alle drie monsters bevatten belangrijke hoeveelheden alkylfenolen met een oorsprong anders dan lignine.

De moleculaire karakterisering m.b.v. analytische pyrolyse van UDOM uit Noordzeewater, gedurende een algenbloei van *Phaeocystis* wordt beschreven in hoofdstuk 3. Enkele belangrijke verschillen werden waargenomen tussen UDOM van het water waarin de algenbloei plaatsvond en UDOM van het water waar geen algenbloei plaats had. Het belangrijkste verschil was de aanwezigheid van relatief grote hoeveelheden onveranderde polysacchariden in het algenbloei-UDOM-monster en de afwezigheid van deze verbindingen in de andere UDOM-monsters.

Een voorstudie aan UDOM uit de Eems-Dollard werd uitgevoerd om de bijdragen van DOM afkomstig van het land en de rivier te onderzoeken (hoofdstuk 4). Er werden vier verschillende UDOM-monsters uit water met een saliniteit, variërend van 0.43 tot 20‰ geïsoleerd. De monsters werden geanalyseerd m.b.v. analytische

pyrolyse, ^{13}C-NMR en stabiele en radioactieve koolstofisotopen. Alle UDOM-monsters uit de monding toonden gelijkende pyrolysaten en ^{14}C-activiteiten. Alleen het ^{13}C-NMR spectrum van het UDOM-monster van het water met de hoogste saliniteit toonde enig verschil met de andere monsters. De stabiele-isotoopwaarden wijzen op menging van rivierwater-DOM met zeewater-DOM van vergelijkbare leeftijden. Ook in het UDOM van de Eems-Dollard werden geen pyrolyseprodukten van onveranderde polysacchariden, lignines, eiwitten en lipiden aangetroffen.

Een hypothese is, dat de stikstofbevattende verbindingen, die in de pyrolysaten van UDOM uit de Grote Oceaan gevonden waren (hoofdstuk 2), afkomstig zijn van chitine. Om deze hypothese te testen werden experimenten met chitinebevattende garnalen uitgevoerd (hoofdsuk 5). Vaste-stof ^{13}C-NMR en analytische pyrolyse werden toegepast om de garnalen, de gedegradeerde garnalen en een chitinestandaard te bestuderen. Vergelijking van de pyrolyseprodukten, die in deze studie werden gevonden, met die, die in de pyrolysaten in het DOM van de Grote Oceaan, aangetroffen waren (hoofdstuk 2), leidde tot de conclusie, dat deze stikstofbevattende verbindingen waarschijnlijk een andere oorsprong hebben.

Om de hypothese te testen, dat polyfenolische bestanddelen, die in DOM en particulair organisch materiaal (POM) aanwezig zijn, afkomstig zijn van algen, werd een aantal macroalgen bestudeerd m.b.v. analytische pyrolyse (hoofdstuk 5 en 6). De algen werden onderworpen aan een aantal chemische-degradatiebehandelingen om de chemische resistentie van de polyfenolische deelstructuren, die aanwezig zijn in de algen, te bestuderen. Er werd aangetoond, dat zowel bruine, groene als rode macroalgen chemisch en mogelijk biologisch zeer resistente macromoleculen bevatten, die gedurende pyrolyse een aantal alkylfenolen produceren. Eén van de conclusies uit deze hoofdstukken is, dat deze van algen afkomstige polyfenolische macromoleculen in belangrijke mate bijdragen aan DOM en POM.

Er werd echter ook aangetoond, dat alkylfenolen in pyrolysaten vele verschillende precursors kunnen hebben (hoofdstuk 7). Een aantal verschillende monsters, die organisch materiaal bevatten, werd bestudeerd. Deze monsters bestonden uit UDOM, POM, sedimenten, polysaccharide/eiwit-standaarden, algen, hydrolyseerbare tanninen, ligninen, bruinkolen, kolen, bodems en insectencuticlae. De monsters werden onderworpen aan verschillende chemische-degradatiebehandelingen. De verdelingspatronen van de alkylfenolen in de pyrolysaten kunnen tot op zekere hoogte gebruikt worden om de verschillende precursors te onderscheiden, te weten: lignines, eiwitten en veranderd eiwit. De conclusie van deze studie was, dat alkylfenolen in pyrolysaten van UDOM, POM en sedimenten polymeren weerspiegelen, die gevormd zijn door cross-linking van tyrosinebevattende eiwiteenheden met polysaccharide-eenheden d.m.v. de vorming van non-amidebindingen als een gevolg van de hydrolyse van eiwit/polysaccharidematerialen in de waterkolom. Ook werden in deze studie

experimenten uitgevoerd, waarbij zouten werden toegevoegd aan de polysaccharide/eiwit-standaarden. Er werd aangetoond, dat de aanwezigheid van een minerale matrix niet van invloed is op de vorming van artifacten gedurende pyrolyse van deze standaarden.

De moleculaire karakterisering van UDOM in Noordzeewater m.b.v. analytische pyrolyse, ^{13}C-NMR en stabiele en radioactieve koolstofisotopen wordt beschreven in hoofdstuk 9 en 10. Er werden weinig verschillen in samenstelling waargenomen in het UDOM van de Noordzee. Het bestond voornamelijk uit refractair organisch materiaal, samengesteld uit koolhydraat- en eiwitachtige deelstructuren.

De karakterisering van de UDOM-monsters van de Eems-Dollardmonsters en nog een aantal andere riviermonsters m.b.v. thermochemolyse, gebruikmakend van tetramethylammoniumhydroxyde (TMAH), wordt beschreven in hoofdstuk 11. Deze methode is een krachtige methode om lignine-eenheden aanwezig in macromoleculair materiaal te bestuderen. Er werden slechts sporen van lignine (2-4 gew.%) in de UDOM-monsters van de Eems-Dollard waargenomen. Zoals al eerder werd geconcludeerd kan een belangrijke bijdrage van "vers" lignine aan UDOM worden uitgesloten.

CHAPTER 1

Introduction

1.1 THE GLOBAL CARBON CYCLE

A number of different types of carbon reservoirs exist on Earth (Hedges, 1992; Figure 1.1). The major carbon reservoir is comprised of sedimentary rocks, both inorganic (~60000 x 10^{18} g C) in the form of carbonates and organic (~15000 x 10^{18} g C) in the form of kerogen, coals, etc. The cycling of carbon in these reservoirs is in the order of geological time scales. Therefore, these carbon reservoirs are of minor importance with respect to the carbon cycling on the short term.

In addition to these sedimentary rocks, there is a number of active (or surface)

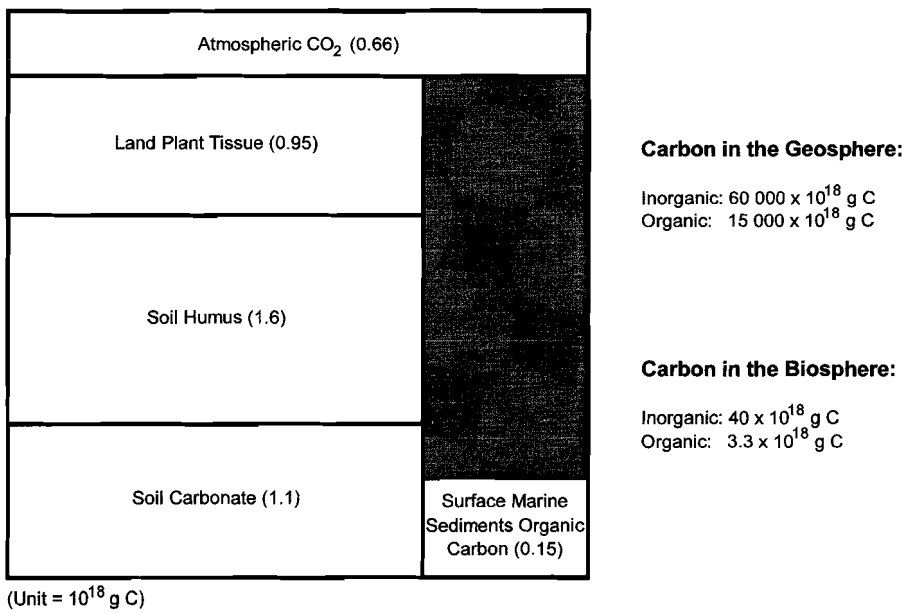

Figure 1.1 Active carbon reservoirs (after Hedges, 1992). N.B. Note that marine inorganic carbon (DIC) is the major reservoir (38 x 10^{18} g C).

carbon pools, which are more dynamic and of more direct importance to the global carbon cycle. These active carbon pools mainly consist of inorganic carbon and some organic carbon. Marine dissolved inorganic carbon (DIC), mainly in the form of carbonate and bicarbonate and relatively minor amounts of CO_2, is the largest of this pool, which holds about 40 x 10^{18} g carbon. The other active inorganic carbon reservoirs are soil carbonate (1.1 x 10^{18} g C) and atmospheric carbon dioxide (0.66 x 10^{18} g C). The organic carbon in these active carbon reservoirs exists both on land as in the marine environment. On land, the major reservoirs are land plants tissue (0.95 x 10^{18} g C) and soil organic matter (or "humus"; 1.6 x 10^{18} g C). In the marine environment, there are two types of active organic carbon reservoirs, *i.e.* surface marine sediment organic carbon (0.15 x 10^{18} g C) and seawater dissolved organic carbon (DOC; 0.60 x 10^{18} g C). The amount of organic matter, present in living marine organisms (0.002 x 10^{18} g C) is negligible compared with that of seawater DOC.

1.2 Dissolved Organic Matter

Water in all it diverse natural environments contains organic matter present in a range of particles with different sizes. Only the particles that are smaller than 1 cm in diameter are considered in this thesis (Figure 1.2). They are dispersed over two major size fractions of organic matter. The size limit of these two fractions is operationally defined as 0.45 μm. Particles that are retained by a 0.45 μm-filter are defined as particulate organic matter (POM) and particles that pass a 0.45 μm-filter are defined as dissolved organic matter (DOM). POM contains suspended particles, large colloids and living organic matter such as bacteria and (micro)algae. DOM however, contains mainly non-living organic matter, like small colloids and truly dissolved molecules. Also viruses are part of DOM. In this thesis, the size limit between POM and DOM has been defined as 0.2 μm. This implies that ultra-microbacteria, which have a diameter between 0.2 and 0.45 μm will be part of POM as are the other bacteria and not of DOM. However, viruses will still be part of DOM.

1.3 Age and Origin of Dissolved Organic Matter in Seawater

Carbon isotope analyses indicate that DOC in the deep ocean is relatively old ($\Delta^{14}C$ = 46% pMC; Williams and Druffel, 1987) and predominantly autochthonous ($\delta^{13}C$ = -21 to -24‰; Williams and Gordon, 1970; Benner *et al.,* 1997). Only extremely low concentrations of terrestrial biomarkers such as lignins could be detected in the humic fraction of DOM from the pelagic Pacific Ocean (Meyers-Schulte and Hedges, 1986;

Opsahl and Benner, 1997). Such results further suggest a primarily marine origin of DOM.

The mean average age of oceanic DOC is more than 1000 years (Kirchman *et al.*, 1991). DOC consists of a labile fraction with a high turnover rate and a refractory fraction. The labile fraction is a possible source of nutrition for the microbial loop (Kirchman *et al.*, 1991; Amon and Benner, 1994, 1996). The turnover rate of marine DOC is highly variable. It varies from less than a day up to more than 1000 years. Williams and Druffel (1987) calculated that in the central part of the North Pacific Ocean upper mixed layer water, 56% of the DOC was less than 30 years old and that the remaining 44% was up to more than 6000 years old. The refractory fraction thus probably consists mainly of constituents, who have been selectively preserved in the water column during diagenesis. Studies by Meyers-Schulte and Hedges (1986) and Williams and Druffel (1988) have shown that marine DOM is comprised mainly of polymeric materials derived from marine sources. Direct molecular-level analyses have provided important information on the concentrations and dynamics of non-polymeric compounds, but these comprise a relatively small fraction (<15%) of DOM (Williams and Druffel, 1988).

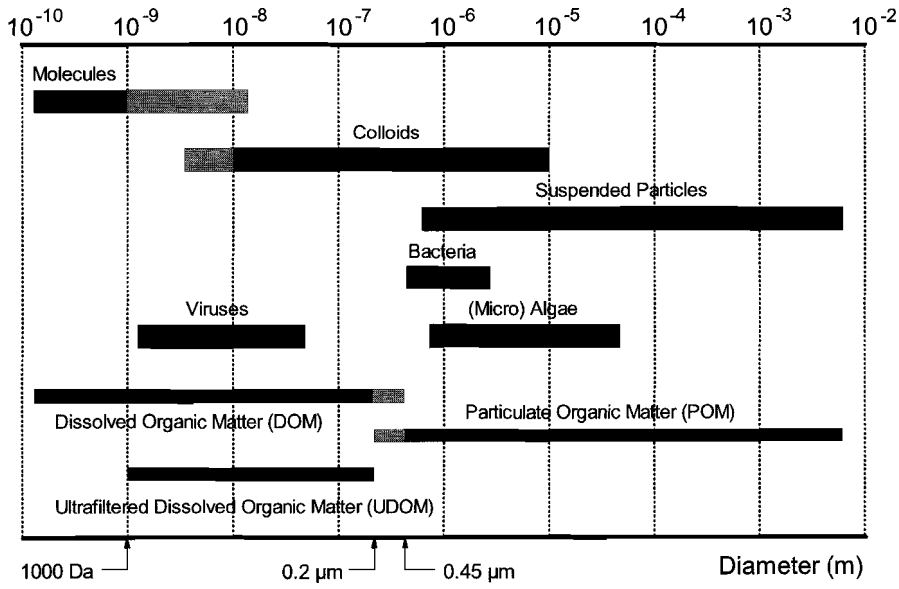

Figure 1.2 Size spectrum of waterborne particles (after Stumm *et al.*, 1979).

Phytoplankton cells excrete numerous different organic compounds that end up in the water column and may coagulate forming large complex compounds. Phytoplankton cells are often grazed by zooplankton, but not always consumed in an efficient way. Because of this so called "sloppy feeding", parts of the cells may become part of POM and even DOM. Furthermore, large particles (fecal pellets) are excreted by zooplankton after feeding on phytoplankton. These particles are a main source of POM. Breakdown or decay of these particles is another important source of the formation of DOM (Libes, 1992). "Marine snow" is a type of detrital material that is amorphous and flocculent. It may be found everywhere in the oceans and serves as a microhabitat for many bacteria, microzooplankton and their detrital products. Marine snow also represents a significant reservoir for numerous inorganic as well as organic compounds, such as proteins, carbohydrates and lipids. Decay of marine snow results in the formation of DOM and POM (Fowler and Knauer, 1986). Furthermore, bacteria may contribute significantly to marine DOM due to the suspected presence of peptidoglycan in DOM (McCarthy *et al.*, 1998).

1.4 Isolation of Dissolved Organic Matter

One of the main problems to characterize DOM on a molecular level is the isolation of DOM. The concentration of DOM is usually expressed in the amount of organic carbon (DOC) per liter. In seawater, the concentration of DOC is typically about 1 mg C/L. Seawater also contains large amounts of salts, typically about 32 g/L. Therefore, simply evaporating water from 1 liter of seawater would yield about 32 g of salt and only 1 mg of organic carbon assuming that DOM is not volatile. The carbon content of the solid sample would therefore be 100% x 0.001 g C / 32 g = 0.0031% C. This is a carbon content that is far too low for any type of molecular analysis to give reliable and significant results.

In the past, researchers have tried to isolate DOM by co-precipitation with metal oxides (especially Fe^{2+}), adsorption onto active carbon and natural solids, adsorption onto synthetic resins (*e.g.* XAD), anion exchange methods (to isolate hydrophobic acids) and reverse osmosis. However, all of these methods either induce artifacts or chemical changes by large changes in *p*H or isolate only very specific fractions of DOM. None of these methods is able to isolate a substantial and/or representative fraction of DOM.

In this thesis, tangential-flow ultrafiltration has been used to isolate DOM from seawater. Benner (1991) and Benner *et al.* (1992) have shown that tangential-flow ultrafiltration is a useful method to recover DOM from seawater. Compounds are concentrated on the basis of molecular size and shape. Ultrafiltration is therefore not

as chemically selective as the XAD isolation method, which concentrates mainly compounds with acidic functionalities. Ultrafiltration is also a much more elegant isolation method that does not require large manipulations of *p*H like the XAD isolation method. Ultrafiltration using a 1000 Dalton cut-off filter has been shown to isolate a much larger fraction (up to ~30%) of seawater DOM (Benner *et al.,* 1992) than conventional DOM isolation methods.

The fraction of DOM isolated from seawater using tangential-flow ultrafiltration will be referred to in this thesis as ultrafiltered dissolved organic matter (UDOM); it represents the fraction of DOM that has a diameter smaller than 0.2 μm and (using 1000 Dalton cut-off filters) larger than 1000 Dalton or approximately 1 nm in diameter (Figure 1.2). This fraction therefore represents the macromolecular fraction of DOM, which thus represents less than 30% of the total DOM.

1.5 Scope and Framework of the Thesis

The objective of the research described in this thesis is to gain more knowledge about the chemical composition of DOM on the molecular level. This knowledge is necessary to determine origin and fate of DOM. Although DOM plays an important role in the global carbon cycle, not much is known about the molecular composition and the origin of DOM.

The following approach was used to achieve this goal:

1) Developing a method to isolate DOM (or UDOM) from (sea)water.
2) Testing and optimalization of the isolation and analysis methods using test samples and parallel studies.
3) Performing analytical pyrolysis studies on standards and known biomacromolecules or appropriate mixtures thereof in order to explain the presence of specific compounds encountered in the pyrolyzates of UDOM samples.
4) Collecting samples from different locations and different types of water and isolating UDOM and analyzing UDOM, mainly by analytical pyrolysis, NMR and carbon isotopes.

1) During the first phase of the research, much effort was put in developing an optimal method to isolate UDOM using cross-flow ultrafiltration. In order to isolate a fraction as large as possible, filters were used with a cut-off of 500 Dalton. However, using the filters with this particulate cut-off, large amounts of salts were retained in the UDOM samples. In fact, the amounts of salts retained by these filters were too much to even be able to analyze any of the organic carbon present in the samples. The amount of salts can

be reduced by diafiltration for a longer period of time with a larger volume of distilled water. However, adjusting the procedure in such a way dramatically reduces the amount of DOC that can be isolated because together with salts also DOC is being removed by the diafiltration procedure. Therefore, filters with a cut-off of 1000 Dalton were chosen to perform following UDOM isolations.

2) Apart from the samples that were collected during the course of the studies described in this thesis, UDOM samples from the Pacific Ocean (chapter 2) and the North Sea (chapter 3) were analyzed that were provided by Dr. Ronald Benner (Marine Science Institute, Port Aransas, Texas, USA). These parallel studies were used to compare results and evaluate UDOM analysis and isolation methods.

The first UDOM samples that were isolated successfully using the method developed in the first phase of the research described in this thesis, originated from the Ems-Dollart estuary (chapter 4). In this pilot study, UDOM from the Ems-Dollart was analyzed using analytical pyrolysis, NMR and carbon isotope analyses.

3) The major part of the studies described in this thesis was focussed on determining the origin and precursors of the pyrolysis products encountered in the pyrolyzates of the UDOM samples (chapters 5-8). To determine the origin of alkylphenols in pyrolyzates of UDOM samples, algal samples (chapters 5 and 6) and protein/polysaccharide standards (chapter 7) were analyzed. After analyzing the algal samples, it was concluded that the precursors of the alkylphenols in pyrolyzates of UDOM are polyphenolic macromolecules of algal origin. However, this conclusion was abandoned after conducting the study on the protein/polysaccharide standards. This study made clear that alkylphenols may be derived from many precursors and that the alkylphenols detected in pyrolyzates of UDOM samples probably were from proteinaceous origin.

To determine the origin of nitrogen-containing pyrolysis products, chitin standards and the decay of chitin containing samples were studied (chapter 8). It could be concluded that the nitrogen-containing pyrolysis products in pyrolyzates of UDOM samples were mainly not derived from chitin, but possibly from proteins and may originate from bacterial lipopolysaccharides.

4) Various UDOM samples were isolated from the North Sea during different cruises (chapters 9 and 10). The North Sea was chosen as a study area for a number of reasons. First of all, the North Sea contains regions of water with different properties of organic matter, varying from a large to a minor terrestrial contribution. Also, the North Sea is a well-studied shelf sea with relatively high DOC concentrations. Finally, the occurrence of algal blooms in parts of the North Sea was a unique opportunity to isolate fresh

UDOM of algal origin, as well as old refractory UDOM. As a result of this, a comparison between labile and refractory UDOM could be made (chapter 3).

The UDOM samples that were isolated were analyzed using analytical pyrolysis, ^{13}C-NMR and stable and radiocarbon isotopes (chapters 4, 9 and 10). However, the focus of these studies was based on analytical pyrolysis.

As an addition to these analyses methods, UDOM samples from the Ems-Dollart were analyzed by thermochemolysis using tetramethylammonium hydroxide (chapter 11) in order to analyze and quantify lignin moieties present in these samples.

CHAPTER 2

Molecular Characterization of Pacific Ocean Dissolved Organic Matter

2.1 INTRODUCTION

Dissolved organic carbon (DOC) in the oceans is one of the major reactive reservoirs of organic matter on Earth (Hedges, 1992). The measurement of marine DOC, its composition and reactivity have been important research topics in oceanography for several decades, but these topics have gained increasing attention in recent years because of international efforts to better understand the global carbon cycle. The study of DOC has been particularly challenging because it occurs in very dilute concentrations (1 mg C/L) in the oceans and is comprised of a complex mixture of organic compounds. The presence of relatively high concentrations (36 g/L) of salts in seawater also complicates the measurement and chemical characterization of marine DOC. Given the importance of marine DOC to the global carbon cycle, we undertook the following study of the chemical composition of DOC to better understand its concentration, source, reactivity and fate in the oceans.

Classical methods for the characterization of marine DOC include measurement of its elemental composition (C, N, P), direct analysis of specific monomers (amino acids, sugars), and spectral analyses (NMR, IR) of material isolated from seawater. There has been renewed controversy over bulk measurements of the total dissolved organic C and N since the introduction of new high temperature combustion methods (Suzuki *et al.,* 1985; Sugimura and Suzuki, 1988). At issue are the estimates of DOC and DON concentrations as well as the bulk atomic ratios of these elements in dissolved organic matter (DOM). Direct molecular-level analyses have provided important information on the concentrations and dynamics of monomers, but these compounds comprise a relatively small fraction (<15%) of the DOM (Williams and Druffel, 1988). Methods for the concentration and isolation of DOM from seawater are needed for more complete characterization of this complex mixture of compounds. The standard method for DOM isolation from seawater, adsorption onto XAD resins, recovers about 5 to 15% of the DOC. Spectral analyses of this material indicate that it is rich in unsubstituted alkyl and carboxyl carbon (Stuermer and Payne, 1976).

In the present study we used a new method, tangential-flow ultrafiltration, for the concentration and isolation of DOM from seawater. This method concentrates

compounds on the basis of molecular size and shape. Ultrafiltration is therefore not chemically selective like the XAD isolation method which concentrates hydrophobic components of the DOM. Ultrafiltration is also a much gentler isolation method that does not require large manipulations of pH like the XAD isolation method. Ultrafiltration (1000 Dalton cut-off filter) has been shown to isolate a much larger fraction (~30%) of seawater DOM, and the isolated DOM is rich in carbohydrates (Benner *et al.,* 1992) To further characterize the ultrafiltered DOM, we analyzed three samples collected from the North Pacific Ocean by pyrolysis-GC-MS.

2.2 RESULTS AND DISCUSSION

Three DOM ocean samples were analyzed by Curie point Pyrolysis-Gas chromatography Mass spectroscopy (Py-GC-MS). The Curie temperature of the ferromagnetic wires used was 358°C and 610°C.

The samples were taken at Aloha Station (22°45.0'N 158°00.0'W) at depths of respectively 10 m, 765 m and 4000 m. A chitin sample (isolated from crab shells, Sigma Chemical Company) was also analyzed.

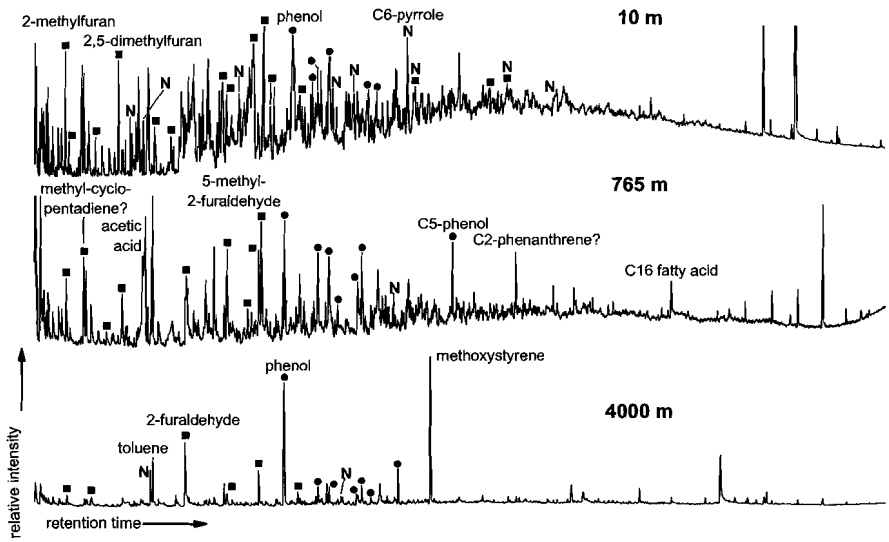

Figure 2.1 Chromatograms of the pyrolyzates of Pacific Ocean DOM samples taken at depth of 10 m, 765 m and 4000 m. The carbohydrate pyrolysis products are indicated by the squares, the alkylphenols by the circles and the nitrogen-containing pyrolysis products are marked with N.

The total ion current traces (TIC) of the pyrolyzates of the DOM samples are shown in Figure 2.1. Inspection of the GC-MS data indicated that specific pyrolysis products of proteins, lignins and resistant aliphatic biomacromolecules were absent from all samples. All pyrolyzates contained furan derivatives originating from partly altered polysaccharides.

Relatively large amounts of alkylphenols are present in the pyrolyzates, especially in the 765 m and 4000 m samples. One possible source of these alkylphenols could be highly degraded lignin. However, no other specific lignin pyrolysis products such as methoxyphenols and dihydroxyphenols are present. 4-Methoxystyrene in the pyrolyzate of the 4000 m sample cannot be ascribed to lignin either. Hence, the precursors of the alkylphenols are presently unknown.

A range of alkylbenzenes (benzene, toluene, C_2-alkylbenzenes) is also present in the pyrolyzates. Their precursors are presently unknown, but they appeared to be real pyrolysis products. They are not found in the pyrolyzate at a Curie temperature of 358°C. As mentioned before, there are no protein pyrolysis products found in the DOM samples. There are, however, significant amounts of nitrogen containing products present in the 10 m sample. These nitrogen compounds have been encountered in some extent in pyrolyzates of chitin, lipopolysaccharides (LPS) and peptidoglycan of bacterial cell walls. Comparison of the nitrogen containing compounds in the pyrolyzate of the 10 m sample with that of the chitin sample isolated from crab shells indicated that only a small part of the nitrogen containing compounds may originate from chitin. The N-containing pyrolysis products of LPS and peptidoglycan (J.J. Boon, unpubl. results) match significantly better with many of those of the DOM sample at 10 m. To evaluate this, a bacterial cell wall sample derived from Pseudomonas sp. Strain RB2256 (ultramicrobacteria) was pyrolyzed under the same conditions. However, only some of the N-containing compounds present in the DOM sample at 10 m were found in this pyrolyzate. An origin from chlorophyll-derived products was excluded based on comparison of the alkylpyrrole distributions in the DOM samples with those in the appropriate standards (Sinninghe Damsté *et al.*, 1992). Hence, additional research is required to unambiguously determine the origin of the N-containing compounds present in the DOM pyrolyzates.

Man-made products, such as fungicides, are also present in the 4000 m sample. Pyrolysis of this sample at a Curie temperature of 358°C revealed that these products are present in the sample and are no pyrolysis products. It is not clear if these products are present in the sea or if they are introduced during the sampling or analysis procedure. To this end "blanks" have been processed, using the same procedure as was used for the seawater samples. For the "blanks" 200 L of "reverse osmosis" water was used. The pyrolyzates of the blanks indicated the presence of a large suite of different compounds.

However, none of the pyrolysis products mentioned before was encountered in these blanks.

2.3 CONCLUSIONS

The method used to isolate DOM from seawater is a relatively good method compared to other methods known; it is not chemically selective and the pH remains constant during the filtration process (Benner *et al.,* 1992). However, the maximum yield is about 30% of the total DOM.

From blank runs it can be concluded that no contaminants are present as main products in the pyrolyzates of the DOM samples. The nitrogen containing pyrolysis products in the 10 m sample may be partly derived from aminopolysaccharides originating from LPS and peptidoglycan.

The origin of the alkylphenols in the 765 m and the 4000 m sample is presently unknown. The origin of the alkylbenzenes in all DOM samples remains unknown as well.

CHAPTER 3

Molecular Characterization of the Macromolecular Fraction of North Sea Dissolved Organic Matter using Pyrolysis-Gas Chromatography/Mass Spectrometry

3.1 ABSTRACT

Ultrafiltered dissolved organic matter (UDOM) isolates from North Sea water was selected for a molecular characterization study. Five samples were isolated from different geographical sites, one sample was isolated from a *Phaeocystis* bloom area. The UDOM in these samples was concentrated using cross-flow ultrafiltration and represented the size fraction between 1000 Dalton and 0.2 μm. The samples were analyzed by analytical pyrolysis for their elemental composition. All samples showed the presence of altered polysaccharides and proteinaceous constituents. One sample contained nitrogen-containing saccharide moieties, which are thought to be derived from peptidoglycan. The UDOM from the algal bloom area also contained unaltered polysaccharides. These results suggest that unaltered polysaccharides are part of the labile dissolved organic matter and altered polysaccharides and proteinaceous moieties are more representative for the refractory dissolved organic matter.

3.2 INTRODUCTION

Marine dissolved organic matter (DOM) is one of the major reactive reservoirs of organic matter on Earth (Hedges, 1992). Given the importance of marine DOM to the global carbon cycle, the aim of the present study is to contribute to a better understanding of the origin and fate of DOM by molecular analysis.

Stable carbon isotope measurements have indicated that dissolved organic carbon (DOC) in the open ocean is predominantly autochthonous ($\delta^{13}C$ = -21 to -24‰; Williams and Gordon, 1970). The recognition of extremely low concentrations of terrestrial biomarkers such as lignins in the humic fraction of DOM from the pelagic Pacific Ocean (Meyers-Schulte and Hedges, 1986) further suggests a marine origin of DOM. Studies by Meyers-Schulte and Hedges (1986) and Williams and Druffel (1988) have shown that marine DOM is comprised mainly of polymeric materials derived from marine sources.

Radio carbon isotope analyses show that DOC in the deep ocean is relatively old ($\Delta^{14}C$ = -540‰; Williams and Druffel, 1987). The average age of DOC is over 1000 years (Kirchman *et al.,* 1991). It is assumed that DOC consists of a labile fraction with a high turnover rate and a refractory fraction. The labile fraction may serve as a source of nutrition for the microbial loop (Kirchman *et al.,* 1991). Williams and Druffel (1987) calculated that in the central North Pacific Ocean upper mixed layer 56% of the DOC was less than 30 years old and that the remaining 44% was up to more than 6000 years old. The refractory fraction thus probably consists mainly of constituents, which have been selectively preserved in the water column. In recent studies of estuarine ultrafiltered DOM (UDOM) by van Heemst *et al.* (2000a) and oceanic colloidal organic matter by Santschi *et al.* (1995), it was suggested that the age (and thus degree of refractivity) of the different size fractions of DOM is reciprocally proportional with its fraction size.

Benner *et al.* (1992) and van Heemst *et al.* (2000a) have shown that tangential-flow ultrafiltration is a successful method used to recover UDOM from seawater and estuarine waters. It concentrates compounds on the basis of molecular size and shape. Ultrafiltration using 1000 Dalton cut-off filters has been shown to isolate a significantly larger fraction (up to ~30%) of seawater DOM (Benner *et al.,* 1992) than conventional DOM isolation methods. The remaining part contains organic matter smaller than 1000 Dalton. Therefore, the isolated ultrafiltered dissolved organic matter (UDOM) is not actually dissolved, but more of a colloidal nature.

As a site for this study the North Sea (a shelf sea) has been selected for a number of reasons: the North Sea is a well studied area, the DOC concentrations are relatively high and one of the samples was also analyzed in a parallel study (McCarthy *et al.,* 1998). UDOM from this site was studied using analytical pyrolysis in combination with gas chromatography/mass spectrometry (Py-GC/MS).

3.3 EXPERIMENTAL

Site and sample description

During a North Sea cruise with the RV *Pelagia* in April 1995, five surface water samples were taken at different sites and processed on-board. Samples 1-3 represent UDOM samples, taken from random locations throughout the North Sea. Sample 4 represented 1000 L of seawater to isolate large amounts of UDOM. This sample was also analyzed in detail in a study by McCarthy *et al.* (1998). Sample 5 was taken from an area during a *Phaeocystis* bloom.

Sample collection and processing

Water samples were collected using an on-board plankton pump equipped with a 63-μm mesh in order to remove large particles. Immediately after sampling, the water samples were prefiltered using an Amicon DC10L ultrafiltration system with a 0.1-μm pore sized polysulfone hollow-fiber filter (H5MP01) to separate DOM from particulate organic matter (POM). The prefiltered samples were subsequently concentrated using an Amicon DC-30 tangential-flow ultrafiltration system equipped with nine Amicon spiral-wound polysulfone 1000 Dalton weight cut-off filters (S10N1). The samples were concentrated to about 2 L and were stored frozen at -20°C for transport to the laboratory. In the laboratory, most salts were removed from the concentrated samples by diafiltration with about 20 L of distilled water. Generally, about 10% of the DOC is lost during diafiltration (Benner, 1991). However, it is essential to remove most of the salts for the analyses by analytical pyrolysis. Subsequently, water was removed by evaporation *in vacuo* at 30°C using a rotary evaporator followed by freeze-drying of the samples.

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS)

Pyrolysis-gas chromatography/mass spectrometric (Py-GC/MS) analyses were performed using a Carlo Erba Mega 500 series gas chromatograph operating in split mode, equipped with a Chemical Data Systems (CDS) pyrolysis interface and a 30 m fused silica capillary column coated with chemically bound DB 1701 (0.25 mm i.d., film thickness 0.25 μm). The interface temperature was held at 230°C. Helium was used as a carrier gas with a flow rate of 10 mL/min at a pressure of 50 kPa. The following oven temperature program was used: initial temperature 35°C (1 min); heating rate 7°C/min; final temperature 290°C (10 min). Samples were weighed and transferred to quartz tubes. Both ends of the tubes were plugged with pre-extracted silica wool. The tubes were placed into a platinum resistance CDS-1000 coil pyroprobe, which heated the samples with a rate of 5°C/ms to an indicated temperature of 610°C. This temperature was maintained for 10 s. The gas chromatograph was connected to a Kratos MS-25 RFA mass spectrometer operated at 70 eV with a mass range of m/z 550-30 and a cycle time of 0.33 s (beam current 150 μA, source temperature 150°C). Data acquisition and analysis were performed using a Dart / Kratos Mach 3 data system. Pyrolysis products were identified based on their mass spectra and GC retention times (van der Kaaden *et al.,* 1984; Pouwels *et al.,* 1989).

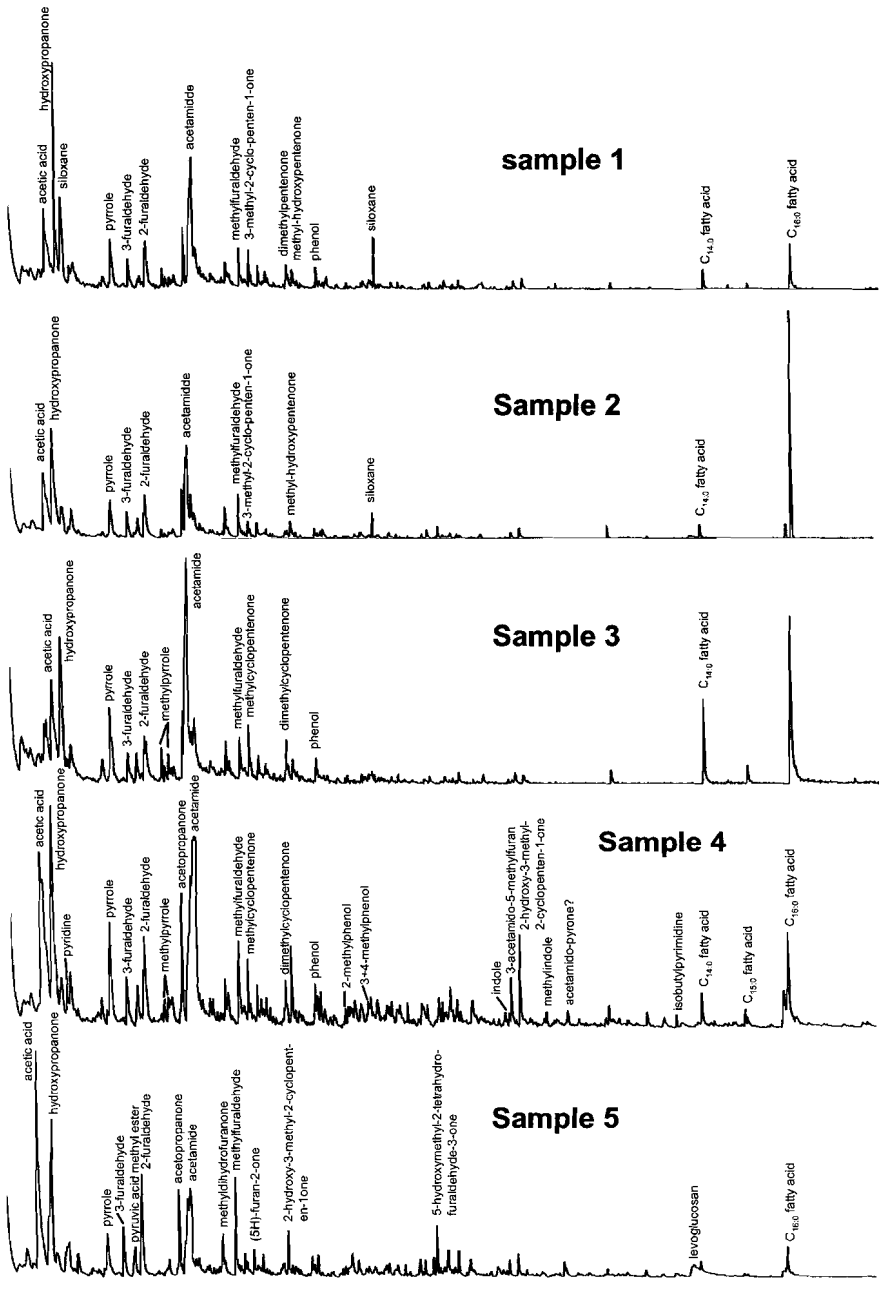

Figure 3.1 Partial Py-GC/MS traces of the North Sea samples.

Determination of C/N ratios

Weighed UDOM samples were combusted using a Carlo Erba 1106 CHN analyzer. Atomic C/N ratios were calculated from the weight percentages of carbon and nitrogen after combustion.

3.4 Results and Discussion

The chromatograms of the pyrolyzates of the five North Sea UDOM samples are shown in Figure 3.1. The presence of acetic acid, furans and furaldehydes in all the pyrolyzates of the North Sea UDOM samples (Figure 3.1) reflects the presence of non-altered or partly altered polysaccharide moieties (Pouwels *et al.,* 1989). Large amounts of acetamide in the pyrolyzates very likely reveals the presence of aminosugar moieties containing N-acetyl units in the UDOM. In samples 3 and 4 acetamide is the major pyrolysis product (Figure 3.1). Also other pyrolysis products of aminosugar moieties, derived from chitin were detected (van der Kaaden *et al.,* 1984; Baas *et al.,* 1995). The aminosugar moieties could therefore be partly derived from chitin. As was mentioned in the experimental section, sample 4 was also analyzed in detail in a parallel study by McCarthy *et al.* (1998). The DOC concentration in the water sample was 0.91 mg C/L (or 76 μM). The concentration of UDOC in this sample was 0.121 mg C/L (or 10.1 μM). The amount of UDOC thus represented 13% of the total DOC. McCarthy *et al.* (1998) concluded that there was a major bacterial contribution to this UDOM sample due to the suspected presence of peptidoglycan. This observation was based on D/L enrichments of four different amino acids (Asp, Glu, Ser and Ala). Bacterial peptidoglycan contains a significant aminosugar component. Therefore, the most likely precursor of the aminosugars encountered in the pyrolyzate of sample 4 is peptidoglycan (McCarthy *et al.,* 1998).

In the pyrolyzate of the *Phaeocystis* bloom sample (sample 5) levoglucosan is encountered (Figure 3.1). This compound is a pyrolysis product of polysaccharidic glucose and it indicates the presence of unaltered polysaccharides in the UDOM sample (Pouwels *et al.,* 1989; Pastorova *et al.,* 1994). These unaltered polysaccharides are probably derived from the algae and may represent algal excretion products. These unaltered polysaccharide macromolecular constituents appear to be part of the labile DOM, since pyrolysis products of these compounds were not encountered in the pyrolyzates of the other UDOM samples (1-4; Figure 3.1).

Furthermore, in the pyrolyzate of all UDOM samples, cyclopentenones were encountered (Figure 3.1). Cyclopentenones are found in relatively small amounts in pyrolyzates of soil organic matter (Saiz-Jimenez and de Leeuw, 1986a) and thus may represent altered polysaccharide precursors. These compounds have also been detected

in relatively larger amounts in the pyrolyzates of estuarine UDOM (van Heemst *et al.*, 2000a).

Aromatic pyrolysis products such as simple phenols are encountered in all pyrolyzates (Figure 3.1). These compounds indicate the presence of aromatic moieties in the macromolecular matrix of the North Sea UDOM. The precursors of phenols, present in pyrolyzates are not always unambiguous (van Heemst *et al.*, 1999). They may be derived from lignins, but lignins (Meuzelaar *et al.*, 1982; Saiz-Jimenez and de Leeuw, 1986b) and even degraded lignins (*e.g.* Saiz-Jimenez and de Leeuw, 1984a) typically yield very characteristic pyrolysis products like dihydrobenzenes and methoxyphenols, that have not been detected in any of the UDOM samples. Therefore, the contribution of lignins to the North Sea UDOM seems to be negligible. It has been indicated by Tsuge and Matsubara (1985) and van Heemst *et al.* (1999) that phenols may very well be derived from tyrosine units in proteins or proteinaceous organic matter. This proteinaceous origin seems to be a valid assumption, based on the additional presence of pyrolysis products like pyrrole, methylpyrrole, indole and methylindole.

Pyrrole and methylpyrrole may be derived from proteins or proteinaceous moieties (Tsuge and Matsubara, 1985; van Heemst *et al.*, 1999), as well as indole and methylindole. The pyrolyzates of all samples contain pyrrole, thus indicating the presence of proteinaceous moieties.

A high relative abundance of various fatty acids, especially C_{14} and C_{16} fatty acids was observed in the pyrolyzates of the UDOM samples. It is likely that these fatty acids are contaminants and represent evaporation products instead of pyrolysis products. Other contaminants, like siloxanes, have also been encountered in the pyrolyzates of the UDOM samples.

All C/N ratios (Table 3.1) of the North Sea UDOM samples are in the range of C/N ratios typical for bulk seawater DOM, *i.e.* between 10 and 25 (Benner *et al.*, 1992). The C/N ratios of the UDOM samples show a similar trend when compared to the pyrolysis results. The C/N ratios of samples 2 and 3 are very similar (18.9 and 18.2 respectively). The C/N ratio of sample 1 is different from the other samples with a value of 16.4. The lowest C/N ratio was determined for sample 4 (14.2). This is in agreement with the detection of the large amount of nitrogen-containing compounds in the pyrolyzate of this sample. The C/N ratio of sample 5 is the highest (21.5). Again, this is in agreement with the pyrolysis results. The pyrolzate of this sample was dominated by pyrolysis products of polysaccharides whereas nitrogen containing products were only detected in relatively low amounts. This further suggests the presence of excreted algal polysaccharides as a result of the *Phaeocystis* bloom.

Table 3.1 C/N ratios of the North Sea UDOM samples.

UDOM sample	*C/N ratio*
1	16.4
2	18.9
3	18.2
4	14.2
5	21.5

3.5 CONCLUSIONS

Polysaccharide moieties contribute to a large extent to the UDOM of the North Sea. A number of these moieties contain nitrogen in the form of N-acetyl units, possibly derived from bacterially derived peptidoglycan.

Unaltered polysaccharides may be part of the labile DOM and they are derived from autochthonous sources, possibly excreted by algae during algal blooms.

Altered polysaccharides and proteinaceous moieties are suspected to be part of the refractory DOM, since they were detected in both the UDOM samples from the *Phaeocystis* bloom area and the remaining North Sea UDOM samples.

3.6 ACKNOWLEDGEMENTS

The authors wish to thank the Netherlands Organisation for Scientific Research (NWO) for providing a studentship to J. D. H. van Heemst and the captain and crew of the RV *Pelagia* for assistance during sampling. Dr. Ronald Benner is acknowledged for donating the UDOM samples and Mike M. Medlin for performing the Py-GC/MS analyses.

CHAPTER 4

Nature, Origin and Average Age of Estuarine Ultrafiltered Dissolved Organic Matter as Determined by Molecular and Carbon Isotope Characterization

4.1 ABSTRACT

The Ems-Dollart estuary (on the border of the Netherlands and Germany) was chosen for a pilot study to characterize ultrafiltered dissolved organic matter (UDOM) in estuarine systems. UDOM samples were taken from four locations with salinities varying from 0.43 to 20‰. The UDOM in these samples was concentrated using cross-flow ultrafiltration and represents the size fraction of DOM between 1000 Dalton and 0.2 μm. The samples were analyzed by analytical pyrolysis, stable carbon isotope and radiocarbon analysis and solid state ^{13}C NMR. All UDOM samples throughout the estuary showed relatively similar pyrolyzates and ^{14}C activities (*c.* 87%). Solid-state ^{13}C NMR spectra were also similar, except for the sample with the highest salinity. UDOM $\delta^{13}C$ values ranged from -27.78 to -25.40‰ with increasing salinity. This suggests mixing of river DOM with seawater DOM of comparable ages. The absence of pyrolysis products of unaltered polysaccharides, lignins, proteins and lipids indicates that the Ems-Dollart estuary UDOM consists of a large fraction of refractory organic matter and a very small fraction of fresh organic matter.

4.2 INTRODUCTION

One of the major reactive reservoirs of organic carbon on Earth is dissolved organic carbon (DOC) in the oceans (Hedges, 1992). Studies on DOC have been important research topics in oceanography for several decades and these topics have gained increasing attention because of efforts to better understand the global carbon cycle. The study of DOC is particularly difficult because DOC occurs in very low concentrations (ca. 1 mg C/L) in the oceans and is comprised of a complex mixture of organic compounds. Given the importance of marine dissolved organic matter (DOM) to the global carbon cycle, we investigated the chemical composition of DOM to better understand its source, reactivity and fate.

The mean average age of oceanic dissolved organic carbon (DOC) is over 1000 years (Kirchman *et al.,* 1991). DOC consists of a labile fraction with a high turnover rate and a refractory fraction. The labile fraction is a possible source of nutrition for the microbial loop (Kirchman *et al.,* 1991; Amon and Benner, 1994, 1996). The turnover rate of marine DOC is highly variable. It varies from less than a day up to more than 1000 years. Williams and Druffel (1987) calculated that in the central North Pacific Ocean upper mixed layer water 56% of the DOC was less than 30 years old and that the remaining 44% was older than 6000 years. The refractory fraction thus probably consists mainly of constituents that have been selectively preserved in the water column. Studies by Meyers-Schulte and Hedges (1986) and Williams and Druffel (1988) have shown that marine DOM is comprised mainly of polymeric materials derived from marine sources. Only very low concentrations of terrestrial biomarkers such as lignins could be detected in the humic fraction of DOM from the pelagic Pacific Ocean (Meyers-Schulte and Hedges, 1986; Opsahl and Benner, 1997). Furthermore, carbon isotope analyses indicated that marine DOC is predominantly autochthonous ($\delta^{13}C$ = -21 to -24‰; Williams and Gordon, 1970; Benner *et al.,* 1997). Such results further suggest a marine origin of DOM. Direct molecular-level analyses have provided important information on the concentrations and dynamics of non-polymeric compounds, but these comprise a relatively small fraction (<15%) of the DOM (Williams and Druffel, 1988).

In estuaries, DOM can originate from a number of autochthonous as well as allochthonous sources (Menzel, 1974; Duce and Duursma, 1977; Handa, 1977; Whittle, 1977). Autochthonous sources are associated with processes such as excretion by organisms living in the estuary, autolysis of dead organisms and microbial decomposition. Allochthonous sources include organic matter from the sea and rivers, marshes and precipitation of low-molecular weight DOM thus forming colloidal DOM. Removal of DOM in an estuary is caused by decomposition, heterotrophic intake by living organisms, transformation of DOM to POM and transport towards the sea (Williams, 1975). Studies concerning the sources of organic matter and processes within the estuaries will provide more insight in the mechanisms that control the global carbon cycle and the oxygen cycle, since 80% of the global organic matter burial and 90% of the global sedimentary mineralization are believed to be concentrated in the coastal zone (Pernetta and Milliman, 1995).

DOC concentrations in estuarine and deltaic waters are generally relatively high. In many cases an estuarine system is characterized by high DOC concentrations in the river water and decreasing DOC concentrations towards the marine end of the estuary. In the Ems-Dollart estuary (Laane, 1980) as well as in other estuaries, like Galveston Bay (Guo and Santschi, 1997), an inverse linear relationship exists between DOC concentration and salinity. Therefore DOC in the Ems-Dollart estuary seems to be

biologically inactive and behaves conservatively during mixing and transport processes. This would suggest that marine DOM contains an important terrestrial component. However, based on stable carbon isotope and lignin contributions, most of the land-derived organic matter discharged by rivers to the marine environment is deposited close to shore (Hedges and Mann, 1979b; Gearing *et al.,* 1977). It is not clear what happens to terrestrial DOM in estuaries. On the one hand, indications exist that a fraction of riverine DOM is removed by flocculation, precipitation and adsorption to particles, thus reducing the impact of riverine DOM to the ocean carbon cycle. On the other hand, experiments have shown that DOM behaves more conservatively (*e.g.* Mantoura and Woodward, 1983), suggesting a more significant contribution of riverine DOM to oceanic DOC (Lee and Wakeham, 1992).

Although data exist on the quantification and composition of suspended organic matter in estuaries (*e.g.* Laane, 1982; Eisma *et al.,* 1991; Qian *et al.,* 1996), molecular composition of colloidal material in estuaries (Sigleo *et al.,* 1982) and bulk chemical characterization of DOC in estuaries (Laane, 1980), no studies concerning the molecular composition of DOM in estuaries have been conducted. Therefore a pilot study was initiated to investigate the molecular composition of DOM in estuaries aiming to determine the contribution of terrestrial DOM to marine DOM in estuaries. The Ems-Dollart estuary has been chosen for this pilot study. It is very well characterized and, compared with other estuaries like those of the Scheldt, Meuse and/or Rhine, it represents a relatively unpolluted area.

The aim of the present study is to determine the origin and fate of organic matter in the Ems-Dollart estuary by means of stable carbon isotope measurements, ^{14}C dating, solid-state ^{13}C-NMR and molecular characterization using analytical pyrolysis.

4.3 EXPERIMENTAL

Site description

The Ems-Dollart estuary is part of the Wadden Sea, a coastal sea separated from the North Sea by a large number of barrier islands. The Wadden Sea is located along the northern coasts of the Netherlands and Germany and the western coast of Denmark.

The salinity at the freshwater end of the estuary is less than or equal to 0.5‰ whereas the salinity at the mouth of the estuary is greater than 35‰. For most of its length, the Ems-Dollart is a fully mixed estuary, both horizontally and vertically (Dorrestein, 1960; Dorrestein and Otto, 1960). Stratification only occurs in parts of the river Ems and in the southern part of the Dollart. Here the estuary is partly mixed. In a one-dimensional model of the Ems estuary Dorrestein and Otto (1960) calculated the

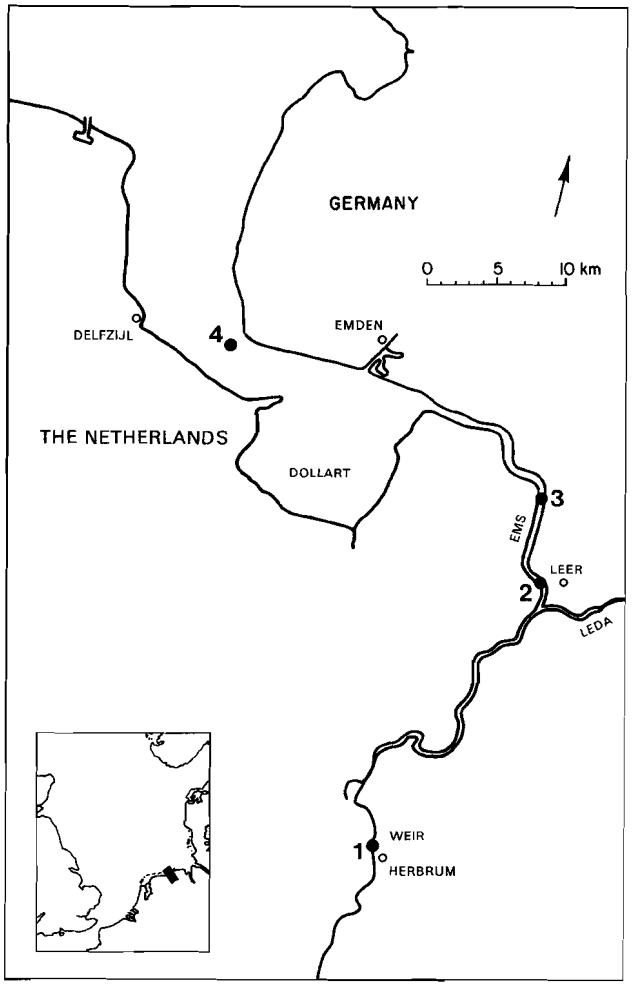

Figure 4.1 Map of the sampling area.

average freshwater flushing time to be 84 tidal periods, assuming no freshwater inflow from the Dollart and a mean freshwater inflow from the Ems of 100 m^3s^{-1}.

Sampling and sample processing

During a cruise with the RV *Navicula* at the end of September 1995, surface water samples were collected using an air-pressure driven membrane pump and a

polyethylene-coated tube at four different locations in the Ems-Dollart estuary (Figure 4.1), representing different water characteristics. Sample 1 was taken from the Ems river upstream from a lock. It represents a pure riverine water sample without any tidal and thus seawater influence. Sample 2 represents a riverine water sample with tidal influence. Sample 3 was taken from a point in the river Ems that was reported by Eisma *et al.* (1991) to be a suspended matter maximum. This point is the boundary between water masses with low salinity and water masses with higher salinity. Finally, sample 4 was taken from the sea end of the estuary, representing river water that has been mixed thoroughly with seawater.

Immediately following sampling, water samples were prefiltered to separate DOM from POM using a Sartocon II Concentrator tangential-flow system equipped with a polypropylene 0.2 μm pore sized Sartorius membrane filter cartridge.

Ultrafiltered dissolved organic matter (UDOM) was obtained by concentrating these prefiltered samples using a custom-made Amicon SP-60 tangential-flow ultrafiltration system equipped with two Amicon S10N1 spiral-wound polysulfone 1000 Dalton cut-off filters. Tangential-flow ultrafiltration is a relatively new method that has been applied successfully to concentrate a fraction of DOM from seawater (Benner, 1991; Benner *et al,* 1992). The samples were concentrated to about 2 L and were stored frozen at -20°C for transport to the laboratory. In the laboratory, most salts were removed from the concentrated samples by diafiltration with 10 volumes (*i.e.* 20 L) of distilled water. Although generally about 10% of the DOC is lost during diafiltration (Benner, 1991), it is essential to remove most of the salts before the analyses by analytical pyrolysis. Subsequently, water was removed by evaporation *in vacuo* at 30°C using a rotary evaporator followed by freeze-drying of the samples.

Solid-state ^{13}C NMR

Dried samples of UDOM were subjected to solid-state ^{13}C NMR in a 9-mm diameter ceramic rotor packed with Teflon tape as filler. The small amount of material, generally 50 mg dry weight per sample (TOC of only 7 to 19%; Table 4.1), necessitated such an approach to optimize the weak signals. The rotor was spun at 3.5 kHz at the magic angle (54.7°) in the probe of a Chemagnetics, Inc., M-100 spectrometer operating at 25.2 MHz for carbon. The standard cross-polarization pulse sequence with 1 ms contact time and 0.7 s cycle time was used and approximately 10^5 acquisitions were accumulated to obtain the spectra. Exactly 512 data points were acquired and zero-filled to 4096 data points prior to filtering with 75 Hz linebroadening and Fourier transformation. The chemical shifts were referenced externally to hexamethylbenzene.

Table 4.1 Hydrographic data.

sample	*salinity*	*sample volume*	*dry weight UDOM sample*	C_{org} *UDOM sample*	*ultrafiltered [DOC]*
	(‰)	*(L)*	*(mg)*	*(%)*	*(µmol/L)*
1	0.43	50	316	15.3±1	80.6
2	1.5	20	288	10.6±1	127
3	2.1	25	270	19.3±1	174
4	20	50	356	7.1±1	42.1

Determination of C_{org}, $\delta^{13}C$ values and ^{14}a values (AMS)

Dried samples of UDOM were acidified with dilute hydrochloric acid to remove carbonates, dried over KOH and placed in quartz tubes together with oxidized copper wire. The tubes were evacuated and sealed and placed in a furnace at 800°C for 6 hours. The combustion gases were led over a copper oven at 600°C and a silver oven at 400°C to remove nitrogen oxide, sulfur oxides and halogens. Carbon dioxide was trapped in liquid air. The amount of CO_2 was determined by expanding the gas into a known volume and measuring its pressure.

$\delta^{13}C$ Values were determined with a VG SIRA 9 isotope ratio mass spectrometer. Subsequently the carbon dioxide was reduced to graphite with hydrogen in the presence of an iron catalyst at 600°C. The resulting graphite/catalyst mixture was pressed into a target for the Tandem-Accelerator Mass Spectrometer (AMS; HVEE BV, Amersfoort; Aerts-Bijma *et al.*, 1997; Wijma and van der Plicht, 1997).

From the measured ^{14}C:^{12}C ratio (^{14}R) of the sample and a known standard the ^{14}a value, expressed as percent of modern carbon (pMC), is calculated as: $^{14}a = (^{14}R_{sample}/^{14}R_{standard}) * \{(1- 25\text{ ‰})/(1 + \delta^{13}C)\}^2 * 100$ pMC. The standard to which ^{14}C data are referred to is oxalic acid from 1950. According to the official definition by Stuiver and Pollach (1977) a correction has to be made for the time of measurement after 1950, but the effect of this is negligible. The ^{14}a value is related to the age of the sample, since due to radioactive decay ($T_{1/2}$ = 5730), ^{14}C is disappearing from the sample during time. Because the ^{14}C concentration is also affected by isotopic fractionation, a correction for this is made with the factor $(1-25‰)/(1+\delta^{13}C)$.

When comparing marine and terrestrial samples, samples with the same age do not have the same ^{14}a value because the carbon source for organisms in the ocean usually has a lower ^{14}C:^{12}C ratio than CO_2 in the atmosphere, due to the so called reservoir effect. Therefore, and because the measured samples are probably mixtures of components with different ages, ^{14}a values are reported in addition to the average

ages, which are merely reported in order to compare the ^{14}C isotope data presented in this study with the existing oceanographic literature.

Curie-point pyrolysis-gas chromatography (CuPy-GC)

Curie-point pyrolysis-gas chromatography (CuPy-GC) analyses were performed using a Hewlett-Packard 5890 gas chromatograph, equipped with a FOM-4LX pyrolysis unit (Boon *et al.,* 1987) and a cryogenic unit. A 30 m fused silica capillary column coated with chemically bound DB 1701 (0.25 mm i.d., film thickness 0.25 μm) was used for all samples. Some selected samples were also studied using a 25 m fused silica capillary column coated with chemically bound CP Sil-5 (0.32 mm i.d., film thickness 0.45 μm). Helium was used as a carrier gas. A flame ionization detector (FID) at 320 °C was used for detection. The GC was temperature programmed as follows: initial temperature 0°C (5 min); heating rate 3°C/min; final temperature 300°C (10 min). Samples were pressed onto flattened ferromagnetic wires (Curie temperatures of 610°C) and placed into the pyrolysis unit. The pyrolysis unit was connected to a FOM high frequency generator that heated the wires inductively in 0.15 s to the Curie temperature. This temperature was maintained for 9 s.

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC/MS)

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC-MS) analyses were carried out in the same way as the CuPy-GC analyses. A Hewlett-Packard 5890 gas chromatograph was connected to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of *m/z* 700-35 and a cycle time of 1 s.

4.4 Results and Discussion

DOM from the Ems-Dollart estuary was concentrated and isolated using tangential-flow ultrafiltration. Benner *et al.* (1992) have shown that tangential-flow ultrafiltration is a successful method to recover a substantial fraction of DOM from seawater. It concentrates compounds on the basis of molecular size and shape. Ultrafiltration is therefore not as chemically selective as the XAD isolation method which concentrates mainly hydrophobic components of DOM. Ultrafiltration is also a much gentler isolation method that does not require large manipulations of pH like the XAD isolation method. Ultrafiltration (1000 Dalton cut-off filter) has been shown to isolate a much larger fraction (up to ~30%) of seawater DOM (Benner *et al.,* 1992) than conventional DOM isolation methods. The 70% that could not be isolated contains

particles smaller than 1000 Dalton. Therefore, the isolated UDOM is not actually dissolved, but more of colloidal nature. In Table 4.1, dry weights of the recovered Ems-Dollart UDOM samples are listed together with the ultrafiltered DOC concentrations calculated from the percentages of organic carbon present in the UDOM samples. No DOC concentrations were measured in this study, so no estimates were made for the recovery of the UDOM. It should be realized, however, that the data obtained do not represent the total size spectrum of the DOM, but the size fraction of DOM ranging from 1000 Dalton to 0.2 μm. From the study by Santschi *et al.* (1995) it has become clear that this fraction may be relatively less refractory than the smaller DOM size fraction.

From Table 4.1, it may be observed that the concentration of ultrafiltered DOC (UDOC) increases with increasing salinity up to the suspended matter maximum (sample point 3). After the suspended matter maximum, the concentration of UDOC decreases rapidly. This may be explained by coagulation of low-molecular weight (LMW) DOM to high-molecular weight (HMW) DOM and particulate organic matter (POM) across the salinity gradient and dilution of UDOM with seawater and seawater UDOM after this point.

Solid-state ^{13}C NMR data

The solid-state ^{13}C NMR spectra shown in Figure 4.2 for three samples (the spectrum of sample 3 was nearly identical to that of sample 2) show a remarkable degree of similarity. Although the poor signal-to-noise ratio precludes more precise comparisons and reporting of integrated intensities, the three spectra appear to be composed mainly of aliphatic carbons (0-100 ppm; Table 4.2). The next most intense signals are those assigned to carboxyl/amide carbons at 175 ppm. The generally high intensity for these signals is indicative of the fact that the UDOM is composed of either highly carboxylated materials or that amide functionalities such as found in proteinaceous materials are important. Aromatic carbons are represented between 100 and 160 ppm, and their intensity is generally greater than would be observed in oceanic UDOM (Benner *et al.,* 1992) and similar to what is expected for riverine DOM (Hedges *et al.,* 1992; Malcolm, 1990). In the aliphatic region, a significant signal is observed at about 72 ppm, attributable to hydroxylated aliphatic carbons as would be found in carbohydrates or alcohols.

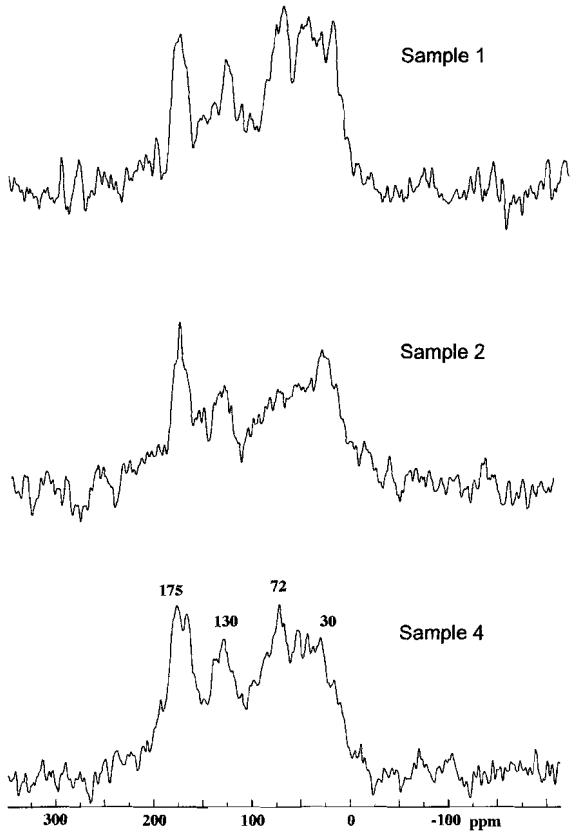

Figure 4.2 Solid-state ^{13}C NMR spectra.

Table 4.2 Relative intensities of the various NMR regions for UDOM samples.

UDOM sample	*aldehyde/ketone (190-220 ppm) (%)*	*carboxyl/amide (160-190 ppm) (%)*	*aromatic (100-160 ppm) (%)*	*alkoxyl (60-110 ppm) (%)*	*paraffinic (0-60 ppm) (%)*
1	1	19	18	23	38
2	4	19	20	23	34
3	2	19	20	24	35
4	4	21	19	27	30

Carbon isotope data

The $\delta^{13}C$ and ^{14}C data of the UDOM samples are shown in Table 4.3. Sample 1 has been taken from a location in the river Ems without any tidal influence. Therefore, the UDOM in this sample is assumed to consist exclusively of terrestrial UDOM. It may therefore be regarded as a terrestrial end member, its $\delta^{13}C$ value (-27.8‰) thus represents the value of terrestrial UDOM present in the Ems-Dollart estuary. The $\delta^{13}C$ values show that from the most upstream station to the turbidity maximum (station 3), there is no significant increase in $\delta^{13}C$ values. This implies that there is almost no mixing of riverine UDOM with marine UDOM. A clear marine contribution is seen in the UDOM from station 4 ($\delta^{13}C$ = –25.4‰). The $\delta^{13}C$ value of UDOM from this station indicates mixing of fluvial UDOM with *c.* 45% marine UDOM, assuming that North Sea UDOM has a $\delta^{13}C$ value of -22 to -23‰ (le Clercq *et al.,* 1997; chapter 9). These results are in agreement with those of Eisma *et al.* (1991), who also found an increase in $\delta^{13}C$ values of suspended organic matter with increasing salinity, indicating mixing of river organic matter with North Sea organic matter. This kind of conservative behavior of UDOM does not mean that is the case in all estuaries. Guo *et al.* (1996) measured $\delta^{13}C$ values of UDOM isolated from the surface waters from the Middle Atlantic Bight of Cape Hatteras. They determined $\delta^{13}C$ values of –24.9‰, -26.6‰, -30.8‰, -30.1‰, -26.8‰, -27.8‰ and -22.9‰ for the UDOM samples collected from water with salinities of 5.0‰, 9.1‰, 10.4‰, 15.0‰, 18.2‰, 25.0‰ and 35.28‰ respectively. In contrast with the present study, the $\delta^{13}C$ values that were determined by Guo *et al.* (1996) seem to indicate a non-conservative behavior of UDOM in the Middle Atlantic Bight.

Samples 1 to 3 also show no change in their ^{14}a values (87 and 86% pMC; Table 4.3). Furthermore, the ^{14}C activity in the fluvial UDOM (sample 1) is equal to that of dissolved inorganic carbon (DIC) at this station, at first sight suggesting that it is recently formed material by photosynthetic assimilation of DIC. However, these equal ^{14}a values may be better explained by assuming that the UDOM consists of old organic material giving accidentally the same ^{14}a value as the DIC. The downstream UDOM sample has a ^{14}a value of 87% pMC, equal to the upstream samples. Thus, the marine UDOM component must have a very similar age as the riverine component (around 87% pMC), since no change in ^{14}a is observed. Although a significant change in $^{14}a_{inorg}$ is observed throughout the estuary, the $^{14}a_{org}$ shows relatively similar values. This means that no relationship exists between the ^{14}C of the organic and the inorganic matter. This implies the refractory nature of the UDOM present in the Ems-Dollart estuary.

Table 4.3 UDOM sample isotope data.

UDOM sample	$\delta^{13}C_{org}$	$\delta^{13}C_{inorg}$
	(‰)	*(‰)*
1	-27.78±0.01	-11.40±0.02
2	-27.15±0.02	n.d.[1]
3	-27.12±0.02	-10.23±0.02
4	-25.40±0.02	-7.43±0.02

[1] n.d. = not determined

UDOM sample	$^{14}a_{org}$	$\Delta^{14}C_{org}$	$^{14}C_{org}$ *age*	$^{14}a_{inorg}$	$\Delta^{14}C_{inorg}$	$^{14}Ci_{norg}$ *age*
	(%pMC)	*(‰)*	*(yr BP)*	*(%pMC)*	*(‰)*	*(yr BP)*
1	87±1	-130±10	1080±100	87±1	-130±10	1080±100
2	87±1	-130±10	1080±100	n.d.[1]	--	--
3	86±1	-140±10	1200±100	90±1	-100±10	850±100
4	87±1	-130±10	1080±100	108±1	80±10	>modern

[1] n.d. = not determined

CuPy-GC-MS results

Chromatograms of the pyrolyzates of the Ems-Dollart UDOM samples are shown in Figure 4.3. These pyrolyzates are dominated by alkylphenols, alkylbenzenes, alkylpyrroles and pyrolysis products of carbohydrates (alkylcyclopentenones, alkylcyclopentanones, alkylfurans and alkylcyclopentadienes). Unaltered polysaccharides yield vast amounts of acetic acid, alkylfurans, alkylfuraldehydes and deanhydromonosaccharides like levoglucosenone, 1,4:3,6-dianhydro-α-D-glucopyranose and levoglucosan (Pouwels *et al.,* 1989; Pastorova *et al.,* 1994) upon pyrolysis. None of these compounds except for small amounts of furans were encountered in any of the pyrolyzates of the Ems-Dollart UDOM, thus no unaltered polysaccharides were present in detectable amounts. The presence of relatively large amounts of salts in the UDOM samples might have influenced the formation of specific pyrolysis products, like the deanhydromonosaccharides. However, laboratory experiments involving addition of salt to a mixture of albumin bovine serum and starch still yielded these deanhydromonosaccharides upon pyrolysis of the mixture (van Heemst *et al.,* 1999).

Aliphatic amino acids in peptide and protein material show very characteristic pyrolysis products which can be monitored by mass chromatography of masses *m/z* 195+209 (Boon and De Leeuw, 1987). These pyrolysis products are indicative for the presence of intact proteins. None of these compounds were encountered in the pyrolyzates of the Ems-Dollart samples, therefore the contribution of fresh proteins to the samples was negligible.

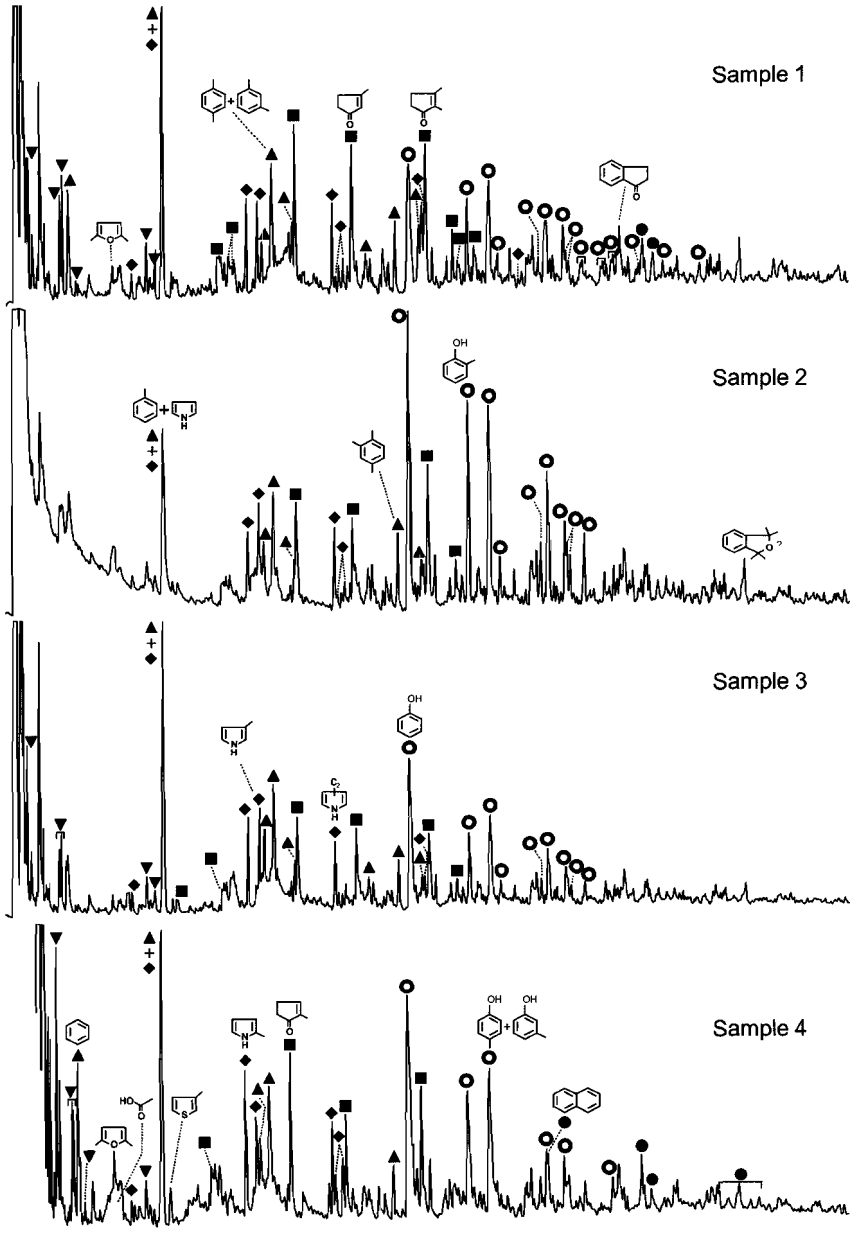

Figure 4.3 Chromatograms of the pyrolyzates (610°C) of the UDOM samples from the Ems-Dollart estuary. Key: ▼ = alkylcyclopentenes; ▲ = alkylbenzenes; ◆ = alkylpyrroles; ■ = alkylcyclopentanones and alkylcyclopentenones; O = alkylphenols.

No large amounts of lipids like fatty acids, phytadienes and sterols were encountered in any of the UDOM pyrolyzates. Overall, no major differences between the different chromatograms of the pyrolyzates of the samples are observed. These data correspond to earlier findings by Duursma (1961) and Laane (1980) that DOM behaves conservatively during transport and mixing in the Ems-Dollart estuary and that it is biochemically inert. Furthermore, Sigleo *et al.* (1982) concluded that the molecular composition of estuarine colloidal matter was very similar throughout the estuary. As was already concluded from the carbon isotope data, the pyrolysis data seem to imply as well that the Ems-Dollart UDOM appears to be refractory in nature, because of the absence of unaltered polysaccharides, proteins and lipids.

Mass chromatography was used to monitor specific chemical classes of compounds, like alkylphenols (Figure 4.4), pyrolysis products of (partly) altered polysaccharides (Figure 4.5) and alkylpyrroles (Figure 4.5). The origin and geochemical importance of the different compound classes is discussed in the following paragraphs.

Alkylphenols

Mass chromatograms reflecting C_0-C_2 alkylphenols in the pyrolyzates of all four Ems-Dollart UDOM samples are shown in Figure 4.4. The samples show very similar alkylphenol distribution patterns. These patterns are very similar to those of alkylphenols in the pyrolyzates of oceanic UDOM like the Pacific Ocean UDOM (van Heemst *et al.,* 1993; 1996), suggesting similar precursors of algal, and thus non-lignin, origin. Alkylphenols in pyrolyzates are generally formed upon pyrolysis of lignins (Meuzelaar *et al.,* 1982; Saiz-Jimenez and de Leeuw, 1986b) or degraded lignins (*e.g.* Saiz-Jimenez and de Leeuw, 1984a). In pyrolyzates of lignins and degraded lignins, methoxyalkylphenols and dihydroxyalkylbenzenes are encountered in addition to alkylphenols. However, in the pyrolyzates of the UDOM samples, neither methoxyalkylphenols nor dihydroxyalkylbenzenes were observed. No specific lignin pyrolysis products were encountered in the pyrolyzates of the Ems-Dollart estuarine samples, despite the relatively large or exclusive contribution of terrestrial organic matter brought in by the river Ems. Thermochemolysis results using tetramethylammonium hydroxide on these samples by van Heemst *et al.* (2000b) reveal the presence of only traces of contributions from lignin, mostly the oxidized lignin forms (weight percentages of approximately 2-4%). Fresh lignin can thus be excluded as major precursor of the alkylphenols present in the UDOM pyrolyzates studied.

It has been shown that in addition to lignins, polyphenolic type macromolecules rich in tyrosine moieties resulting from degradation of proteins also produce alkylphenols upon pyrolysis (van Heemst *et al.,* 1999). An origin from cross-linked proteins or proteins/polysaccharides seems to be the best option, since the findings by van Heemst *et al.* (1999). On the basis of the presence of these non-lignin phenols in the pyrolyzates

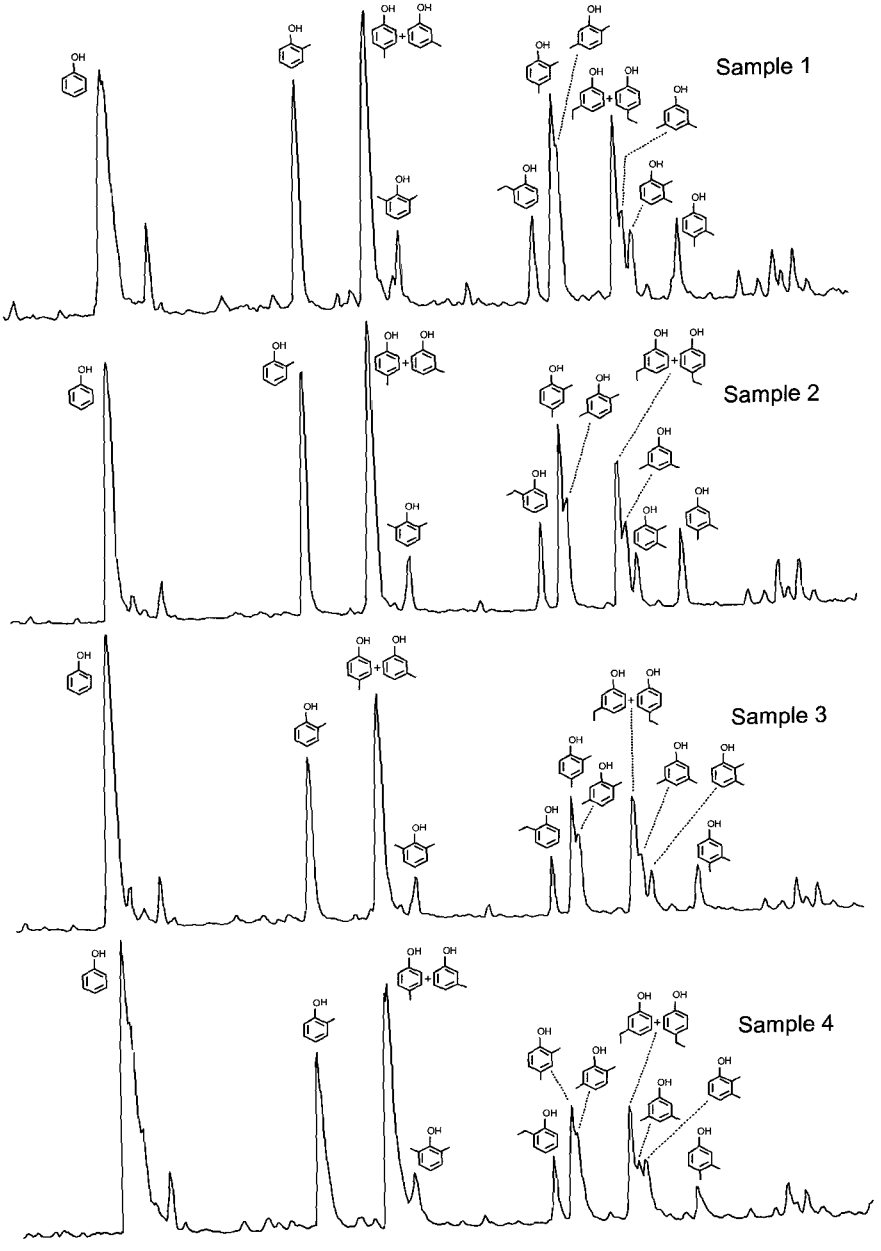

Figure 4.4 Mass chromatograms (*m/z* 94+107+108+121+122) of C_0-C_2 alkylphenols.

of the samples, it may be concluded that the UDOM from the Ems-Dollart is old and transformed. However, it is difficult to determine a terrestrial or marine origin, only on the basis of the pyrolysis results, because the precursors (polysaccharides, proteins, etc.) are present in organic matter both on land and in the sea. The precursors of the alkylphenols may thus represent heavily degraded proteins and/or lignins originating from peat and soil organic matter in the hinterland.

Alkylpyrroles

The mass chromatograms shown in Figure 4.5 reveal distribution patterns of C_0-C_2 alkylpyrroles in the Ems-Dollart UDOM pyrolyzates. The precursors of the alkylpyrroles are yet unknown nitrogen containing compounds. These compounds are not likely to be derived from the degradation products of pigment molecules produced by bacteria and plants (*e.g.* chlorophyll-a) based on comparison of the alkylpyrrole distributions in pyrolyzates of the UDOM samples with those in the pyrolyzates of appropriate standards (Sinninghe Damsté *et al.,* 1992). Nitrogen-containing compounds are encountered in pyrolyzates of a number of macromolecules, like chitin, proteins and macromolecules present in bacterial cell walls. A specific pyrolysis product of chitin is 2-(N-acetylamino)-levoglucosan (van der Kaaden *et al.,* 1984; Baas *et al.,* 1995). This compound was not encountered in the pyrolyzates of the UDOM samples. Therefore, chitin was excluded as precursor for the nitrogen-containing compounds encountered in the Ems-Dollart UDOM samples. No fresh proteins are present in the Ems-Dollart UDOM samples because of the absence of the specific protein pyrolysis products mentioned before. However, in pyrolyzates of albumin bovine serum, pyrrole and methyl-pyrroles have been encountered (van Heemst *et al.,* 1999). It is unlikely that proteins have survived serious biological degradation, but some moieties present in proteins could have been preserved.

Alkylcyclopentanones

In the pyrolyzates of all Ems-Dollart UDOM samples alkylcyclopentanones are encountered as important pyrolysis products (Figs. 3 and 5). The nature of the precursor of these compounds is not completely understood. Alkylcyclopentenones have been encountered in pyrolyzates of soil organic matter (Saiz-Jimenez and de Leeuw, 1984b and 1986a). They have also been encountered in pyrolyzates of amylose (van der Kaaden *et al.,* 1983) and as burned sugar aroma components (Mills and Hodge, 1976). It is believed that the precursors of the alkylcyclopentanones in the UDOM samples are related to refractory organic matter, possibly of polysaccharide origin.

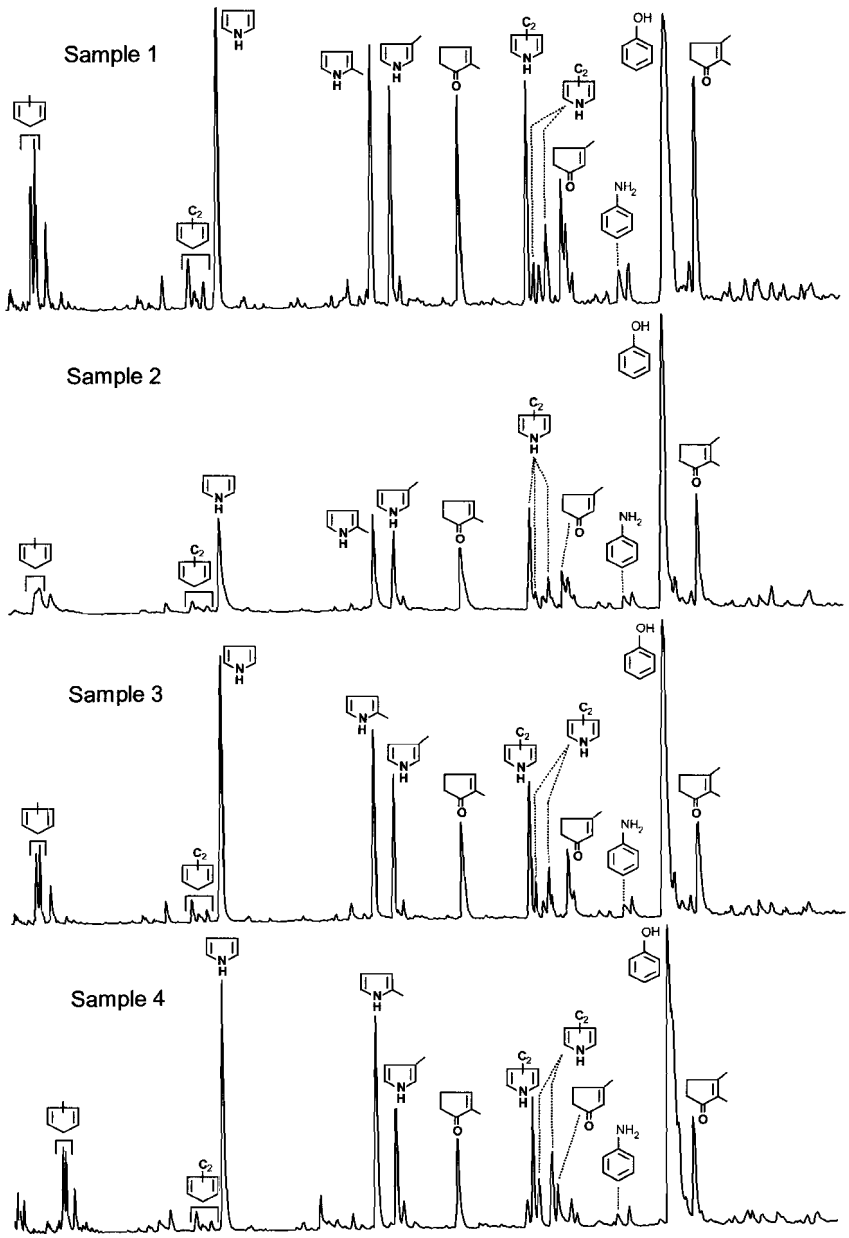

Figure 4.5 Mass chromatograms (*m/z* 67+80+94) of alkylcyclopentenones, alkylpyrroles and alkylcyclopentenes.

Alkylbenzenes

All pyrolyzates contain relatively large amounts of alkylbenzenes. The mass chromatograms shown in Figure 4.6 reveal distribution patterns of C_0-C_3 alkylbenzenes in the pyrolyzates of the Ems-Dollart UDOM samples. All chromatograms of the pyrolyzates show very similar distribution patterns of the C_1 to C_3 alkylbenzenes, suggesting similar precursors. The exact precursors of the alkylbenzenes are unknown, but they might be related to aromatic moieties present in the macromolecular structure of organic matter (Hartgers *et al.,* 1994). These compounds have been reported to occur in other pyrolyzates of UDOM and POM samples, *e.g.* in pyrolyzates of UDOM from the Pacific Ocean (van Heemst *et al.,* 1993), of sediment trap material from the Mediterranean (Peulvé *et al.,* 1996a) and of organic matter present in algae (van Heemst *et al.,* 1996). In ocean water UDOM, and thus probably also in Ems-Dollart UDOM, the precursor of the alkylbenzenes is part of the refractory UDOM. This is based on increasing relative amounts of the alkylbenzenes in pyrolyzates of UDOM samples of increasing depth (van Heemst *et al.,* 1993), suggesting selective preservation of the precursors of these alkylbenzenes.

4.5 CONCLUSIONS

The chemical composition of UDOM (fraction of 1000 Dalton to 0.2 μm) from the Ems-Dollart estuary does not change significantly throughout the estuary. However, an increase in $\delta^{13}C$ values of UDOM is observed with increasing salinity, suggesting mixing of river UDOM with seawater UDOM of similar molecular composition in the estuary. This change in $\delta^{13}C$ is not paralleled by a change in the ^{14}a values of the UDOM samples. The ^{14}C activities are the same for all four stations, probably because the UDOM from the North Sea has the same ^{14}C activity.

UDOM from the Ems-Dollart estuary contains aromatic moieties of unknown origin and moieties derived from altered polysaccharides and proteins.

The negligible contribution of unaltered polysaccharides, proteins and lipids emphasizes the refractory nature of UDOM from the Ems-Dollart estuary.

This refractory nature may be due to cross-linking reactions between proteins and polysaccharides, resulting in the formation of refractory organic matter in marine and terrestrial (soil) organic matter.

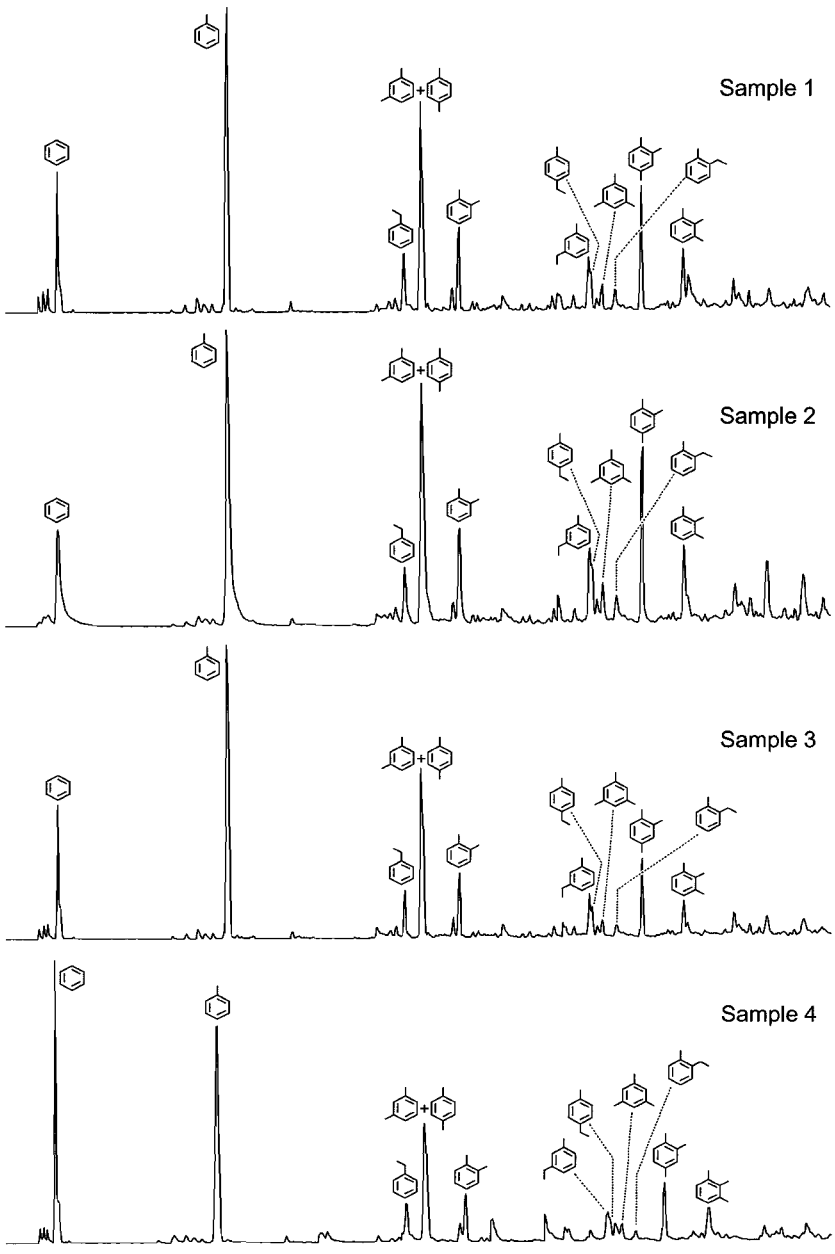

Figure 4.6 Mass chromatograms (*m/z* 78+92+106+120) of C_0-C_3 alkylbenzenes.

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5

Algal Polyphenolic Resistant Macromolecules in Marine Dissolved and Particulate Organic Matter

5.1 INTRODUCTION

In analytical pyrolysis studies of dissolved organic matter (DOM, van Heemst *et al.,* 1993) and particulate organic matter (POM, Peulvé *et al.,* 1996a) alkylphenols were encountered in relatively large amounts in the pyrolyzates, particularly in the deeper water samples. In the pyrolyzate of a DOM sample of 4000-m depth from the Pacific Ocean, alkylphenols are the most abundant pyrolysis products. This suggests that these alkylphenols in the DOM pyrolyzates are related to a highly resistant polyphenolic type precursor, because it seems to be selectively preserved with increasing depth. Similar distributions of these alkylphenols, recently encountered in pyrolyzates of sediment-trap samples from the Mediterranean further indicated the presence of such precursors in POM (Peulvé *et al.,* 1995).

Alkylphenols in pyrolyzates are generally formed upon pyrolysis of lignins (Meuzelaar *et al.,* 1982; Saiz-Jimenez and de Leeuw, 1986b). In pyrolyzates of lignins, methoxyalkylphenols and dihydroxyalkylbenzenes are always encountered in addition to alkylphenols. However, in the studies by van Heemst *et al.* (1993) and Peulvé *et al.* (1995) neither methoxyalkylphenols nor dihydroxyalkylbenzenes were observed. Therefore lignin, and hence terrestrial input was excluded as a contributor to the DOM and POM samples studied.

Brown algae are known to produce polyphenolic polymers (phlorotannins) up to 10% of their dry weight as external metabolites for the chelation of metal ions (Ragan and Glombitza, 1986) and as defense chemicals against grazing herbivores (Boettcher and Targett, 1993). Therefore, a number of brown algae, but also a green, a red alga and a microalga were subjected to analytical pyrolysis to validate the origin of the alkylphenols in DOM and POM.

5.2 RESULTS AND DISCUSSION

The brown macroalgae *Nereocystis luetkeana, Fucus gardneri, Costaria costata, Sargassum muticum,* the green macroalga *Ulva fenestrata* and the red macroalga *Opuntiella californica* were received from Dr. M. A. Goñi (1992) and were collected at

Eagle Point (Washington State, USA), except for the *Sargassum muticum,* which was taken from a continuous-flow holding tank near Eagle Point. The microalga *Rhodomonas sp.* was grown in a culture. Figure 5.1 shows the processing scheme for the algae.

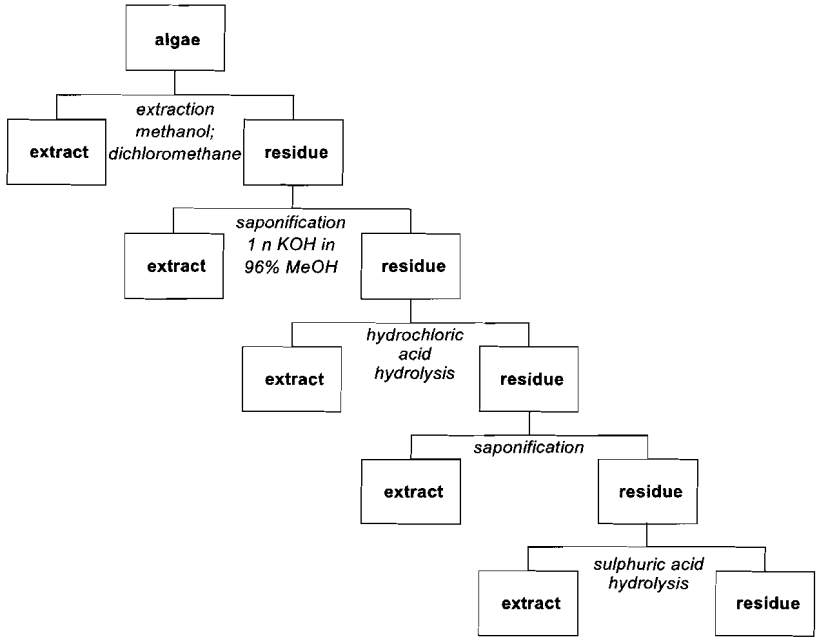

Figure 5.1 Algae processing scheme.

In order to remove lipids and ester-bound moieties, the algae were saponified. In the case of *Nereocystis luetkeana* and *Ulva fenestrata* proteins were removed from the residue by treatment with hydrochloric acid. The carbohydrates were removed by treatment with sulfuric acid. All residues were analyzed by Curie point Pyrolysis-Gas chromatography-Mass spectroscopy (CuPy-GC-MS). In this study also DOM samples from the Pacific Ocean at depths of 10 m, 765 m and 4000 m (Benner *et al.,* 1992; van Heemst *et al.,* 1993) and POM samples taken from sediment traps in the Ligurian Sea (Mediterranean, Peulvé *et al.,* 1995) at depths of 100 m, 200 m, 1000 m and 2000 m are included. DOM and POM samples were analyzed by CuPy-GC-MS under the same conditions as the algal residues.

In Figure 5.2 the total ion current trace (TIC) of the algal residue after extraction of the green alga *Ulva fenestrata* is shown as well as the mass chromatogram, revealing the abundance of ions specific for alkylphenols. Proteins are present in the residues of the algae after extraction. As expected they were not removed by saponification, but after acid treatment they were not encountered in the residues.

Figure 5.2 reflects the presence of indole and methylindole as major pyrolysis products. Their precursors are not ester-bound, because these pyrolysis products are still present in the pyrolyzates of the residues after saponification. The precursor is probably related to relatively abundant tryptophan moieties in proteins, because in the pyrolyzates of the residues after acid treatment with hydrochloric acid indole and methylindole were not present anymore.

The precursor of the alkylphenols in the pyrolyzates of all algal residues seems to be resistant to the chemical treatments of these residues. The alkylphenols are found in all residues of the algae, including the acid treated algal residues of the red alga Nereocystis luetkeana and the green alga *Ulva fenestrata.*

5.3 CONCLUSIONS

Polyphenolic macromolecules are not exclusively present in brown macroalgae, but occur also in green macroalgae, red macroalgae and a microalga.
Complexation of these phlorotannin-type macromolecules with metal ions results in insoluble phlorotannin-type constituents. These complexes are thought to contribute significantly to DOM and POM.

The algal polyphenolic macromolecules may represent an important part of the refractory part of DOM and POM through the selective preservation pathway.

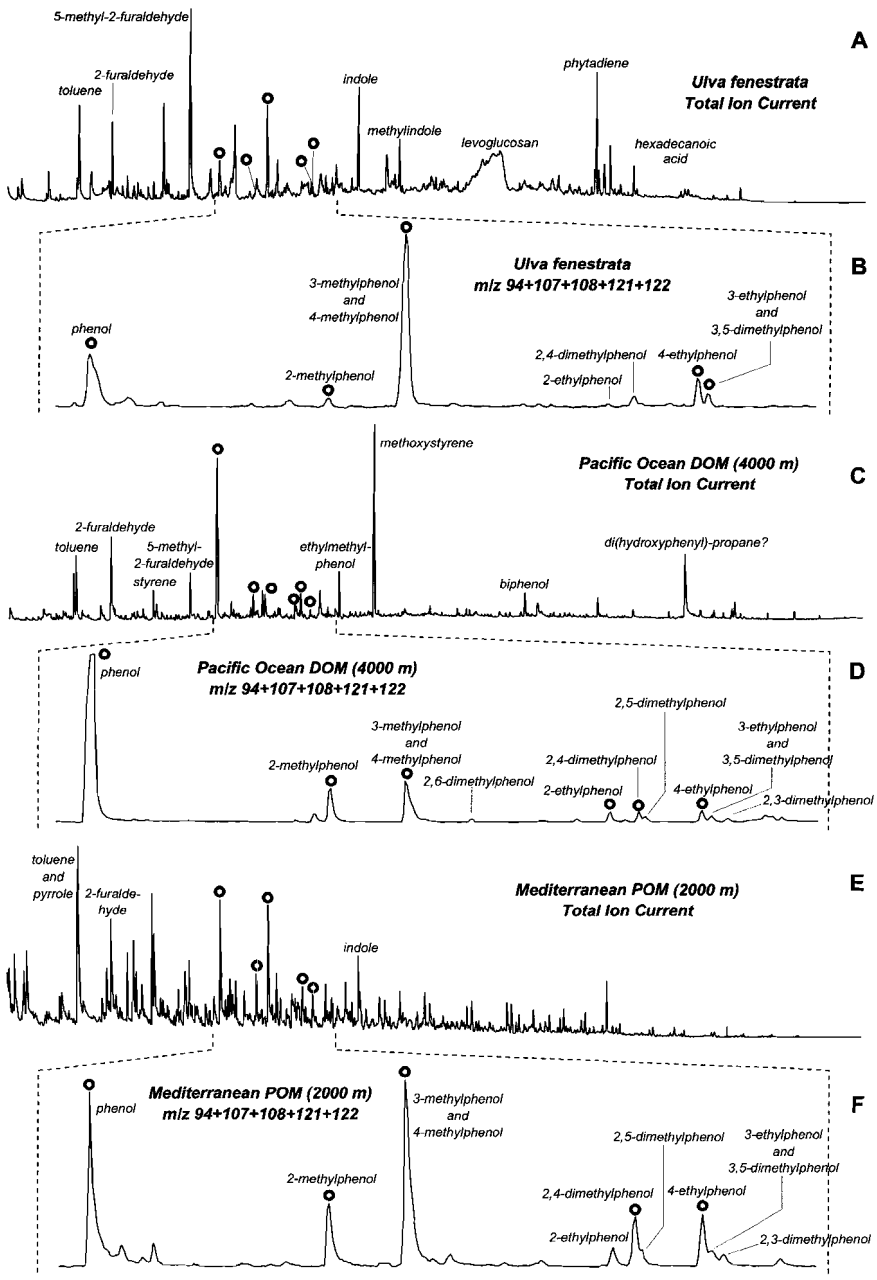

Figure 5.2 CuPy-GC-MS TIC's of a DOM sample, a POM sample and the residue after extraction of *Ulva fenestrata* (A, C and E respectively). The mass chromatograms (A, D and F) reveal the relative abundance of alkylphenols in the pyrolyzates.

CHAPTER 6

Novel Algal Polyphenolic Biomacromolecules as Significant Contributors to Resistant Fractions of Marine Dissolved and Particulate Organic Matter

6.1 ABSTRACT

To investigate whether novel polyphenolic constituents, that have been reported to contribute considerably to dissolved organic matter (DOM) and particulate organic matter (POM), are of algal origin, a number of algae, *i.e.* the brown macroalgae *Nereocystis luetkeana, Fucus gardneri, Costaria costata, Sargassum muticum*, a green macroalga *Ulva fenestrata,* a red macroalga *Opuntiella californica* and a microalga *Rhodomonas* sp. were subjected to analytical pyrolysis. The freeze-dried algae were extracted and saponified. In the case of *N. luetkeana* and *U. fenestrata* proteins and carbohydrates were removed by acid treatments with HCl and H_2SO_4. All fractions were analyzed by Curie-point Pyrolysis-Gas chromatography-Mass spectroscopy (CuPy-GC-MS).

Alkylphenols were encountered in the pyrolyzates of all algae, and in the residues obtained after acid treatment of *N. luetkeana* and *U. fenestrata*. It is thought that these alkylphenols are generated from possibly alkylated phlorotannin-type compounds. Though non-alkylated phlorotannins are known to occur almost exclusively in brown macroalgae, these postulated alkylated counterparts occur in both brown macroalgae and other algae. Their selective preservation during water column transport causes a significant enrichment of these substances in DOM and POM.

6.2 INTRODUCTION

In a recent study van Heemst *et al.* (1993) reported on the occurrence of relatively large amounts of alkylphenols in analytical pyrolyzates of Pacific Ocean dissolved organic matter (DOM) sampled at Station ALOHA (22°45.0'N, 158°00.0'W) at depths of 10, 765 and 4000 m (Benner *et al.,* 1992). The abundance of alkylphenols in these pyrolyzates increased dramatically with increasing depth. In the pyrolyzate of the deepest (4000 m) DOM sample the alkylphenols are the most abundant pyrolysis products, indicating that these alkylphenols are generated from a relatively resistant polyphenolic type precursor, that is selectively preserved. Similar distributions of these

alkylphenols have been found in pyrolyzates of particulate organic matter (POM) present in sediment-trap samples from the Mediterranean (Peulvé *et al.*, 1996a) and in pyrolyzates of the organic fraction of suspended matter in the surface waters and bottom nepheloid layer of the Rhone delta (Sicre *et al.*, 1994). These data suggest that similar types of polyphenolic biomacromolecules are present in all size fractions of marine organic matter.

Alkylphenols are formed upon pyrolysis of lignin (Meuzelaar *et al.*, 1982; Saiz-Jimenez and de Leeuw, 1986b) in addition to methoxyalkylphenols and hydroxyalkylbenzenes. Neither methoxyalkylphenols nor hydroxyalkylbenzenes were however detected in the DOM pyrolyzates analyzed by van Heemst *et al.* (1993). Based on their absence and the unlikely possibility that higher plant constituents such as lignin are present as major components in the deep ocean, lignin was excluded as the precursor of the pyrolytically formed alkylphenols. Brown macroalgae are known to biosynthesize polyphenolic substances, *e.g.* phlorotannins, as external metabolites to chelate metal ions (Ragan *et al.*, 1979 and 1980; Ragan and Glombitza, 1986) and as defence chemicals against grazing herbivores (Tugwell and Branch, 1992; Boettcher and Targett, 1993). Hence phlorotannins, alkylated phlorotannins or similar compounds may be considered as possible precursors of the alkylphenols obtained in the DOM pyrolyzates. Other biomacromolecules possibly generating alkylphenols upon pyrolysis are as yet unknown polyphenolic molecules and siderophores. These latter compounds are encountered in bacteria and contain catechol moieties that scavenge ferric ions (Reid and Butler, 1991; Crumbliss, 1991).

To substantiate a possible algal origin of the alkylphenols produced upon pyrolysis of DOM and POM samples a number of algae, *i.e.* four brown macroalgae, a green macroalga, a red macroalga and a microalga were studied using analytical pyrolysis methods. The macroalgae were selected based on the CuO oxidation data reported by Goñi (1992) and Goñi and Hedges (1995) indicating the presence of phenolic-type substances in these algae.

6.3 EXPERIMENTAL

Algae

The brown macroalgae *Nereocystis luetkeana*, *Fucus gardneri*, *Costaria costata*, the green macroalga *Ulva fenestrata* and the red macroalga *Opuntiella californica* were collected at Eagle Point (Washington State, USA). The brown alga *Sargassum muticum* was taken from a continuous-flow holding tank near Eagle Point (Goñi, 1992). The microalga *Rhodomonas* sp. was grown on f/2-medium in a continuous culture (Guillard, 1975) with a cell concentration of $2 \cdot 10^6$ cells/ml, a light intensity of 150 $\mu E/m^2 s$ and a dilution rate of 0.16 d^{-1}.

Solvent extraction

The freeze-dried algae were extracted ultrasonically with methanol. The suspensions were centrifuged (1 min at 3000 rpm) and the supernatants (extracts) were removed. The residues were extracted another three times with methanol and three times with dichloromethane. The residues were dried and stored at room temperature.

Saponification

Before saponification aliquots of the residues after solvent extraction of all algae, except for *Rhodomonas* sp., were again extracted three times with methanol/water (1:1 v/v). A 1 N potassium hydroxide solution in 96% methanol was added to the residues after extraction. The suspensions were refluxed and stirred for one hour using a magnetic stirrer. After cooling, the reaction mixtures were acidified to pH 3 using 2 N hydrochloric acid in methanol/water (1:1 v/v). After centrifugation (1 min at 3000 rpm), the residues were extracted several times with water, once with methanol/water (1:1 v/v), two times with methanol and three times with dichloromethane. Centrifugation and removal of the supernatant was performed after each extraction step. The final residues were dried and stored at room temperature.

Acid hydrolyses

To remove proteins and to some extent polysaccharides, aliquots of the residues after saponification of *N. luetkeana* and *U. fenestrata* were suspended in a 4 N hydrochloric acid solution in water. The samples were heated at 105°C for six hours. After cooling the samples were neutralized using a 16 N potassium hydroxide solution in water. The mixtures were centrifuged (1 min at 3000 rpm) and the residues were freeze-dried, saponified and extracted as described above.

In order to remove remaining polysaccharides, aliquots of the residues obtained after this acid treatment were suspended in a 24 N sulfuric acid solution in water. After two hours at room temperature, the suspension was diluted with water until the equivalence of sulfuric acid in solution was 2 N. The samples were heated at 105°C for 4.5 hours. After cooling the samples were centrifuged (5 min at 4500 rpm) and the residues were washed with water until complete removal of acid. The residues were freeze-dried and stored at room temperature.

Curie-point pyrolysis-gas chromatography (CuPy-GC)

Curie-point pyrolysis-gas chromatography (CuPy-GC) analyses were performed using a Hewlett-Packard 5890 gas chromatograph, equipped with a cryogenic unit and a 25 m fused silica capillary column coated with chemically bound CP Sil-5 (0.32 mm internal

diameter and a film thickness of 0.45 μm). Helium was used as a carrier gas. A flame ionization detector (FID) at 320 °C was used for detection. The temperature program was as follows: initial temperature 0°C (5 min); heating rate 3°C/min; final temperature 300°C (10 min).

Samples were pressed onto flattened ferromagnetic wires and placed into a pyrolysis unit (FOM -4LX; Boon *et al.,* 1987). The pyrolysis unit was connected to a Fisher 9425 high frequency generator that heated the wires inductively in 0.15 s to the Curie-point temperature. This temperature was maintained for 10 s.

Ferromagnetic wires with Curie-point temperatures of 300, 358, 450, 510, 610 and 770°C were used. A Curie-point temperature of 300°C is too low to generate pyrolysis. Therefore, "pyrolysis" at this temperature is used to discriminate between pyrolysis products and free, low-molecular-weight compounds, which evaporate.

Curie-point pyrolysis-gas chromatography-mass spectrometry (CuPy-GC-MS)

Curie-point pyrolysis-gas chromatography-mass spectrometry (CuPy-GC-MS) analyses were carried out in a similar way to the CuPy-GC-FID analyses. A Hewlett-Packard 5890 gas chromatograph was connected to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of *m/z* 50-800 and a cycle time of 1 s.

6.4 RESULTS AND DISCUSSION

The total ion current traces (TIC's) of pyrolyzates (610°C) of the algal residues obtained after solvent extraction of the brown macroalga *N. luetkeana*, the green macroalga *U. fenestrata* and the red macroalga *O. californica* are shown in Figure 6.1. The pyrolysis products were identified based on their mass spectra and GC-retention times. The polysaccharide pyrolysis products (mainly furan derivatives) were identified by comparison of GC and GC-MS data with those of Pouwels *et al.* (1989) and van der Heijden (1994). The assignments of all numbered peaks are given in Table 6.1. All three pyrolyzates contain a large suite of polysaccharide pyrolysis products, alkylphenols, alkylindoles and lipids. Large differences are evident in the pyrolyzates of the brown macroalga, the green macroalga and the red macroalga. Though present in the pyrolyzate of the red alga, phenol (**39**) and the alkylphenols (**46-48** and **53-56**) are not as abundant as in the pyrolyzates of the green and brown macroalgae. Indole (**61**) and methylindoles (**63**) are clearly more abundant in the pyrolyzates of the green and brown macroalgae than in the pyrolyzate of the red alga.

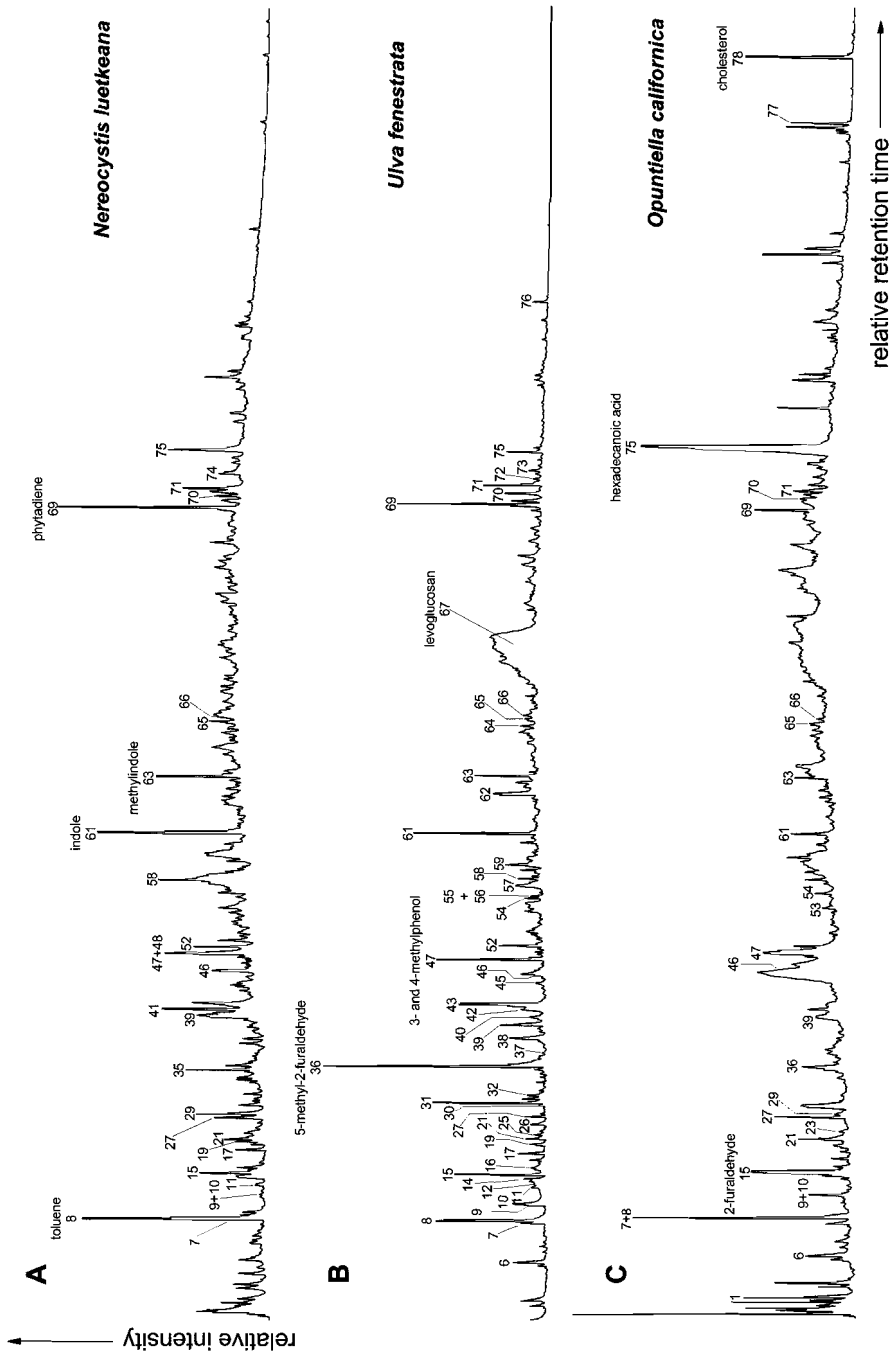

Figure 6.1 Partial TIC-traces of pyrolyzates (610°) of the residues after extraction of (A) *N. luetkeana*, (B) *U. fenestrata* and (C) *O. californica.*

Table 6.1 Identified compounds present in the pyrolyzates of algal residues.

Nr.	Compound	MW	Source
1	methylfuran	82	PS
2	3-methylbutanal	86	
3	hydroxypropanone	74	PS
4	2-methylbutanal	86	
5	acetic acid	60	
6	2,5-dimethylfuran	96	PS
7	pyrrole	67	
8	toluene	92	
9	2,3-dihydrofuran-2-one	84	PS
10	2,3-dihydrofuran-3-one	84	PS
11	3-furaldehyde	96	PS
12	1,2(or 3?)-dimethylpyrrole	95	
13	2-cyclopentenone	82	PS
14	2,4-pentadienal	82	PS
15	2-furaldehyde	96	PS
16	1-cyclopentene-3,4-dione ?	96	PS
17	2-methylpyrrole	81	
18	5-methyl-2,3-dihydrofuran-2-one	98	PS
19	3-methylpyrrole	81	
20	2-propylfuran	110	PS
21	ethylbenzene	106	
22	acetic acid hydroxypropanonyl ester	116	PS
23	1,3-dimethylbenzene	106	
24	1,4-dimethylbenzene	106	
25	2-hydroxymethylfuran	98	PS
26	2,5-dihydrofuran-2-one	84	PS
27	styrene	104	
28	2-methyl-2-cyclopentene	96	PS
29	1,2-dimethylbenzene	106	
30	2-acetylfuran	110	PS
31	methylfuraldehyde ?	110	PS
32	methylfuraldehyde ?	110	PS
33	2-hydroxy-2-cyclopentenone	98	PS
34	3-methyl-2-cyclopentene	96	PS
35	2,4-dimethylfuran ?	96	PS
36	5-methyl-2-furaldehyde	110	PS
37	ethyltoluene	120	
38	4-hydroxy-5,6-dihydro-2H-pyran-2-one	114	PS
39	phenol	94	PH
40	ethylmethylpyrrole	109	
41	2,3-dimethylcyclopent-2-en-1-one	110	PS
42	3-(or 2-)hydroxy-2-(or 3-)methyl-2-cyclopentene-1-one	112	PS
43	4-hydroxy-5-methyl-5,6-dihydro-2H-pyran-2-one (dianhydrorhamnose)	128	PS
44	benzeneacetaldehyde	120	
45	2-acetylpyrrole	109	
46	2-methylphenol	108	PH
47	3-methylphenol	108	PH
48	4-methylphenol	108	PH
49	(tetrahydro-2-furyl)-propanone ?	128	PS
50	3-furancarboxylic acid methyl ester	126	PS
51	levoglucosenone	126	PS
52	benzyl cyanide	117	
53	2,4-dimethylphenol	122	PH
54	4-ethylphenol	122	PH
55	3-ethylphenol	122	PH
56	3,5-dimethylphenol	122	PH
57	1,4:3,6-dianhydro-ß-D-glucopyranose	144	PS
58	2-phenylethyl cyanide	131	
59	2,3-dihydrobenzofuran	120	PS
60	5-(hydroxymethyl)-2-furaldehyde ?	126	PS
61	indole	117	
62	amino acid dimer (ile-val and/or val-leu)	195	PR
63	methylindole	131	
64	2-hydroxy-3H-indole	133	
65	ethylindole	145	
66	dimethylindole	145	
67	levoglucosan	162	PS
68	an anhydrohexose	162	PS
69	phytadiene	278	CH
70	phytadiene	278	CH
71	phytadiene	278	CH
72	phytadiene	278	CH
73	dibutylphthalate		*
74	tetradecanoic acid	228	LIP
75	hexadecanoic acid	256	LIP
76	icosene ?	280	LIP
77	cholesta-3,5-diene	368	LIP
78	cholesterol	386	LIP

CH = chlorophyll, LIP = lipids, PH = polyphenolic macromolecule, PR = protein, PS = poly-saccharide, * = contamination

Lipids

A large suite of lipids is encountered in the pyrolyzates of the algal residues after solvent extraction. These lipids consist of chlorophyll pyrolysis products (phytadienes (**69-72**)), fatty acids (**74, 75**) and sterols (**78**). Because these lipids are virtually absent in the pyrolyzates of the residues obtained after saponification (Figure 6.2) they mainly represent ester-bound moieties.

The same macroalgae as used in this study were also subjected to cupric oxide (CuO) oxidation by Goñi (1992). The alkaline CuO oxidation was originally developed to characterize and quantify lignin in a variety of geochemical samples (Hedges and Ertel, 1982). This technique involves oxidation under alkaline conditions with CuO at elevated temperatures (155-170°C) for three hours, followed by acidification with concentrated HCl and extraction with ether. The resulting product mixtures were analyzed by gas chromatography and gas chromatography-mass spectrometry. Because of the alkaline reaction conditions used in this method saponification occurs simultaneously. The reaction products of the "CuO oxidation" could thus be compared to some extent with those released by saponification. Large amounts of lipids like C_{14} and C_{16} fatty acids were encountered in all algal CuO oxidation mixtures (Goñi, 1992). This is in agreement with the presence of these compounds in the pyrolyzates of the residues before saponification and their lower amounts in the pyrolyzates of the residues after saponification (Figure 6.2).

Protein pyrolysis products

Protein pyrolysis products were revealed by mass chromatography of the ions *m/z* 195+209 corresponding to pyrolysis products of aliphatic amino acids in peptide and protein material (Boon and de Leeuw, 1987). The pyrolyzates of the residues after extraction and after saponification show the characteristic pattern of protein pyrolysis products reported by Boon and de Leeuw (1987), thus clearly indicating the presence of proteins. In the TIC traces of the residues after extraction (Figure 6.1) the protein pyrolysis product (**62**) is present in minor quantities. As expected, after acid hydrolysis using hydrochloric acid no protein pyrolysis products were detected in the pyrolyzates of the residues of *N. luetkeana* and *U. fenestrata*.

Polysaccharide pyrolysis products

Figures 6.1 and 6.2 indicate large differences in polysaccharide compositions of the different algae. Figure 6.2 shows that levoglucosan (**67**) is the most important polysaccharide pyrolysis product of *U. fenestrata* in the pyrolyzate of the residue after saponification. This compound, together with another anhydrohexose (**68**), is one of the most abundant pyrolysis products of the residue after saponification of *O. californica*. Whereas in the case of *N. luetkeana* it is 2-furaldehyde (**15**). This compound is an important pyrolysis product of the other algal samples as well. The relative abundance of 5-methyl-2-furaldehyde (**36**) is larger in *U. fenestrata* and *O. californica* than in *N. luetkeana*. Dianhydrorhamnose (**43**) is only abundant in the pyrolyzate of *U. fenestrata*. This compound indicates the presence of a polysaccharide with rhamnose as an important building block. 3-Furancarboxylic acid methyl ester (**50**) indicates an unique polysaccharide present in *O. californica*, not present in the other algae.

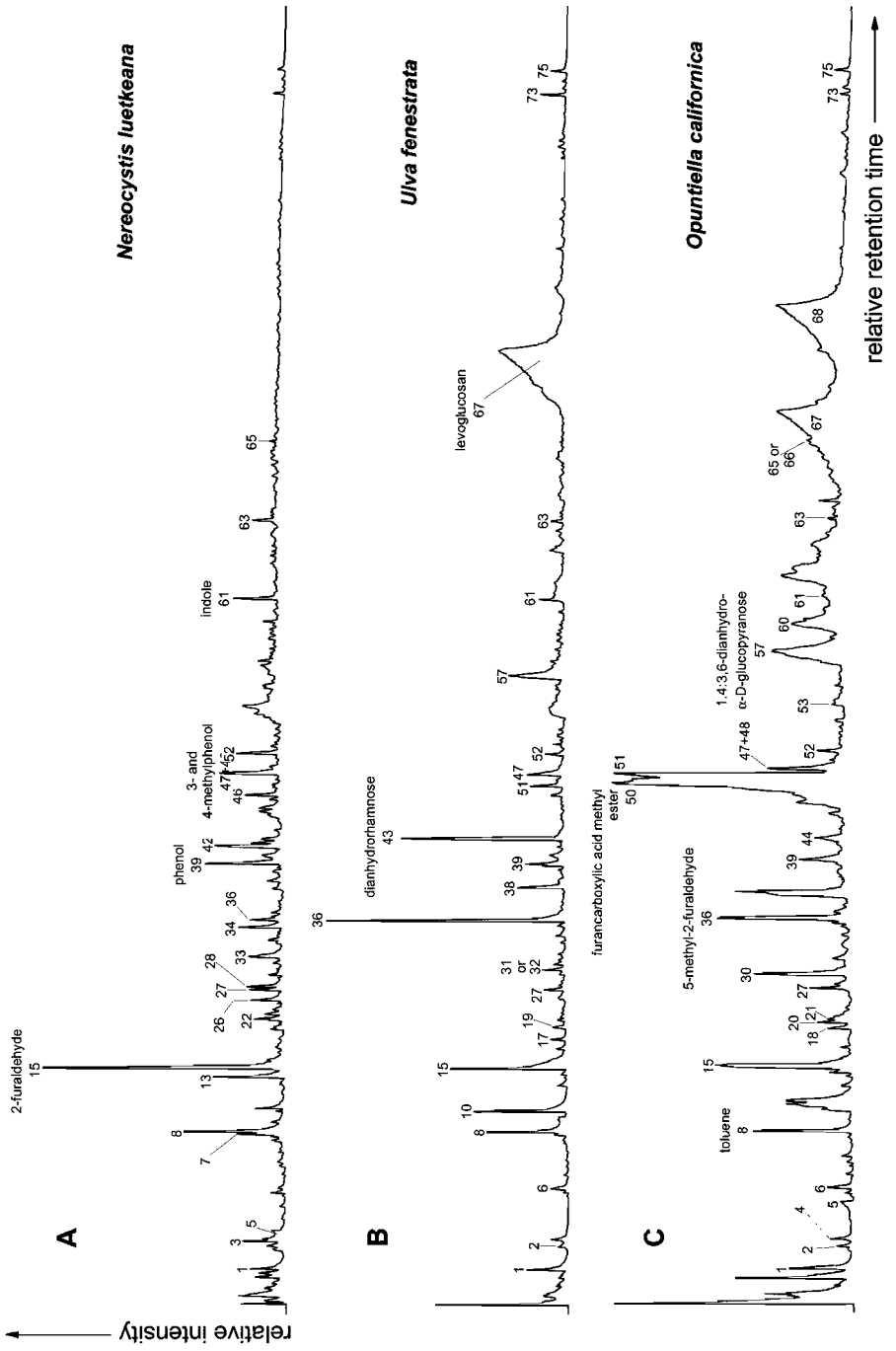

Figure 6.2 Partial TIC-traces of pyrolyzates (610°) of the residues after saponification of (A) *N. luetkeana*, (B) *U. fenestrata* and (C) *O. californica*. Note: compounds 51 and 67 are very polar compounds, which show important variations in retention times

Different algal polysaccharides produce different pyrolysis products or similar pyrolysis products with different distributions (Helleur *et al.,* 1985a and 1985b). The differences between the algal polysaccharide pyrolysis products are therefore explained by the diverse compositions of algal polysaccharides. CuO oxidation of the same macroalgae (Goñi, 1992) resulted in the detection of various polysaccharide CuO products related to the precursors cellulose, xylan and pectin. However, based on the non-specific CuO oxidation products of such precursors, no discrimination between the different polysaccharides could be made.

Alkylindoles

All pyrolyzates of the residues obtained after solvent extraction and saponification contain a large number of nitrogen-containing pyrolysis products apart from those described earlier as the specific protein pyrolysis products. Indole (**61**) and C_1 and C_2 alkylindoles (**63-66**) are relatively abundant.

Figure 6.3 shows the mass chromatograms of indole and methylindole (*m/z* 117+131) and ethylindole and dimethylindole (*m/z* 145) in the pyrolyzates (610°C) of the residues after saponification. Except for an increase of methylindole relative to indole in the pyrolyzate of *O. californica*, no major differences exist in these distribution patterns in the pyrolyzates of the other algal samples. After hydrolysis with hydrochloric acid the relative abundance of the alkylindoles in the pyrolyzates of the residue becomes negligible. This implies that the alkylindole precursor probably is the tryptophan amino acid moiety, which is present in relatively large amounts in algal proteins (Tsuge and Matsubara, 1985).

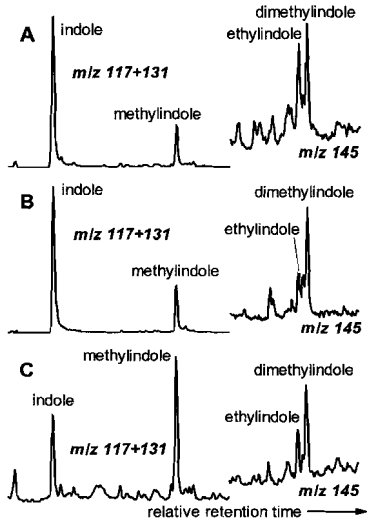

Figure 6.3 Alkylindole distributions in pyrolyzates (610°) of the algal residues after saponification of (A) *N. luetkeana*, (B) *U. fenestrata* and (C) *O. californica.*

Alkylphenols

The pyrolyzates of the residues of *N. luetkeana, F. gardneri, C. costata, S. muticum, U. fenestrata, O. californica* and *Rhodomonas* sp. after solvent extraction all contain alkylphenols. These alkylphenols are not derived from lignin or a lignin-type macromolecule, because of the absence of methoxyalkylphenols in the pyrolyzates. The mass chromatograms shown in Figure 6.4 reflect the distributions of the alkylphenols in the pyrolyzates of the residues of *N. luetkeana, U. fenestrata* and *O.*

californica after saponification. The mass chromatograms indicate that the alkylphenol distribution patterns in all algae studied are similar. They obviously reflect the presence of a non-lignin polyphenolic type constituent. Analyses of the CuO oxidation products of these algae also indicated the presence of such phenolic macromolecules in these algae (Goñi, 1992).

The alkylphenol distributions in the pyrolyzates of the algal residues after saponification show very similar distribution patterns to those observed in the pyrolyzates after solvent extraction. This further indicates that the polyphenolic precursor in all algae is very similar and that it is not ester-bound to the matrix since saponification did not affect the chemical nature of these constituents. The alkylphenol distribution patterns are very similar to those of the alkylphenols generated from the Pacific Ocean DOM sample at 765 m (van Heemst *et al.,* 1993) as shown in Figure 6.5. This indicates that the polyphenolic precursor in the DOM sample is of algal origin. As mentioned previously, brown algae are known to produce large amounts of phlorotannins (Ragan and Glombitza, 1986). It is therefore likely that this algal precursor is a phlorotannin-like macromolecule, though it is obviously not water-soluble like phlorotannin. This insoluble alkylated phlorotannin-like component has, to the best of our knowledge, not been observed by other authors, simply because it is not soluble and cannot be extracted.

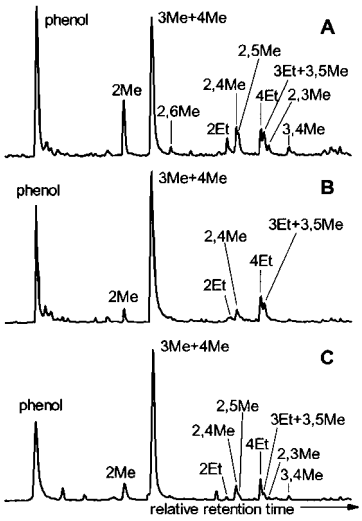

Figure 6.4 Alkylphenol distributions (*m/z* 94+107+108+121+122) in pyrolyzates (610°) of the algal residues after saponification of (A) *N. luetkeana*, (B) *U. fenestrata* and (C) *O. californica*. The abbreviations are explained in Figure 6.5B.

It is known from studies of *Botryococcus braunii* that strains of this green microalga produce phenolic moieties when grown under saline conditions (Sabelle *et al.,* 1993). The cell walls of the algae, grown under saline conditions, show a remarkable difference from the ones, grown under non-saline conditions. The cell walls of the first are of a more "closed" type (Sabelle *et al.,* 1993), *i.e.* the outer cell walls are of a thickened and more pronounced multilayered nature. Therefore it is likely that the phenolic moieties are located in the cell walls.

Also non-hydrolyzable residues (algaenans) of several species of marine green microalgae, *i.e. Chlorella spaerckii, Chlorococcum* sp. and *Nannochloris* sp., produce alkylphenols upon pyrolysis (Gelin *et al.,* 1997). In the case of *Nannochloris* sp. the alkylphenols are the most abundant pyrolysis products. Alkylphenol distribution patterns in the pyrolyzates of the non-hydrolyzable residues of these marine microalgae

are very similar to the alkylphenol distribution patterns in the residues of the algae used in the present study. Therefore it is evident that this type of polyphenolic macromolecule is not restricted only to the macroalgae used in the present study, but is also present in various other, marine, algae. We believe that the polyphenolic macromolecule is located in the cell walls of the algae, although its function remains unclear. The data presented in this paper suggest that this polyphenolic macromolecule is not only present in brown macroalgae, but also in a green macroalga, a red macroalga and a microalga.

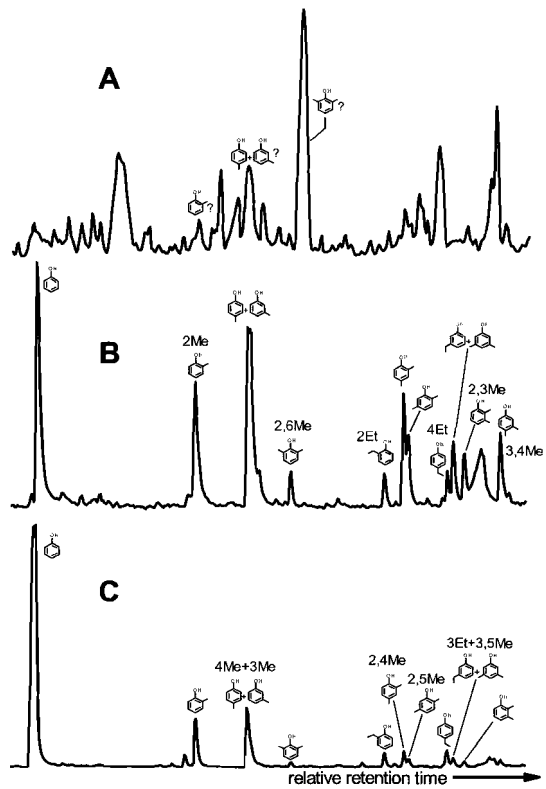

Figure 6.5 Alkylphenol distributions (*m/z* 94+107+ 108+121+122) in pyrolyzates (610°) of DOM samples from the Pacific Ocean (22°45'N; 158°00'W), taken at (A) 10 m, (B) 765 m and (C) 4000 m, based on data by van Heemst *et al.* (1993).

After acid hydrolyses with hydrochloric acid and/or sulfuric acid of the residues after saponification of *N. luetkeana* and *U. fenestrata* the alkylphenols are still present in the pyrolyzates exhibiting very similar distributions (Figure 6.6). This infers that the alkylphenols are neither ester-bound, nor glycosidically or amide-bound.

Having "survived" these chemical treatments the precursor must be highly resistant chemically, although it cannot be completely excluded that the macromolecular network provides a possible steric hinderence of the bonds that bind the phenolic moieties. The resistance against microbial degradation is indicated by the increasing contents to these alkylphenols in the pyrolyzates of the DOM samples with increasing depth (van Heemst *et al.,* 1993).

Figure 6.7 shows partial FID-traces of pyrolyzates of the *U. fenestrata* residue after solvent extraction at Curie-point temperatures 300, 358, 650, 510, 610 and 770°C. Identification of the pyrolysis products was based on CuPy-GC-MS data obtained from the analyses at 358 and 610°C. The chromatogram obtained at 300°C shows that in spite of the extraction procedure the residue contains traces of evaporating products with $C_{16:0}$ fatty acid as the most abundant compound. Therefore it was used as a pseudo-internal

standard to compare the chromatograms obtained at the different Curie-point temperatures. The analysis at 358°C shows that polysaccharide and chlorophyll pyrolysis products are formed at this relatively low pyrolysis temperature. They are low-energy pyrolysis products. Ester-bonds are already broken at a Curie-point temperature of 358°C (de Leeuw and Baas, 1993). The formation of indole and alkylphenols obviously requires more energy since they are generated in relatively increasing amounts at temperatures of 450°C and higher. Figure 6.7 indicates that the energy of formation of benzyl cyanide is similar of those of indole and methylindole. At a Curie-point temperature of 770°C several non-specific low molecular weight pyrolysis product (*e.g.* toluene, pyrrole) are formed.

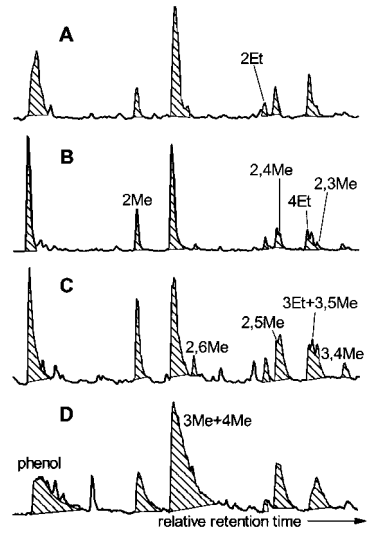

Figure 6.6 Alkylphenol distributions (*m/z* 94+107+108+121+122) in pyrolyzates (610°) of residues of *N. luetkeana*. after (A) solvent extraction, (B) saponification, (C) acid hydrolysis using HCl and (D) acid hydrolysis using H_2SO_4. The abbreviations are explained in Figure 6.5B.

The alkylphenol moieties must have strong chemical bonds to the matrix organic matter, because a high Curie-point temperature is required to generate alkylphenols upon pyrolysis. The alkylphenols are thus generated from the insoluble phlorotannin-like biomacromolecules described above. These insoluble polyphenolic macromolecules may be located in the "physodes", the numerous small vesicles in brown algae containing the soluble phlorotannins (Ragan, 1985). They may also be located at the outside of the cell to scavenge metal ions from the environment.

Dissolved organic carbon (DOC) measurements by Kirchman *et al.* (1991) suggest that DOC (and thus DOM) consists of two fractions, a labile fraction with a high turnover rate being a substrate in the microbial loop, and a refractory fraction. The proposed algal polyphenolic biomacromolecules seem to be important constituents of the refractory component of DOM, since they do not occur in brown macroalgae only and contribute heavily to open ocean DOM.

6.5 CONCLUSIONS

In the pyrolyzates of the algae residues after solvent extraction chlorophyll pyrolysis products, fatty acids and sterols are encountered. These lipids are for the greater part removed from the algal residues by saponification.

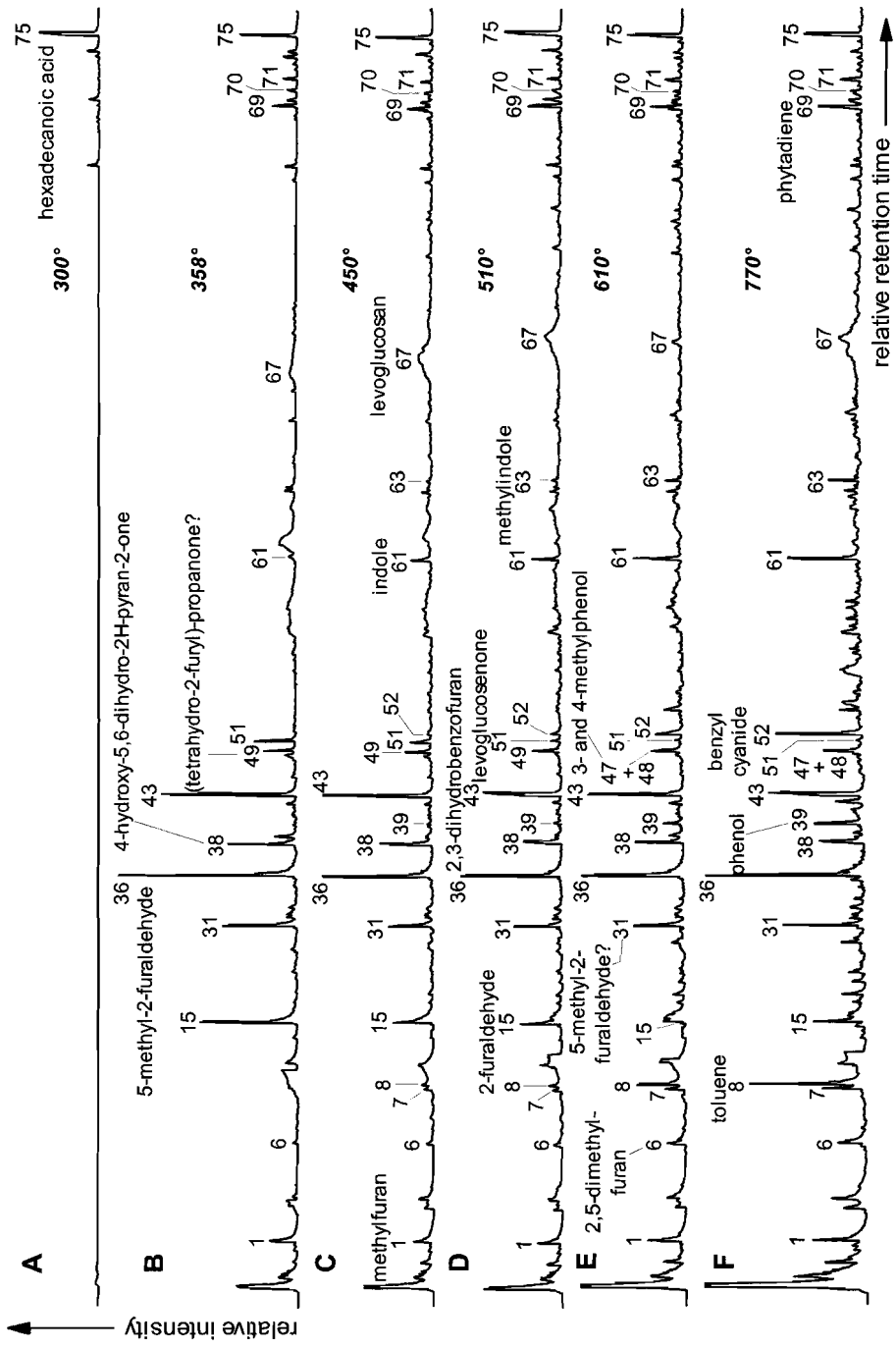

Figure 6.7 Partial chromatograms of pyrolyzates of the residue of *U. fenestrata* after solvent extraction at Curie-point temperatures of (A) 300°C, (B) 358°C, (C) 450°C, (D) 510°C, (E) 610°C and (F) 770°C.

All algae studied show large differences in polysaccharide compositions.

Alkylindoles are present in all pyrolyzates of the residues after solvent extraction and saponification of all algae studied. The precursor of these alkylindoles is removed by acid hydrolysis using HCl, and is therefore probably of proteinaceous nature.
Alkylphenols in pyrolyzates of DOM are not necessarily indicative for the presence of lignins and thus for terrestrial input.

Polyphenolic macromolecules, probably related to possibly alkylated phlorotannin-type components, are not exclusively present in brown macroalgae, but occur also in a green macroalga, a red macroalga and a microalga. These macromolecules are thought to be alkylated insoluble phlorotannin-type macromolecules.

The presence of these polyphenolic macromolecules as significant contributors to DOM and POM is thought to result from colloidal coagulates present in the water column. These algal polyphenolic macromolecules are obviously resistant and therefore become increasingly important in the refractory fraction of DOM through the selective preservation pathway.

The polyphenolic macromolecules, present in both DOM and POM, do probably originate mainly from autochthonous (micro)algae, present in the water column. They do possibly originate only in minor amounts from macroalgae.

6.6 ACKNOWLEDGEMENTS

The authors like to thank Dr. Miguel A. Goñi, Woods Hole Oceanographic Institution (Department of Marine Chemistry and Geochemistry), for providing the macroalgae used in this study. The authors also like to thank Dr. Wim C. M. Klein Breteler, NIOZ (Department of Pelagic Systems), for providing cultures of the *Rhodomonas* sp. algae. The authors would also like to thank the Netherlands Organisation for Scientific research (NWO) for providing a studentship to Jasper van Heemst.

CHAPTER 7

Multiple Sources of Alkylphenols Produced upon Pyrolysis of DOM, POM and Recent Sediments

7.1 ABSTRACT

This study focuses on the different precursors of simple alkylphenols in pyrolyzates of different types of organic matter. Several organic matter samples were studied, *i.e.* dissolved organic matter (DOM), particulate organic matter (POM), sediments, polysaccharide/protein standards, algae, hydrolyzable tannins, lignins, lignites, coals, soils and insect cuticles. Most samples were subjected to saponification and some to hydrolysis using hydrochloric acid. Distribution patterns of the alkylphenols in the pyrolyzates were compared with those in pyrolyzates of natural samples. To some extent, the distribution patterns of these alkylphenols can be used to discriminate their precursors, lignins, protein and transformed protein. Alkylphenols in pyrolyzates of samples like DOM, POM and sediments are probably reflecting polymers which are formed by cross-linking of protein units (tyrosine) forming non-amide bonds on hydrolysis of polysaccharide/protein material in the water column. The polysaccharide part plays a major role in the formation of these polymers.

7.2 INTRODUCTION

Recent analytical pyrolysis studies of dissolved organic matter (DOM) from the Pacific Ocean have revealed relatively large amounts of alkylphenols, particularly in DOM pyrolyzates recovered from greater water depth (van Heemst *et al.*, 1993). In the pyrolyzate of a sample at 4000 m depth alkylphenols were the most important pyrolysis products. Based on this observation it was suggested that alkylphenols in DOM pyrolyzates represent resistant moieties, most probably derived from polyphenolic precursors, which are selectively preserved with increasing depth. Similar distribution patterns of alkylphenols have been encountered in pyrolyzates of particulate organic matter (POM) present in sediment-trap material from the Mediterranean (Peulvé *et al.*, 1996a) and in pyrolyzates of the organic fraction of suspended matter in the surface waters and bottom nepheloid layer of the Rhône delta

(Sicre *et al.,* 1994). These data obtained from DOM and POM, suggest that similar types of polyphenolic moieties are present in all size fractions of marine organic matter.

Apart from their presence in pyrolyzates of marine organic matter, alkylphenols have been encountered in numerous pyrolyzates of sediments (Peulvé *et al.,* 1996b) and soils (Saiz-Jimenez and de Leeuw, 1986a; van Bergen *et al.,* 1997). In many of these cases, it is believed that phenols are derived from intact or degraded lignins (Peulvé *et al.,* 1996b). Although a contribution of lignins to marine organic matter cannot be fully excluded, Meyers-Schulte and Hedges (Meyers-Schulte and Hedges, 1986; Hedges, 1991) have shown that only 0.2% of the bulk organic carbon in the open ocean represents lignin units. Furthermore, pyrolysis of intact and degraded lignins, yields compounds such as methoxyphenols and, especially for degraded lignin, dihydroxybenzenes (Meuzelaar *et al.,* 1982; Saiz-Jimenez and de Leeuw, 1984a; 1986b), with alkylphenols only of relatively minor importance. None of these lignin markers (methoxyphenols and dihydroxybenzenes) were encountered in detectable amounts in the pyrolyzates of the aforementioned DOM, POM and sediment samples. This, therefore, excludes lignins as precursors for the observed alkylphenols.

Van Heemst and co-workers (1996) suggested algal-derived polyphenolic macromolecules, such as phlorotannins and phlorotannin-like materials, as the main precursors of these phenols in marine organic matter. Their conclusion was based on the absence of distinct lignin markers in pyrolyzates of the DOM samples studied, in conjunction with the fact that various algae reveal similar alkylphenol distribution patterns upon pyrolysis to those encountered in pyrolyzates of DOM samples. There are, however, other alternative sources for pyrolytically generated alkylphenols such as phenolic moieties in proteins (e.g. tyrosine). These moieties can be found in all ecological systems (rivers, soils, etc.), thus their supply to the oceans is unlimited.

7.3 MATERIALS AND METHODS

Samples

The origin and characteristics of the samples used in this study and previous studies of relevance are summarized in Table 7.1.

Table 7.1 Sample description

sample type	*composition*	*characteristics /origin*	*column* [a]	*treatments* [b]	*reference*
Standards	BSA[c]	Acros	DB 1701		
	BSA+starch	Acros/Baker	DB 1701	E, S, HH	
	BSA+starch+polygal. acid	Acros/Baker/Sigma	DB 1701	E, S	
	BSA+cellulose	Acros	DB 1701	E, S	
	Polytyrosine	ICN	DB 1701		
	Tannin (tannic acid)	Acros	CP sil-5		
DOM		North Sea	DB 1701		
		Ems-Dollart	CP sil-5		
		Pacific Ocean	CP sil-5		Benner *et al.*, 1992
POM		Mediterranean	CP sil-5		
Sediment		Laptev Sea	CP sil-5		
Brown macroalgae	*Nereocystis luetkeana*	Eagle Point, WA, USA	DB 1701	E, S, HH, HS	Goñi (1992)
	Fucus gardneri	Eagle Point, WA, USA	DB 1701	E, S	Goñi (1992)
	Costaria costata	Eagle Point, WA, USA	DB 1701	E, S	Goñi (1992)
	Sargassum muticum	continuous-flow holding tank, Eagle Point, WA, USA	DB 1701	E, S	Goñi (1992)
Green macroalga	*Ulva fenestrata*	Eagle Point, WA, USA	DB 1701	E, S	Goñi (1992)
Red macroalga	*Opuntiella californica*	Eagle Point, WA, USA	DB 1701	E, S	Goñi (1992)
Microalga	*Rhodomonas* sp.	continuous culture, Texel, The Netherlands	DB 1701	E	
Soil	Mineral soil	Rothamsted Experimental Station, UK	DB 1701	E, S, HH	van Bergen *et al.*, 1998
Lignins	Dicotyledon lignin	Modern cherry wood	DB 1701	E, S, HH	Stankiewicz *et al.*, 1998
	Monocotyledon lignin	Modern grass	DB 1701	E, S, HH	van Bergen *et al.*, 1997
Lignites		Tertiary, Tuplice, Poland	DB 1701	E	
		Tertiary, Legnica, Poland	DB 1701	E	
Coal		Carboniferous, Illinois #6, Argonne Premium Coal	DB 1701	E	van Bergen *et al.*, 1998
Insect cuticle	Chitin-protein complex	Modern cockroach cuticle	DB 1701	E, S, HH	Stankiewicz *et al.*, 1996

[a] column used for Py-GC-MS analyses
[b] E = extracted; S = saponified; HH = hydrolyzed using hydrochloric acid; HS = hydrolyzed, using sulfuric acid
[c] BSA = albumin bovine serum

Solvent Extraction

Samples were extracted ultrasonically with methanol (4 times) and dichloromethane (4 times). The suspensions were centrifuged (1 min at 3000 rpm) and the supernatants (extracts) were removed. The residues were dried over nitrogen.

Saponification

A 1 N potassium hydroxide solution in 96% methanol was added to the residues after extraction. The suspensions were heated to reflux for one hour. After cooling, the reaction mixtures were acidified to pH 3 using 2 N hydrochloric acid in methanol/water (1:1 v/v). After centrifugation (1 min at 3000 rpm), the residues were extracted several times with water, once with methanol/water (1:1 v/v), two times with methanol and three times with dichloromethane. After each extraction step the suspension was centrifuged and the supernatant was removed. The final residues were dried.

Saponification Blank

To evaluate the effect of parameters other than base on the residual organic matter after saponification a mixture of starch and albumin bovine serum (BSA) was treated as described above, except that no potassium hydroxide was added to the 96% methanol solution. The residue was freeze-dried.

Acid Hydrolyses

To remove proteins, aliquots of the residues after saponification were suspended in a 4 N hydrochloric acid solution in water. The samples were heated at 105°C for six hours. After cooling the samples were neutralized using a 16 N potassium hydroxide solution in water. The mixtures were centrifuged (1 min at 3000 rpm) and the residues were again saponified and extracted as described above.

To remove remaining polysaccharides, aliquots of the residues obtained after the hydrochloric acid treatment were suspended in a 24 N sulfuric acid solution in water. After two hours at room temperature, the suspension was diluted with water until the equivalence of sulfuric acid in solution was 2 N. Samples were heated at 105°C for 4.5 hours. After cooling the samples were centrifuged (5 min at 4500 rpm) and the residues were washed with water until complete removal of acid. The residues were freeze-dried.

Curie-Point Pyrolysis-Gas Chromatography (CuPy-GC)

Curie-point pyrolysis-gas chromatography (CuPy-GC) analyses were performed using a Hewlett-Packard 5890 gas chromatograph, equipped with a FOM-3LX pyrolysis unit (Boon *et al.,* 1987) and a cryogenic unit. A 30-m fused silica capillary column coated with chemically bound DB 1701 (0.25 mm i.d., film thickness 0.25 μm) was used for all samples. Some selected samples were also studied using a 25-m fused silica capillary column coated with chemically bound CP Sil-5 (0.32 mm i.d., film thickness 0.45 μm). Helium was used as a carrier gas. A flame ionization detector (FID) at 320 °C was used for detection. The GC was temperature programmed as follows: initial temperature 0°C (5 min); heating rate 3°C/min; final temperature 300°C (10 min). Samples were pressed onto flattened ferromagnetic wires (Curie temperatures of 610°C) and placed into the pyrolysis unit. The pyrolysis unit was connected to a FOM high frequency generator that heated the wires inductively in 0.15 s to the Curie temperature. This temperature was maintained for 9 s.

Curie-Point Pyrolysis-Gas Chromatography/Mass Spectrometry (CuPy-GC-MS)

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC-MS) analyses were carried out in the same way as the CuPy-GC analyses. A Hewlett-Packard 5890 gas chromatograph was connected to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of *m/z* 700-35 and a cycle time of 1 s. Pyrolysis products were identified based on their mass spectra and GC-retention times (Stankiewicz *et al.,* 1996; Pouwels *et al.,* 1989), whereas identification of some of them was based on comparison with standards.

7.4 RESULTS

DOM / POM / Sediments

CuPy-GC/MS chromatograms (TIC traces and alkylphenol traces) of pyrolyzates of DOM, POM and a sediment sample are shown in Figure 7.1. They show numerous pyrolysis products, most of which can be identified to be derived from one particular biomacromolecule. Acetic acid, furan and cyclopentenone derivatives are pyrolysis products of polysaccharides or (partly) altered polysaccharide macromolecules (Pouwels *et al.,* 1989), whereas pyrroles and benzyl nitriles may originate from proteinaceous materials. Alkylphenols are clearly present in all of these pyrolyzates.

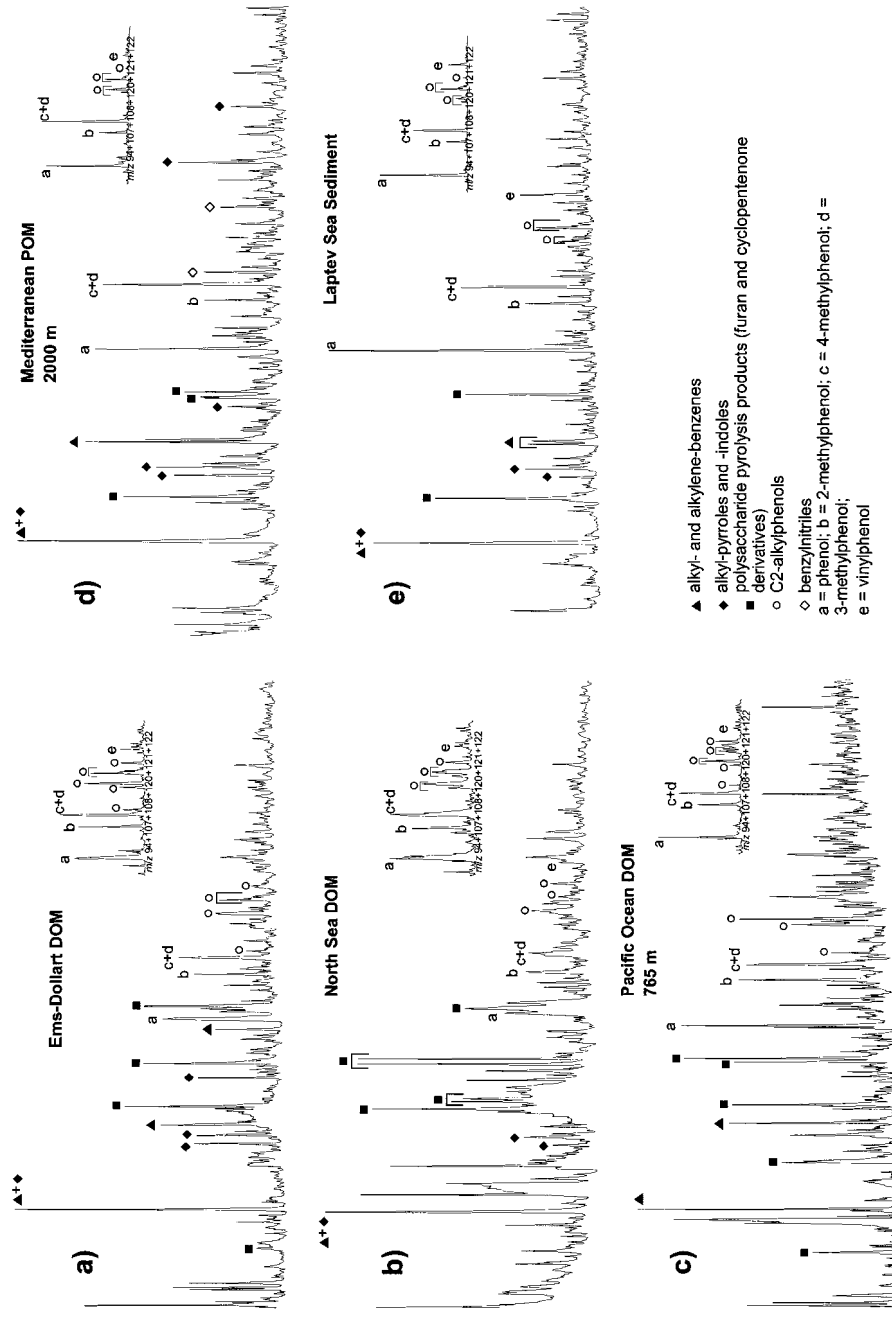

Figure 7.1 Total ion current traces and alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122; inserts) of the pyrolyzates of DOM (a-c), POM (d) and a sediment sample (e) (column used: CP-sil 5).

However, their origin is not entirely straight forward. Their distribution patterns are best visualized using mass chromatography (Figure 7.1; inserts).

Polysaccharide/Protein Standards

Mass chromatograms of alkylphenols in the pyrolyzates of the mixtures of polygalacturonic acid, starch and BSA, in the residue after saponification and in the residue after subsequent acid hydrolysis are shown in Figure 7.2c-e. The pyrolyzates contain a complex mixture of pyrolysis products. Phenol, 4-methylphenol, indole and methylindole are present in the pyrolyzate of the untreated mixture in relatively large amounts (Figure 7.2a). Indole and methylindole (probably 3-methylindole) are the pyrolysis products of tryptophan moieties in proteins (Tsuge and Matsubara, 1985). Only traces of 2-methylphenol are present. Phenol, 4-methylphenol and *p*-vinylphenol are the main pyrolysis products of tyrosine moieties present in the albumin protein. This unit contains a 4-hydroxybenzyl moiety, which produces mainly phenol and 4-methylphenol upon pyrolysis (Tsuge and Matsubara, 1985). These compounds are also the two major components in the pyrolyzate of polytyrosine (data not shown, but is virtually identical to the mass chromatogram shown in Figure 7.2b). The pyrolyzates of the residue of the mixture of polygalacturonic acid, BSA and starch after saponification (Figure 7.2d) and the residue after subsequent acid hydrolysis (Figure 7.2e) show relatively increased amounts of 2-methylphenol and C_2-alkylphenols (2- and 3-ethylphenol and dimethylphenols) relative to 4-methylphenol (Figure 7.2e), when compared with the untreated mixture (Figure 7.2c). These data indicate that base and acid hydrolysis removes (degrades?) moieties from the macromolecular matrix, which produce predominantly 4-methylphenol upon pyrolysis and/or forms moieties yielding predominantly 2-methylphenol and 3-methylphenol. Apart from changes in distribution of particular alkylphenol isomers, their total relative abundance compared with other pyrolysis product has decreased in the residue of the mixture after saponification. Acid hydrolysis using hydrochloric acid has caused an additional decrease of alkylphenol producing moieties as reflected in the reduction of the relative amounts of alkylphenols in the total pyrolyzate (data not shown).

The same experiments performed with mixtures of: i) starch and BSA, ii) cellulose and BSA and iii) starch and polytyrosine, showed very similar results (data not shown). Alkylphenol distribution patterns were similar in all pyrolyzates before (Figure 7.2g-i) and after base (*e.g.* Figure 7.2f) and after acid treatments. This indicates that the enhanced intensity of 2-methylphenol, 3-methylphenol and the C_2-alkylphenols as compared with that of 4-methylphenol in the pyrolyzates of the acid-treated residues is due to changes induced by the entire hydrolysis process, and not by heating, catalysis by methanol or acidification following saponification alone. To

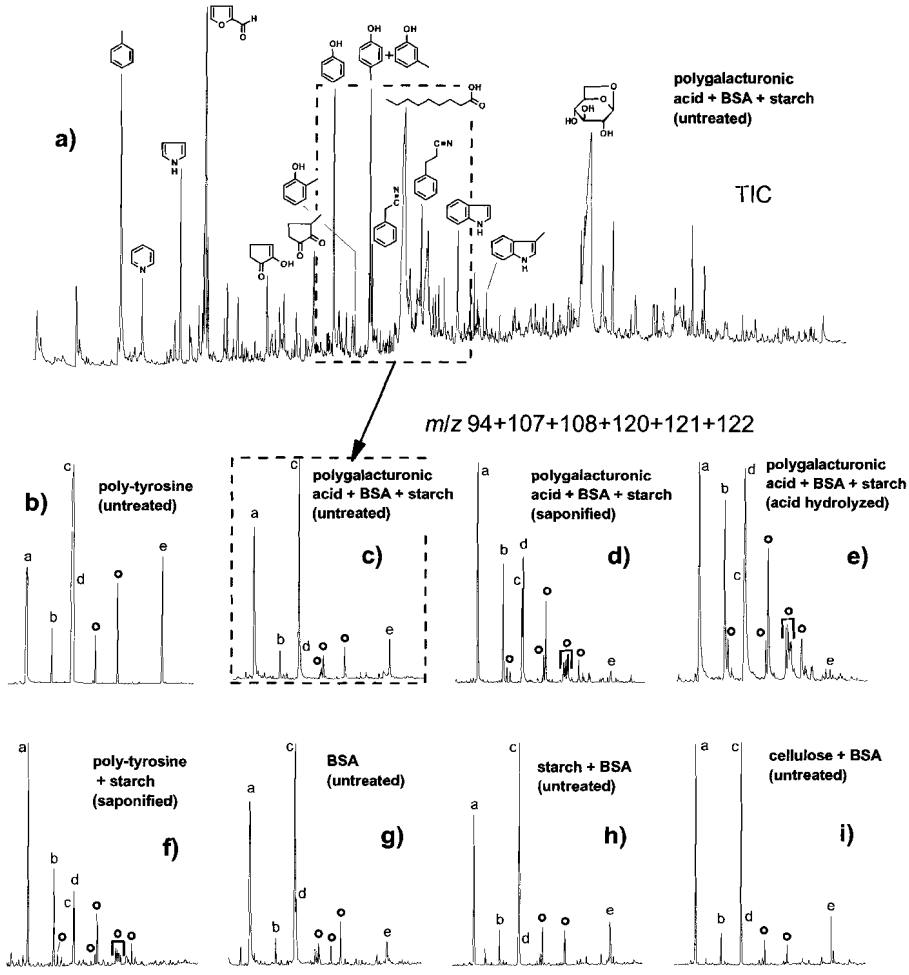

Figure 7.2 Total ion current trace (a) and alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122; b-i) of the pyrolyzates of polysaccharide/protein standard samples. Key: open dots = C_2-phenols, a = phenol, b = 2-methylphenol, c = 4-methylphenol, d = 3-methylphenol, e = vinylphenol.

examine possible catalysis effects of cations on the formation of alkylphenols upon pyrolysis, sodium chloride was added (weight ratio mixture/salt 1:4) to an aliquot of the mixture of starch and BSA. The distribution pattern of the alkylphenols in the pyrolyzate was virtually identical to that in the pyrolyzate of the mixture without salt. BSA itself is completely soluble in water. It was saponified in the same way as the polysaccharide/protein mixtures, but no residue was obtained. Residues of the

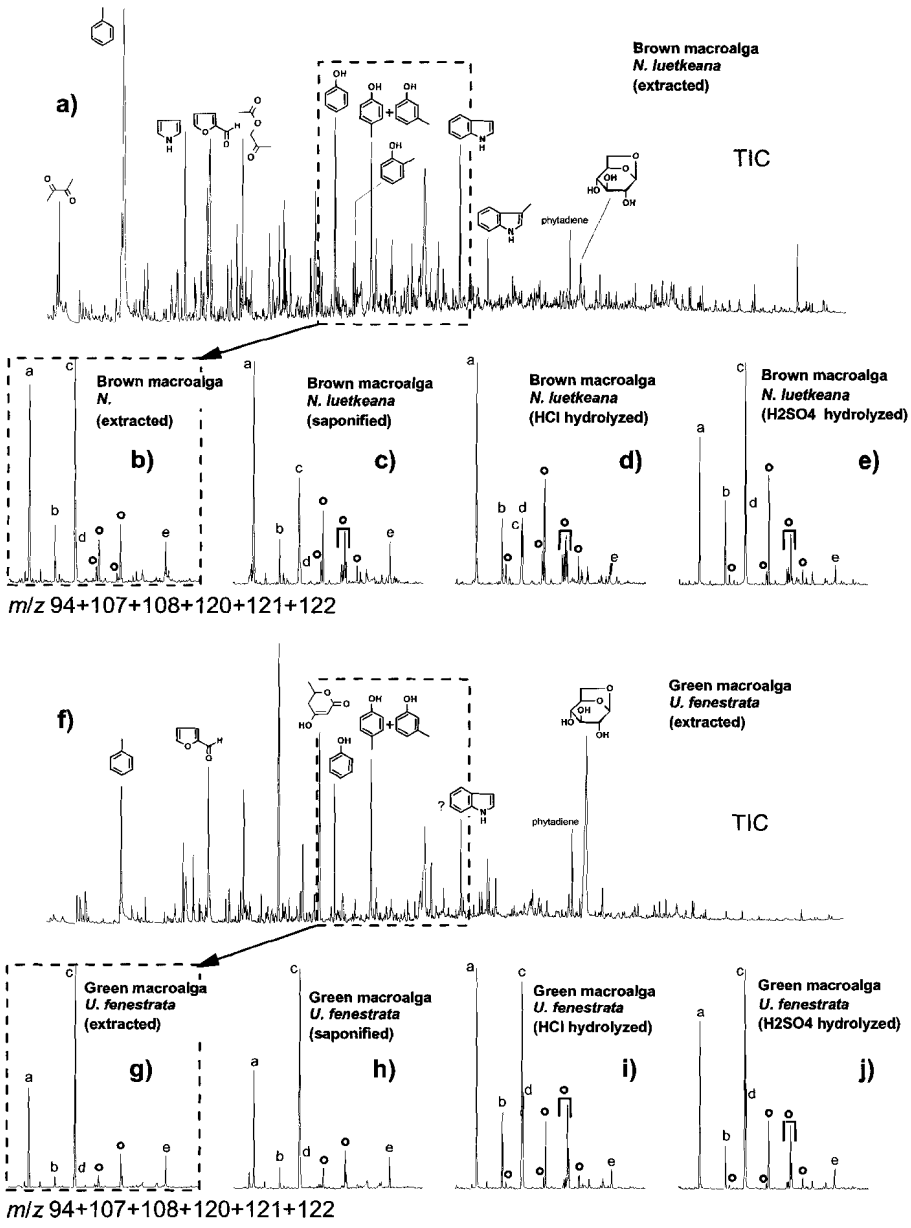

Figure 7.3 Total ion current traces (a,f) and alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122; b-e,g-j) of the pyrolyzates of brown and green macroalgae. Key: open dots = C_2-phenols, a = phenol, b = 2-methylphenol, c = 4-methylphenol, d = 3-methylphenol, e = vinylphenol.

polysaccharide/protein mixtures after saponification typically ranged from 30 to 50% in dry weight.

Algal Polyphenols

In pyrolyzates of the algae studied alkylphenols are encountered in relatively large amounts. The pyrolyzates of the brown macroalgae (Figures 7.3a-e and 7.4a-b) show non-proteinaceous alkylphenol distribution patterns, *i.e.* relatively high abundance of 2-methylphenol and C_2-phenols compared with those of 4-methylphenol. The pyrolyzates of the green macroalga (Figure 7.3f-j), the red macroalga (Figure 7.4c) and the microalga (Figure 7.4d) more closely resemble protein-like distribution patterns of alkylphenols. Thus, the precursors of the alkylphenols in the pyrolyzates of the green and red macroalgae and the microalga are probably proteinaceous in origin, whereas

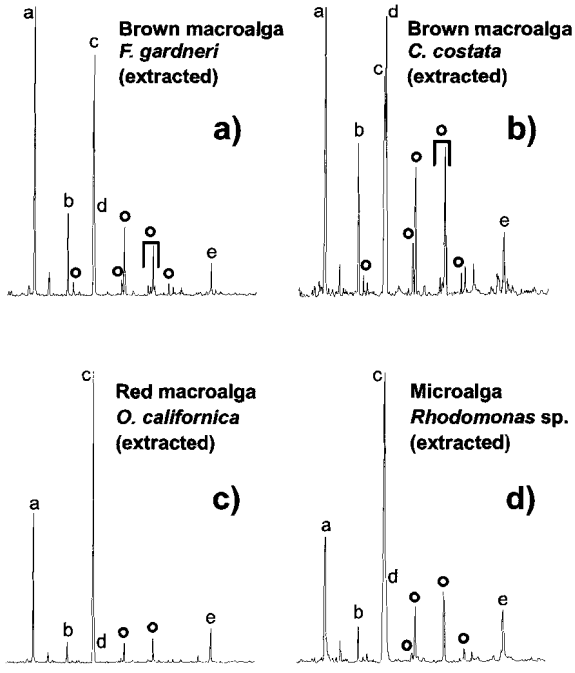

Figure 7.4 Alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122) of the pyrolyzates of brown macroalgae (a-b), a red macroalga (c) and a microalga (d). Key: open dots = C_2-phenols, a = phenol, b = 2-methylphenol, c = 4-methylphenol, d = 3-methylphenol, e = vinylphenol.

the precursors present in the brown macroalgae are probably of both proteinaceous and non-proteinaceous, *i.e.* alkylated phlorotannin-like, origin. This conclusion contrasts that of van Heemst *et al.* (1996) who suggested the precursors of the alkylphenols in pyrolyzates of all of these algae to be of alkylated phlorotannin-like origin.

The alkylphenol distribution in the pyrolyzates of the residue of the green macroalga *U. fenestrata* after saponification and subsequent acid hydrolysis (Figure 7.3i) changes in a similar way as observed in the pyrolyzates of the polysaccharide/protein mixtures (Figure 7.2e). This further supports the idea that alkylphenols in the pyrolyzates of this alga originate from a proteinaceous precursor rather than from an alkylated phlorotannin-like molecule. A similar phenomenon was observed in the residue after saponification of the brown macroalga *N. luetkeana* (Figure 7.3b-c), indicating a significant contribution of a proteinaceous precursor to the algal residues of this brown macroalga.

Hydrolyzable Tannins

The main pyrolysis product of tannic acid is trihydroxybenzene (data not shown). Phenols and dihydroxybenzenes are encountered in the pyrolyzate, but no methyl- or C_2-phenols are detected.

Lignins

Figure 7.5 shows mass chromatograms of alkylphenols in the pyrolyzates of residues of dicotyledonous lignin (cherry wood) after solvent extraction (b), saponification (c) and subsequent acid hydrolysis (d) and a residue of monocotyledonous lignin (grass) after solvent extraction (e). Alkylphenols and the characteristic methoxyphenols are present in the pyrolyzates of both types of lignin. Alkylphenols represent only a minor part of the total pyrolyzates of the lignin samples (Figure 7.5a), with distribution patterns (Figure 7.5b-d) resembling that of non-proteinaceous material (*i.e.* high relative abundance of 2-methylphenol and C_2-alkylphenols compared with that of 4-methylphenol). The distribution pattern of the methylphenols in the pyrolyzate of the wood sample varies only slightly when subjected to chemical degradation treatments such as saponification and subsequent hydrochloric acid hydrolysis.

Lignites / Coals

The lignite of Legnica and the Illinois coal (Figure 7.6c-d) reveal distribution patterns similar to the ones revealed by the lignin samples (Figure 7.5b). This indicates that throughout the degradation (diagenesis) of lignins the distribution of the phenols

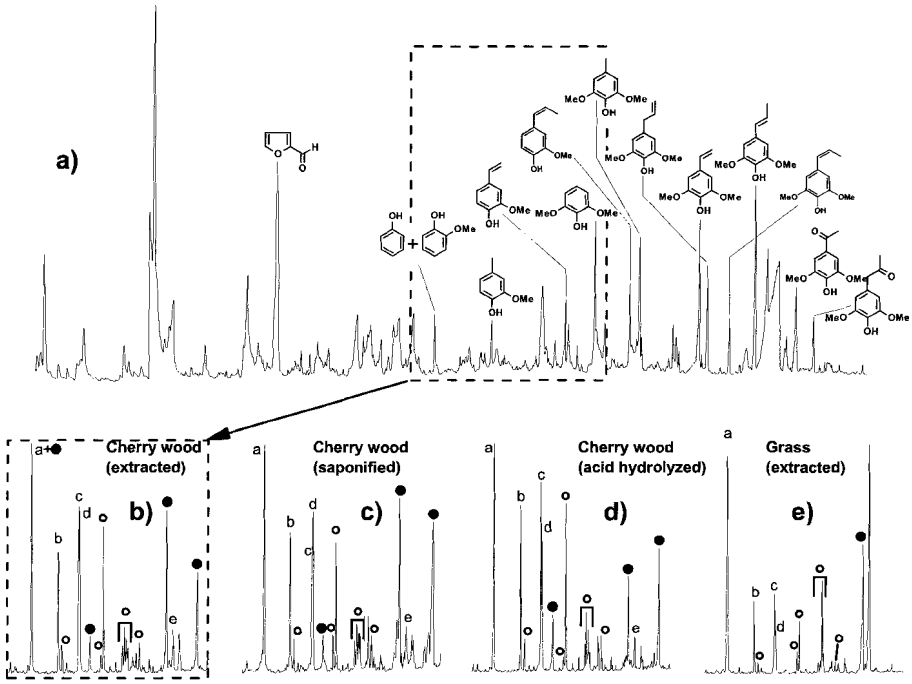

Figure 7.5 Total ion current trace (a) and alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122; b-e) of the pyrolyzates of lignin samples. Key: open dots = C_2-phenols, closed dots = specific lignin pyrolysis products, a = phenol, b = 2-methylphenol, c = 4-methylphenol, d = 3-methylphenol, e = vinylphenol.

remains relatively unchanged. However, it should be noted that lignin as the sole source of phenols in pyrolyzates of Illinois coals is still questionable (cf. van Bergen *et al.* (1995)). In contrast, the Miocene (*c.* 15 Ma) age lignite of Tuplice (Figure 7.6a) reveals an alkylphenol distribution pattern (Figure 7.6b) similar to the ones revealed by non-lignin derived samples, like the brown macroalgae samples (Figures 7.3a and 7.4a). The lignite from Tuplice has a higher amount of alginite and other liptinite macerals than the one from Legnica, which is more xylitic (i.e. containing recognizable wood structures) in composition (Stankiewicz and Mastalerz, unpublished data). According to the maceral percentages, the Legnica lignite has 50% humotelinite (structured huminite), 48% humodetrinite (non-structured huminite), and only 1.5% liptinite; whereas the Tuplice lignite has 10% humotelinite, 72 % humodetrinite (which can have bacterial contribution), 9 % liptinite (in which *c.* 4% is algal biomass), and the rest being inertinite. The higher xylitic influence in the

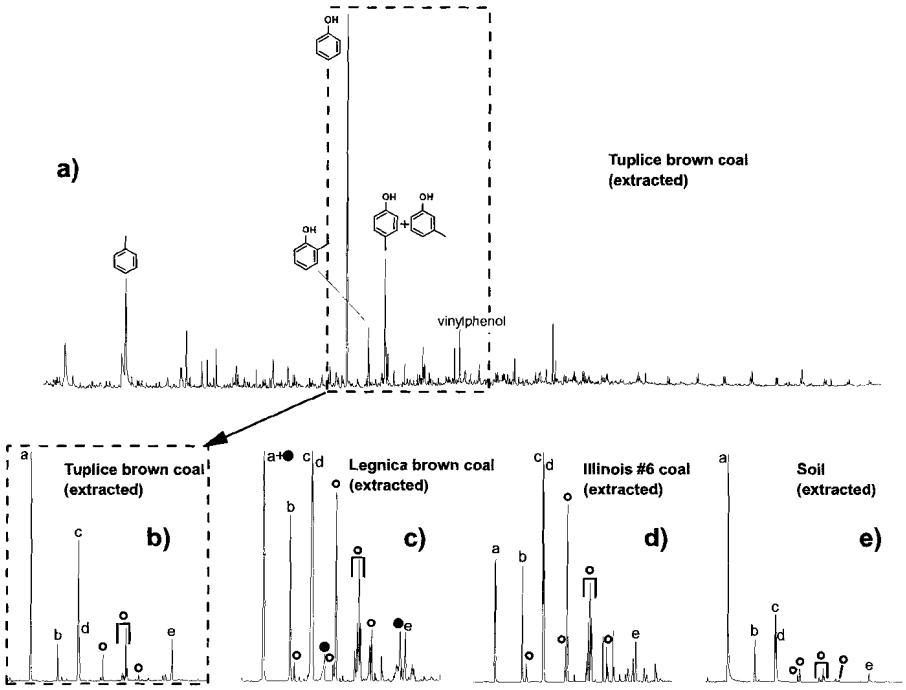

Figure 7.6 Total ion current trace (a) and alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122; b-e) of the pyrolyzates of lignin samples. Key: open dots = C_2-phenols, closed dots = specific lignin pyrolysis products, a = phenol, b = 2-methylphenol, c = 4-methylphenol, d = 3-methylphenol, e = vinylphenol.

Legnica lignite compared with the Tuplice could explain the differences in phenol distributions.

Soils

The distribution pattern of the methylphenols in the pyrolyzates of the soil sample (Figure 7.6e) is similar to the ones encountered in pyrolyzates of the hydrolyzed polysaccharide/protein mixtures. This phenol distribution pattern, in combination with the virtual absence of lignin-derived methoxyphenols, was suggested to be evidence of (modified) proteinaceous moieties as the sole source of the alkyl phenols in this, and related, soil samples (van Bergen *et al.,* 1997; 1998).

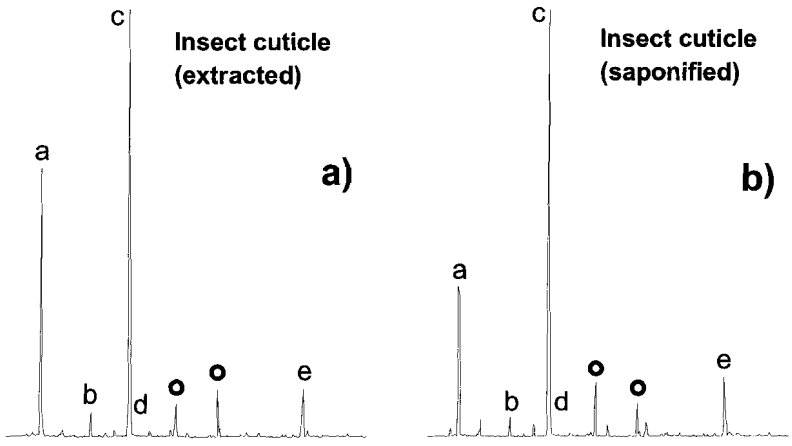

Figure 7.7 Alkylphenols mass chromatograms (*m/z* 94+107+108+120+121+122) of the pyrolyzates of a residue of an insect cuticle after solvent extraction (a) and after saponification (b). Key: open dots = C_2-phenols, a = phenol, b = 2-methylphenol, c = 4-methylphenol, d = 3-methylphenol, e = vinylphenol.

Insect Cuticles

Chromatograms of alkylphenols in the pyrolyzates of the residues of an insect cuticle (cockroach) after solvent extraction and after saponification are shown in Figure 7.7a-b. The residue after subsequent hydrochloric acid hydrolysis revealed an alkylphenol distribution almost identical to the ones shown in Figure 7.7a-b. The alkylphenol distribution patterns of all cuticle residues show a distribution pattern, characteristic of proteinaceous materials. Intensities of 2- and 3-methylphenol and C_2-phenols are much lower than that of 4-methylphenol and do not change in the pyrolyzate of the residue of the insect cuticle after saponification.

7.5 DISCUSSION

Changes in Phenol Distributions

Changes in alkylphenol distribution patterns after saponification of the polysaccharide/protein mixtures may be due to cross-link reactions occurring during base hydrolysis, either between saccharide moieties and the amino acids of the protein or cross-linking in the presence of polysaccharides between different protein units.

These cross-link reactions may promote formation of insoluble organic matter. These type of reactions could be similar to the ones involved in the formation of melanoidins from monosaccharides and amino acids (Rubinsztain *et al.,* 1984; Mills and Hodge, 1976, Boon *et al.,* 1984; Yamamoto and Ishiwatari, 1989; Evershed *et al.,* 1997).

Albumine bovine serum itself is completely soluble in water. It has been saponified in the same way as the polysaccharide/protein mixtures, but no residue was obtained. This implies that the formation of the insoluble material containing phenolic moieties indeed requires additional moieties, i.e. saccharide, to promote cross-linking.

Alkylphenol distributions in the pyrolyzates of the residues of the insect cuticles do not change after saponification and acid hydrolysis. This may be due to the short peptide chain of the protein and its covalent cross-linking to the chitin and mucopolysaccharide macromolecules, thus forming a very rigid structure (Schaefer *et al.,* 1987). Alternatively, it is possible that in the insect cuticle isolate the peptide units are located too far apart to interact which each other and thus do not take part in cross-link reactions during saponification. A third explanation might be that the peptide part of the cuticle in total is water-insoluble and is therefore not removed by the extractions following saponification like in the polysaccharide/protein mixtures, which were performed using a water-soluble protein.

When compared with alkylphenol distribution patterns in pyrolyzates of polysaccharide/protein standards, the alkylphenol distribution patterns in DOM samples closely resemble those in the hydrolyzed (base and acid) residues of the polysaccharide/protein mixtures (Figure 7.2d-e). This is especially evident in the alkylphenol distribution patterns encountered in the pyrolyzates of DOM from the Ems-Dollart estuary and the North Sea DOM (Figure 7.1b-c; inserts). It is therefore suggested that the precursor of the alkylphenols in pyrolyzates of DOM is a preserved macromolecular remnant of proteinaceous organic matter in the water column resulting from hydrolytic transformation. Fractions of organic matter in the water column are easily hydrolyzed by micro-organisms (Amon and Benner, 1994; Kirchman *et al.,* 1991). However, it should be emphasized that hydrolysis in aquatic environments is mainly a biomediated process which is not necessarily identical to the chemical hydrolysis treatment described herein. Alkylphenols with distribution patterns similar to those in pyrolyzates of DOM are also revealed in pyrolyzates of POM, suggesting that the macromolecule containing the phenolic moieties may be of variable size. POM, which contains these phenolic macromolecules, is deposited in marine sediments, since a similar alkylphenol distribution pattern is also observed in the pyrolyzate of the sediment sample (Figure 7.1e; insert). As has been discussed before, the alkylphenols in the DOM, POM and sediment samples are unlikely to be derived from lignins, because of the absence of specific lignin markers (methoxyphenols) from these pyrolyzates. A major algal polyphenolic source is also

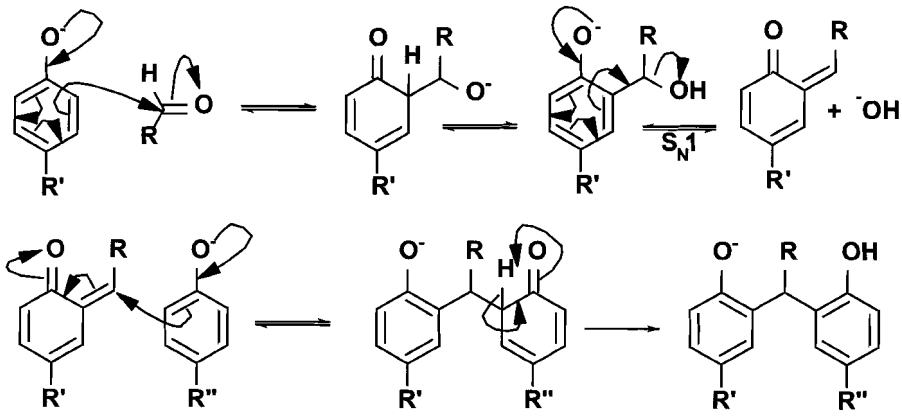

Figure 7.8 Reaction mechanism of the formation of insoluble polymer from proteins and carbohydrate during the saponification reaction.

unlikely as no specific phlorotannin pyrolysis products (like phloroglucinol) were detected in any of the samples. Furthermore, the absence of trihydroxybenzene also excludes tannins based on tannic acid moieties. Therefore, a hydrolyzable tannin source for these alkylphenols may be ruled out.

Cross-Linking Reaction Mechanism

The reaction mechanism of the cross-link reactions is proposed in Figure 7.8. The proton attached to the oxygen of the phenolic group of tyrosine is abstracted by the base used in the saponification reaction. This ion is stabilized by resonance. The other main resonance structures are the *ortho*- and the *para*-phenolic ketone anion. This nucleophilic ion may attack the aldehyde group of *e.g.* a polysaccharide moiety. After keto-enol rearrangement either the *ortho*- or the *para*-bounded alcohol is formed. The hydroxide group is substituted by another deprotonised phenolic moiety via a S_N1 substitution. Further polymerization reactions *via* this mechanism may lead to large phenol-aldehyde-like polymeric structures. However, the occurrence of sufficient amounts of aldehyde functional groups in the polysaccharides used in this study is questionable.

7.6 CONCLUSIONS

Alkylphenols in pyrolyzates of recent and ancient organic matter can reflect different origins. Based on the distribution patterns of these alkylphenols, lignin, protein, transformed protein (in particular tyrosine derivatives) can be discriminated.

Alkylphenols in pyrolyzates of samples like DOM, POM and some marine sediments are probably reflecting polymers which are formed by cross-linking of protein units (tyrosine) forming non-amide bonds as a result of hydrolysis of polysaccharide/protein material. The polysaccharide part plays a major role in the formation of these polymers.

7.7 ACKNOWLEDGEMENTS

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CHAPTER 8

Selective Preservation of Chitin during the Decay of Shrimp

8.1 ABSTRACT

The preservation potential of chitin in the marine environment is a matter of debate. To determine the relative survival of chitin and other organic components the shrimp *Crangon* was decayed under different laboratory conditions. Solid state ^{13}C NMR and Curie point pyrolysis-gas chromatography-high resolution mass spectrometry demonstrated that slightly transformed chitin represents the major component of the remaining biomass after only eight weeks. This selective preservation confirms that the resistance of chitin to decay may be a major factor in accounting for the extensive fossil record of arthropods lacking a biomineralized skeleton. It also suggests that chitin is likely to be an important contributor to the organic content of recent marine sediments. The pyrolyzate of the preserved cuticle of fossil shrimps reveals a homologous series of alkanes and alkenes indicating a substitution of chitin by more resistant organic matter derived from other sources.

8.2 INTRODUCTION

Arthropods are a major component of marine communities and they represent a significant proportion of biomass in the oceans. They are the most important group that uses chitin as a structural biopolymer, but it also occurs in a range of other invertebrates including hydrozoans, annelids, brachiopods and molluscs, as well as fungi and some algae (Muzzarelli, 1977). The annual production of chitin is enormous (see Gooday, 1990) but it has not been detected in large quantities in oceanic sediment; it appears to be absent from all but a thin veneer at the top of the column (van Waveren, 1993; Poulicek and Jeuniaux, 1989). Thus it is normally considered to have a relatively low preservation potential compared to other resistant biomacromolecules, particularly those in vascular plants and algae (Tegelaar *et al.,* 1989a). Nonetheless there is increasing evidence of the preservation of fossil arthropods as an organic residue in a range of environments from marine to non-marine (Butterfield, 1990, 1994; Briggs and Clarkson, 1989; Shear and Kukalova-Peck, 1990). This is a reflection of the decay-resistant properties of the organic exoskeleton (of which chitin is a primary constituent) particularly where it is tanned or sclerotized.

Components of crustacean exoskeletons have recently been reported for the first time among palynomorphs and palynodebris in modern sediments from the Banda Sea (Indonesia) (van Waveren, 1993 and 1994). The total organic particle concentrations in most of the sediment samples were extremely high (up to 1,500,000 particles per gram of dry sediment) and over 50% reflected zooplankton remains. Such a high proportion of zooplankton has not been reported previously. The high levels in this case may be due partly to the fact that the samples were not acetolyzed or oxidized before analysis, so that chemically labile zooplanktonic palynomorphs were not destroyed (van Waveren, 1993). Since the bulk of the zooplankton remains consist of the exoskeletons of planktonic crustaceans it is assumed that chitin, the major building block of these exoskeletons (and, like cellulose, one of the most abundant biomolecules in the biosphere) has been preserved selectively. Van Waveren (1993, 1994) considers that the significant preservation of this relatively labile biomolecule in the Banda Sea reflects high productivity, high sedimentation rates, and short residence times in the uppermost oxic layers. This last aspect is particularly important because chitin, including the most stable alpha form that is present in crustaceans, can be degraded rapidly by oxic bacteria (Gooday, 1990). Experiments monitoring the rate of decay (measured by weight loss) of commercially produced crab chitin in different natural environments (Allison, 1991) demonstrated substantial variability even at individual stations. However, rates of decay were highest in flowing seawater and in sandy sediment, intermediate in marine muddy sand, and lowest in freshwater mud. It is likely that these differences are at least partly a reflection of availability of oxygen.

Although most chitin is clearly recycled by the activities of micro-organisms, arthropods and palynomorphs that lack biomineralized structural tissues, and are assumed to have been originally chitinous, occur in a range of marine deposits through the Phanerozoic (Traverse, 1988; Butterfield, 1990, 1994; Briggs and Clarkson, 1989). It is therefore important to determine to what extent the arthropod fossil record reflects greater decay resistance in chitin than in other biomolecules (and whether it is selectively preserved in depositional environments characterized by high productivity and sedimentation rates). Here we describe experiments designed to evaluate the postulated selective preservation of chitin in the marine environment, and its likely role in the extensive fossil record of arthropods. It should be noted, however, that organic remains of arthropods are also widespread in ancient non-marine deposits (Shear and Kukalova-Peck, 1990; Shear *et al.,* 1984; Jeram *et al.,* 1990). Recent analyses of Quaternary Coleoptera have demonstrated that 50 to 75% of the original chitin survives in beetles from late-glacial and last interglacial deposits (Miller *et al.,* 1993).

Laboratory decomposition experiments were carried out on the shrimp *Crangon* under different conditions. The chemical compositions of appropriate samples were analyzed both by flash pyrolysis-gas chromatography-high resolution mass spectrometry (Py-GC-HRMS) and solid-state ^{13}C-NMR.

8.3 EXPERIMENTAL AND ANALYTICAL PROCEDURES

A range of experiments was carried out to investigate morphological decay and biodegradation in shrimps (Briggs and Kear, 1994). The experimental runs described here, however, were carried out independently to monitor the degradation of biomolecules. Live specimens of the common shrimp *Crangon crangon* were obtained by trawling in Plymouth Sound, southern England. They were killed by anoxia, and each transferred singly to a screw-top glass experimental vessel (ointment jar) with 50 ml of standard artificial seawater (ASW) (Briggs and Kear, 1994). The ASW had first been inoculated with water (50 ml/l) and sediment (ca 0.5 ml/l) from the Tay Estuary, Dundee, Scotland. The inoculated ASW was allowed to incubate for at least 48 hours at room temperature, with added yeast extract (0.1 g/l) as a bacterial substrate, before the carcasses were introduced. pH was adjusted to 8.0 by adding small amounts of dilute HCl or NaOH. The Tay Estuary was used as a source of inoculum as this site is characterized by high rates of organic matter degradation as a result of a range of processes, both aerobic, and anaerobic sulfate-reduction (Parkes and Buckingham, 1986; Parkes *et al.,* 1989). Thus a suite of potential degraders was present including those associated with the carcass and with the seawater and sediment.

The survival of chitin was investigated in carcasses decayed under two different sets of circumstances. In the first the ASW was set up with a 50% oxygen saturation at the outset, and the vessel was covered by a screw cap that allowed slow diffusion. Thus although oxygen reached an effective zero saturation in one day as a result of decay, levels gradually recovered, approaching the starting level within 8 weeks but not necessarily recovering to 50% saturation. The pH value fell from a starting level of 8.0 to 7.34 within 3 days, but recovered to 8.00 between 1 to 2 weeks, rising to 8.83 by 8 weeks. In the second set of circumstances, the ASW was completely anoxic, having been degassed with oxygen-free nitrogen before sealing in an aluminum bag with an anaerocult (Merck A, which reduces the O_2 in the air space to zero within an hour). Thus oxygen levels remained at zero; pH levels fell to 6.49 within 3 days and recovered to 8.00 between 2 to 4 weeks, rising to 8.45 by 8 weeks. (These conditions correspond to 1b and 1d respectively of Briggs and Kear (1994).) All the experiments were incubated at $20 \pm 0.5^{\circ}C$ in the dark.

Decay was allowed to proceed for 8 weeks at which point the experimental vessels and contents were flash frozen in liquid nitrogen and freeze-dried. Freshly killed shrimps were treated in the same way as a basis for comparison.

The freeze-dried samples were powdered by using a micro-dismembrator. Aliquots were extracted with a methanol/dichloromethane 1/1 mixture and subsequently analyzed by flash pyrolysis-gas chromatography (Py-GC) and flash pyrolysis-gas chromatography-high resolution mass spectrometry (Py-GC-HRMS). Aliquots of the residues were saponified by refluxing for 1 hour in a 1 N potassium hydroxide - 96% methanol solution. The mixture was acidified to pH 3 with 2 N

hydrochloric acid and the supernatant was removed. The residue was extracted ultrasonically with methanol/ water (1/1 v/v), methanol and dichloromethane. The residues were dried under reduced pressure and analyzed using Py-GC and Py-GC-HRMS.

Analyses of the homogenized, but further untreated samples were performed using solid state ^{13}C NMR. Py-GC, Py-GC-HRMS and NMR data were compared with those obtained from analyses of a commercially produced available chitin isolated from shrimps [Sigma Chemical Co., chitin (poly-N-acetylglucosamine)]. The commercially produced chitin was prepared by dissolving shrimp cuticle chitin in concentrated HCl. After filtration the filtrate was added to a 50% aqueous ethanol solution. Chitin precipitated and was washed several times with water (Skujins *et al.*, 1965).

In addition we analyzed cuticle from a specimen of the fossil shrimp *Antrimpos* from the Jurassic Solnhofen limestone of Germany and cuticle from a specimen of the fossil shrimp *Pseudotealliocaris* from the lowermost Carboniferous at Willie's Hole in southern Scotland (Cater *et al.*, 1989; Briggs and Clarkson, 1989), together with a sample of the associated matrix. The Willie's Hole section (Cater *et al.*, 1989) is interpreted as representing a marine-influenced delta plain. It is not clear whether the shrimps were carried in from the sea, or inhabited the brackish water habitat. These geological samples were subjected to Py-GC and Py-GC-HRMS after extraction with a mixture of methanol/dichloromethane (1/1 v/v).

Pyrolysis-gas chromatography was performed using a FOM-3LX Curie point pyrolysis unit (Boon *et al.*, 1987) connected to the injector of a Hewlett Packard model 5890 series II gas chromatograph. The Curie temperature of the ferromagnetic wires used was 610^0C. The wires were kept at the final temperature for 10 seconds. The oven was programmed from 0^0C (5 min) to 300^0C (10 min) at a rate of 3^0C/min. Separation was achieved by using a fused silica capillary column (25 m x 0.32 mm id) coated with CP-Sil 5 CB (film thickness 0.42 μm). Helium was used as the carrier gas. The same type of pyrolysis unit, gas chromatograph and capillary column were used for the Py-GC-HRMS analyses. The gas chromatograph was connected to a VG Autospec Ultima (mass range m/z 800-50; scan time 1s /decade; cycle time 1.7s; ionization energy 70eV). Characteristic m/z values were selected to generate mass chromatograms in order to study the occurrences and relative abundances of the major classes of products present in the pyrolyzate. All solid state ^{13}C-NMR spectra were recorded on a Varian VXR-400 S at 100 MHz.

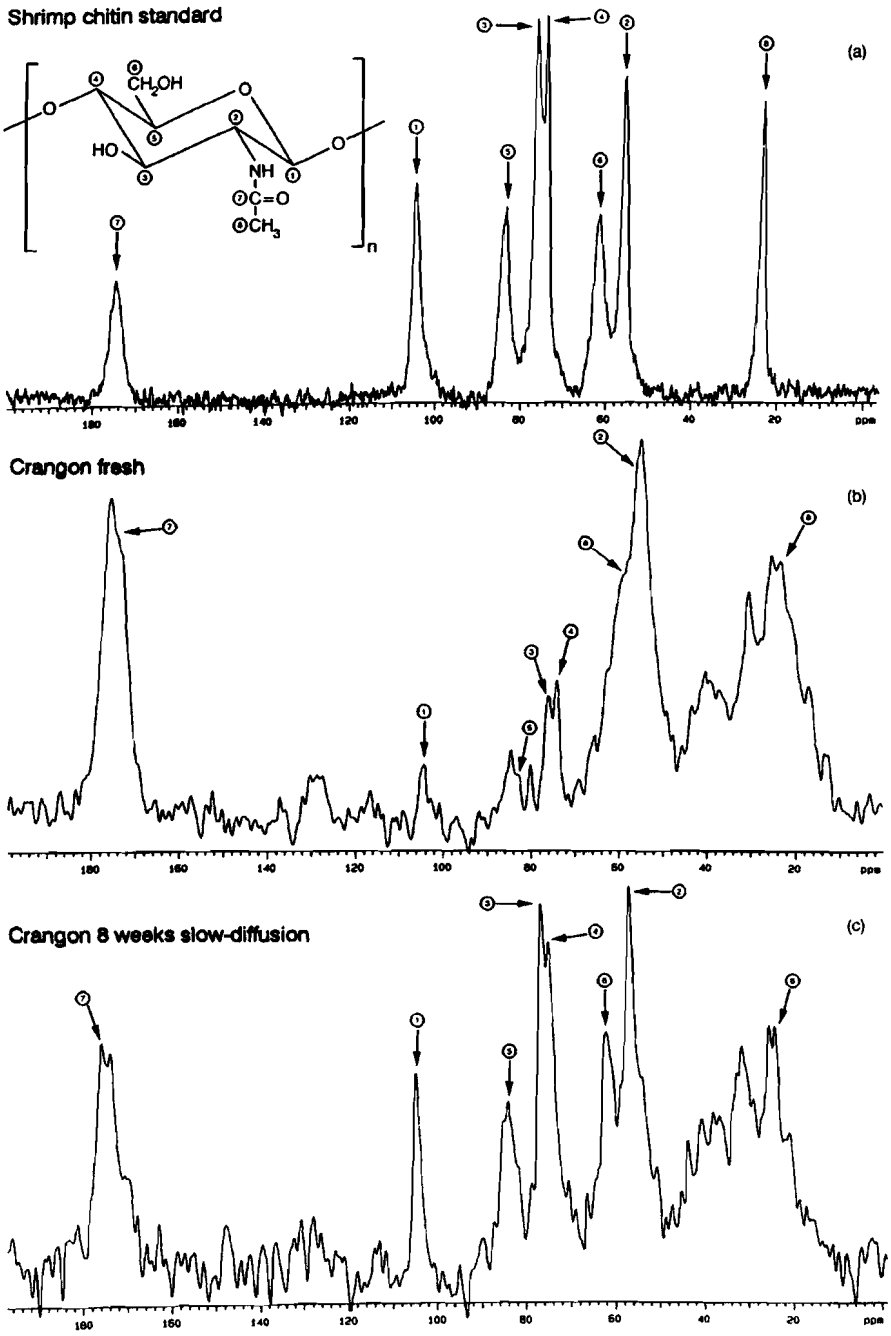

Figure 8.1 ^{13}C-NMR traces of commercially obtained shrimp chitin (a), fresh shrimp (b) and of decayed shrimp after 8 weeks with slow diffusion (c).

8.4 RESULTS AND DISCUSSION

Weight loss and morphological decay

Weight loss was monitored in separate experiments carried out under the same conditions, where the residue was filtered and oven dried (Briggs and Kear, 1994). The major loss occurred during the first week, when the dry weight fell to below 30% of the starting dry weight, and it remained at 20-25% after 8 weeks (anoxic conditions). By this time the exoskeleton of the shrimp had begun to disarticulate and the cuticle had become flaccid and collapsed when removed from the water. The muscles had reached a semi-liquid state and settled to the lowest part of the body cavity. For further details see Briggs and Kear (1994).

^{13}C NMR analysis

Aliquots of the untreated homogenised samples were analysed: a fresh sample, a sample decayed for 8 weeks under conditions of slow diffusion, and a sample of a commercially available chitin standard isolated from shrimps. While peaks with identical chemical shifts in the NMR spectra of all three samples (Figure 8.1) are noted, the NMR spectrum of the standard chitin is much more closely matched by that of the *Crangon* sample following 8 weeks of decay than that of the fresh shrimp. Comparison of the NMR spectra of the fresh *Crangon* sample and that of the sample after 8 weeks of decay clearly indicate that chitin (or a slightly altered decay product) is preserved selectively. Based on these NMR data it is estimated that ca 60% of the residue after 8 weeks represents chitin (this corresponds to 12 to 15% of the original starting dry weight).

Py-GC-HRMS

The shrimp samples were saponified and extracted to remove relatively small amounts of lipids before pyrolysis was performed. This procedure was followed since free lipids, and pyrolysis products of bound lipids, add unnecessarily to the complexity of the pyrolyzate. In a separate experiment we demonstrated that chitin is not affected by this saponification procedure, i.e. the composition of pyrolysis products before and after saponification is identical. Py-GC-HRMS total ion current traces of the pyrolyzate of the shrimp chitin standard, and of the saponified and extracted samples of the fresh shrimp and the shrimp after 8 weeks of anoxic decay, are shown in Figure 8.2. The major pyrolysis compounds obtained from the chitin standard are listed in Table 8.1.

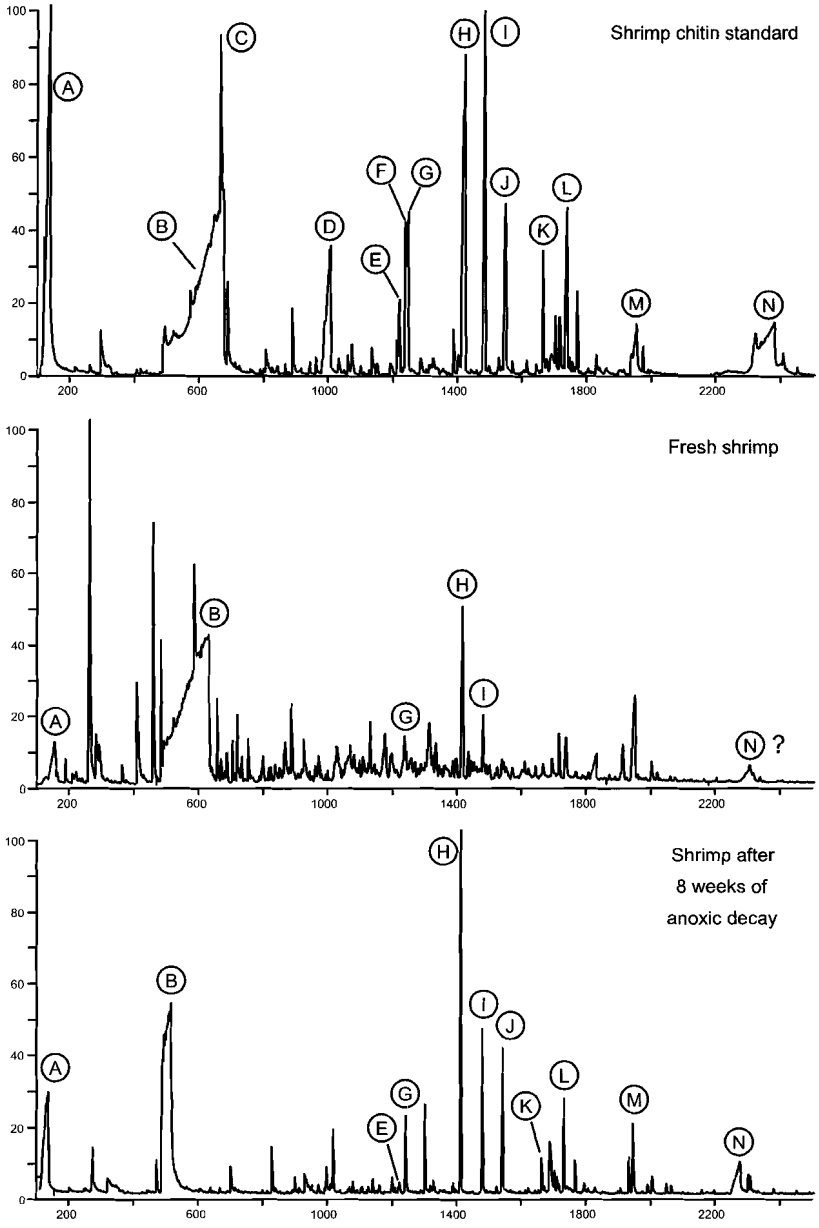

Figure 8.2 Total ion current traces of the shrimp chitin standard, of the fresh shrimp and the shrimp after 8 weeks of anoxic decay.

Table 8.1 Mass spectral data of pyrolysis products obtained via Py-GC-MS of the chitin standard.

com-pound	m/z *base peak*	m/z *values of important fragment ions and their relative abundances (% of base peak intensity)*
A[1]	60	
B[2]	59	
C	79	52 (65), 78 (37), 107 (16)
D[3]	68	98 (98), 96 (82), 53 (74), 97 (50), 70 (34), 52 (33), 69 (21), 55 (13)
E	109	95 (46), 81 (39), 108 (49), 68 (37), 80 (27), 137 (22), 67 (17), 54 (16), 53 (15), 52 (14)
F[4]	108	109 (21), 79 (20), 92 (19), 78 (17), 65 (17)
G	109	95 (60), 137 (50), 68 (44), 81 (43), 108 (27), 80 (26), 67 (20), 54 (18), 52 (14), 53 (10)
H	97	69 (79), 139 (26), 54 (20), 68 (17)
I	111	82 (47), 83 (47), 153 (23), 55 (17), 54 (17), 68 (12)
J	109	80 (80), 81 (40), 151 (21), 53 (14), 108 (14), 54 (14), 52 (12)
K	82	127 (37), 80 (25), 81 (13), 96 (10)
L[5]	125	69 (22), 167 (22), 68 (15), 55 (13)
M	125	110 (96), 167 (63), 84 (37), 83 (30), 124 (21), 96 (19), 54 (18), 55 (15), 69 (14), 126 (12), 53 (14)
N[6]	59	60 (25), 101 (23), 114 (22), 72 (16), 203 (14), 57 (13), 84 (12), 186 (10), 56 (10)

[1] acetic acid; [2] acetamide; [3] levoglucosenone; [4] methyl-*N*-acetyl-2-pyridone; [5] trianhydro-2-acetamido-2-deoxyglucose; [6] 2-(N-acetylamino)levoglucosan

The pyrolyzate of the decayed shrimp sample is remarkably similar to that of the chitin standard; a relatively large number of the major pyrolysis compounds of both are identical. Several compounds are absent, however, (C, D and F) and the relative abundances of the others differ to some extent from the standard.

A direct comparison of the composition of the products of chitin in the pyrolyzates of the fresh and decayed samples is hampered by the enormous complexity of the pyrolyzate of the fresh sample. Hence mass chromatograms selectively indicating the pyrolysis products of chitin were constructed from m/z traces including masses 59, 60, 67, 79, 80, 82, 97, 111 and 125 to facilitate comparison and interpretation (Figure 8.3). These mass chromatograms show that the original chitin has been transformed. New pyrolysis compounds J, K and L and M, which were absent in the original pyrolyzate, are present in the decayed sample. Products J, K, L and M are also present in the pyrolyzate of the chitin standard, where their presence may reflect the treatment used to obtain the standard chitin from shrimps. The manufacturers treatment of shrimps with concentrated HCl may have caused a partial deacetylation of the chitin (van Waveren, 1993; Richards, 1951) together with a possible decrease in molecular weight.

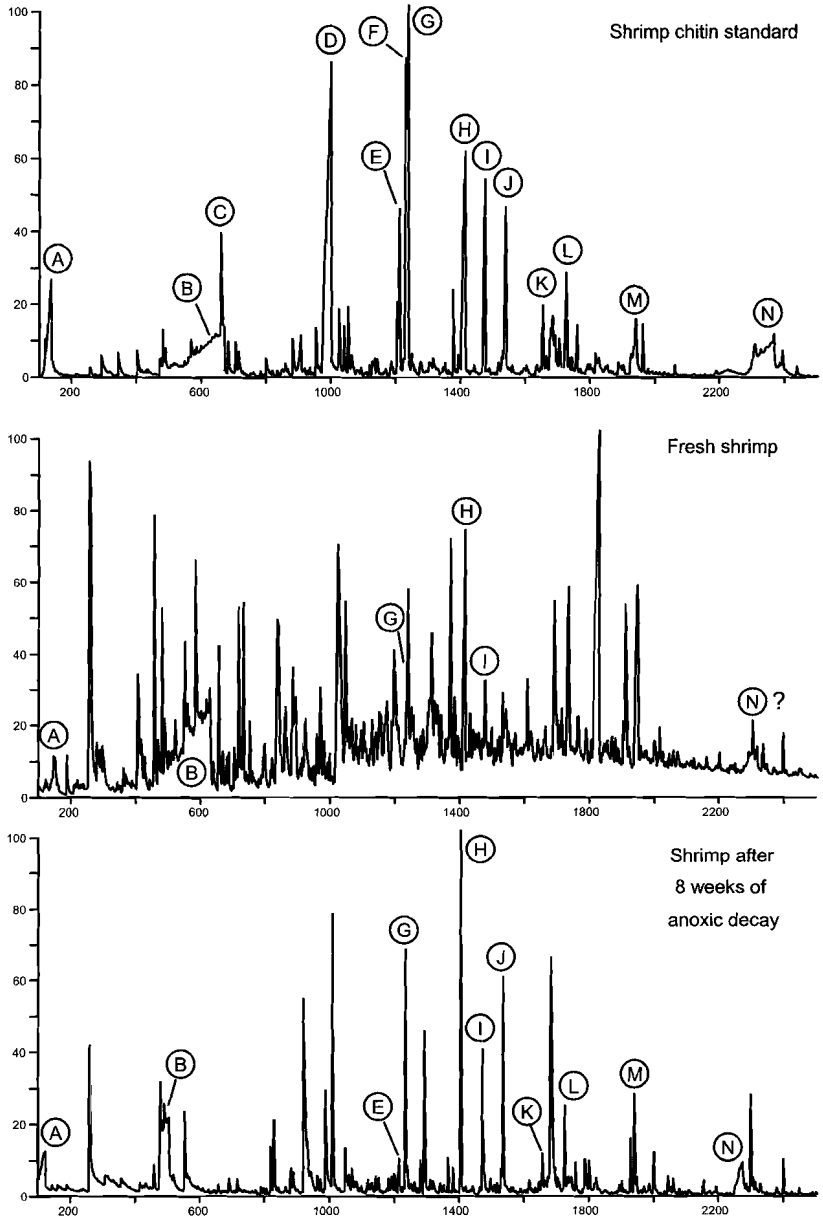

Figure 8.3 Cumulative mass chromatograms of *m/z* 59, 60, 67, 79, 80, 82, 97, 111 and 125 of the shrimp chitin standard, of the fresh shrimp, and the shrimp after 8 weeks of anoxic decay.

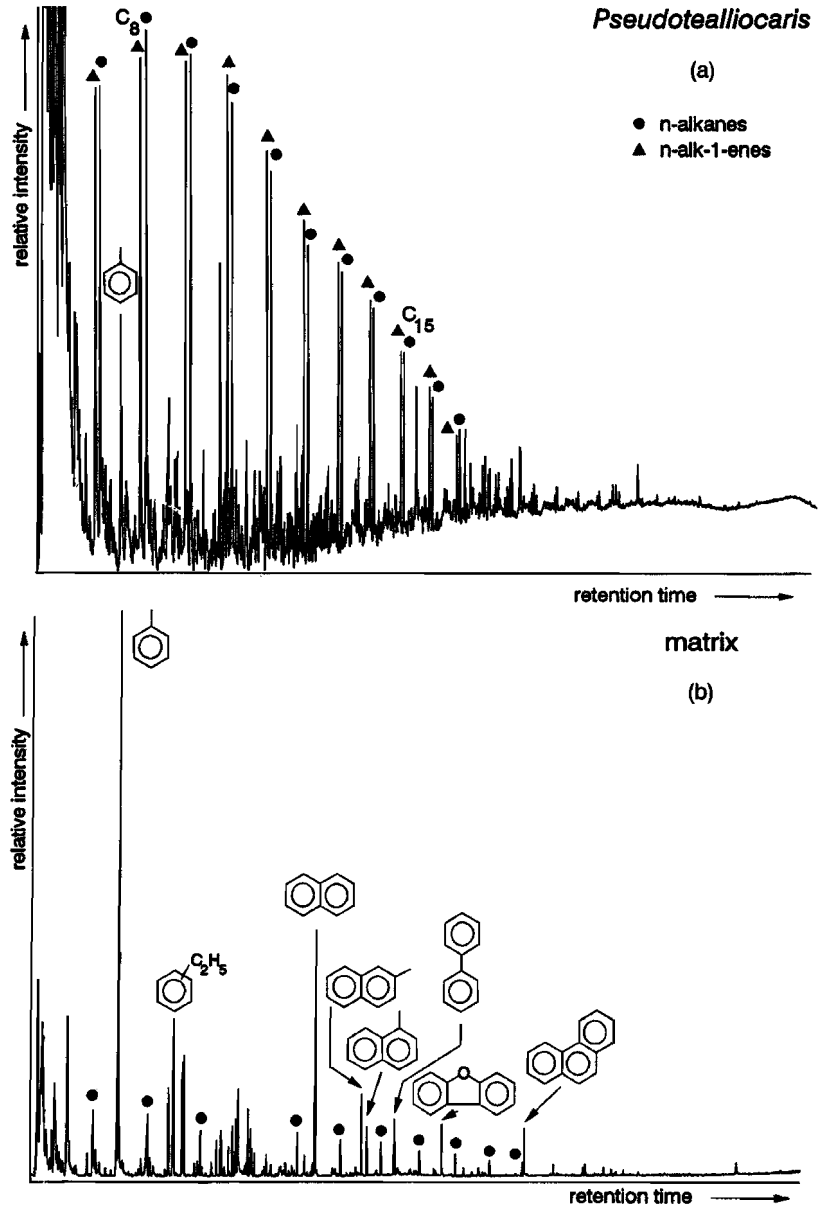

Figure 8.4 Gas chromatogram of the pyrolyzate of the *Pseudotealliocaris* sample (a) and the total ion current trace of the matrix of the *Pseudotealliocaris* sample (b).

The presence of J, K, L and M in the 8 week shrimp sample suggests that decay may also result in some deacetylation and hydrolysis. This interpretation is supported by the decrease of acetamide (compound B) in the pyrolyzate of the decayed shrimp compared to that of the fresh one.

Figure 8.4 shows the GC and TIC traces of the pyrolyzates of the cuticle of *Pseudotealliocaris* (mechanically removed from the fossil arthropod using a modified dental drill) from the lowermost Carboniferous and its sedimentary matrix, respectively. The pyrolyzate of the fossil shrimp is characterized by homologous series of n-alkenes and n-alkanes possibly indicating the presence of a highly aliphatic resistant biomacromolecule (Tegelaar et al, 1989a). The pyrolyzate of the sedimentary matrix is completely different and indicates that the organic matter is relatively aromatic in nature. Thus although the organic matter in the fossil material is different from that in the sedimentary matrix neither shows any characteristic pyrolysis products of chitin. Hence, we must conclude that chitin is absent. Pyrolysis data from the Jurassic *Antrimpos* sample firmly support this conclusion because chitin pyrolysis products are again completely absent.

8.5 CONCLUSIONS

Both the ^{13}C-NMR data and the Py-GC-HRMS data clearly indicate that chitin is preserved selectively during decay, although its structure may change slightly. The lack of evidence for the presence of chitin in the fossil samples suggests that the longer term preservation of chitinous tissues is not associated with chitin or transformed chitin but may involve a gradual substitution of chitin by more resistant organic matter derived from other sources.

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CHAPTER 9

Molecular and Carbon Isotope Characterization of North Sea Macromolecular Dissolved Organic Matter (Part I)

9.1 ABSTRACT

The North Sea was chosen as a study area to molecularly characterize ultrafiltered dissolved organic matter (UDOM). Six different samples were selected, three with a possibly significant terrestrial influence and three with a minor terrestrial contribution. The UDOM in these samples was concentrated using cross-flow ultrafiltration and represented the size fraction between 1000 Dalton and 0.2 μm. The samples were analyzed by analytical pyrolysis, stable and radiocarbon isotopes measurements and by solid-state ^{13}C-NMR. All samples revealed a dominant presence of altered polysaccharides moieties. UDOM was isolated with recoveries before diafiltration between 23 and 50% and final recoveries between 9 and 20% of total DOC. The $\delta^{13}C$ isotope values were in the range of –23 to –21‰, typical for oceanic DOM and excluding a significant terrestrial contribution. The ^{14}a values of the UDOM samples were in the range from 90 to 103% pMC, indicating a very recent origin, pointing to an autochthonous marine origin. Overall, the UDOM analyzed in this study seemed to be mainly refractory in nature, although the UDOM from the samples with a suspected terrestrial influence contained some fresh material.

9.2 INTRODUCTION

Marine dissolved organic matter (DOM) is one of the major reactive reservoirs of organic matter on Earth (Hedges, 1992). Given the importance of marine DOM to the global carbon cycle, the aim of the present study is to determine the origin and fate of DOM. Stable carbon isotope measurements have indicated that DOC in the deep ocean is predominantly autochthonous ($\delta^{13}C$ = -24 to -21‰; Williams and Gordon, 1970). The fact that only extreme low concentrations of terrestrial biomarkers such as lignins could be detected in the humic fraction of DOM from the pelagic Pacific Ocean (Meyers-Schulte and Hedges, 1986), further suggests a marine origin of DOM. Studies by Meyers-Schulte and Hedges (1986) and Williams and Druffel (1988) further show that marine DOM is comprised mainly of polymeric materials derived from marine sources.

Radiocarbon isotope analyses indicated that DOC in the deep ocean is relatively old ($\Delta^{14}C$ = 46% pMC; Williams and Druffel, 1987). The mean average age of (DOC) is over 1000 years (Kirchman *et al.,* 1991). It is assumed that DOC consists of a labile fraction with a high turnover rate and a refractory fraction. The labile fraction could serve as a source of nutrition for the microbial loop (Kirchman *et al.,* 1991). The turnover rate of marine DOC is highly variable. It varies from less than a day up to more than 1000 years. Williams and Druffel (1987) calculated that in the central North Pacific Ocean upper mixed layer water 56% of the DOC was less than 30 years old and that the remaining 44% was up to more than 6000 years old. The refractory fraction thus probably consists mainly of constituents, which have been selectively preserved in the water column. In a recent study of estuarine DOM by van Heemst *et al.* (2000a) it was suggested that the age (and thus degree of refractivity) of the different size fractions of DOM is reciprocally proportional with its fraction size.

In the present study we have investigated DOM from the North Sea. The North Sea was chosen as a study area, mainly because the North Sea is a well-studied shelf sea with relatively high DOC concentrations. At several locations of the North Sea, UDOM was isolated and analyzed by means of stable and radiocarbon isotope measurements, solid-state ^{13}C-NMR and molecular characterization using analytical pyrolysis.

9.3 EXPERIMENTAL

Site description

The North Sea (Figure 9.1) is a semi-enclosed shelf sea with a total upper surface area of 575,000 km^2 located in the northwestern part of Europe (de Haas, 1997). In the southern part of the North Sea, the water depth is in the order of 40-50 m. In the northern part the water depth reaches 200 m, at the shelf edge. Important topological features in the North Sea are the Norwegian Channel and the Skagerrak. The water depth in the northern part of the Norwegian Channel is over 400 m. The Norwegian Channel is separated from the Skagerrak by a sill at 280 m water depth. The deepest point in the Skagerrak is more than 750 m.

Exchange of North Sea and Atlantic Ocean waters occurs through The Channel in the southern part of the North Sea. Between Scotland and the Shetlands and between the Shetlands and Norway, the North Sea has an open connection with the Norwegian Sea. Through the Kattegat and Skagerrak, relatively low saline Baltic Sea outflow waters enter the North Sea. The dominantly westerly winds, the tidal motion and the shape of the North Sea basin result in a counterclockwise residual water circulation. During winter the water mass of the North Sea is totally mixed. In summer the waters in the southern part are mixed as well, while in the northern part the waters are stratified. Together with the water from the bordering seas and from rivers flowing into

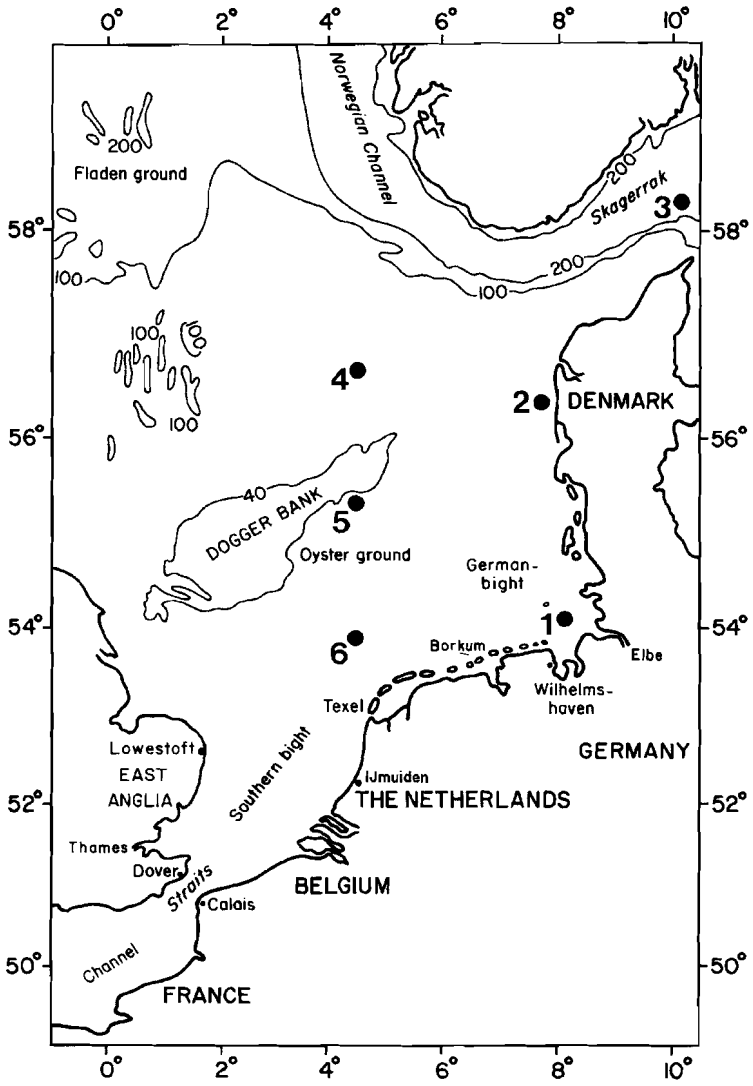

Figure 9.1 Map of the sampling area.

the North Sea, sediments and attached organic matter enter the system. Furthermore, sea floor and coastal erosion, primary production and atmospheric input contribute to the sediment and organic load of the North Sea. Organic matter accumulation, *i.e.* sedimentation, mainly occurs along the eastern margin of the North Sea, because of the counterclockwise residual circulation.

Sample description

During a cruise with the RV *Pelagia* at the North Sea in November 1995, six surface water samples were taken and processed on-board (Figure 9.1; Table 9.1). Samples were taken in the German Bight (sample 1), a few miles west of the coast off Denmark (sample 2), in the Skagerrak (sample 3), in the central North Sea (two stations, samples 5 and 6) and at the Friesian Front (sample 6). In all cases, 160 L of water was collected, except for sample 1, for which 120 L was collected.

Table 9.1 Hydrographic data.

sample	*location*	*volume (L)*	*depth (m)*	*salinity (‰)*	*temperature (°C)*
1	54°06'N; 08°10'E	120	2.3	29.11	7.6
2	56°23'N; 07°51'E	160	2.2	33.22	9.4
3	58°12'N; 10°15'E	160	9.8	32.72	6.8
4	56°40'N; 04°30'E	160	n.a.	n.a.	n.a.
5	55°22'N; 04°30'E	160	5.7	34.95	11.1
6	53°43'N; 04°31'E	160	2.7	34.26	11.5

n.a. = not analyzed

Sample collection and processing

Water samples were collected using a CTD having temperature, conductivity, dissolved oxygen, fluorescence and pressure sensors. An aluminum rosette containing 24 12-L Niskin bottles was used to obtain water samples from predetermined depths. The CTD and rosette were deployed on a 3-conductor cable allowing for the real-time display of data and acquisition, and for tripping the bottles in areas of interest in the water column. Immediately after sampling, the water samples were poured through a 63-μm mesh net in order to remove large particles and prefiltered through 0.2-μm pore sized Sartorius regenerated cellulose filters using 0.5 bar helium pressure in order to separate DOM from POM. The prefiltered samples were subsequently concentrated using a custom-made Amicon SP-60 tangential-flow ultrafiltration system equipped with Amicon spiral-wound polysulfone 1000 Dalton cut-off filters. Tangential-flow ultrafiltration is a relatively new method, that is applied successfully to concentrate high molecular weight DOM from seawater (Benner, 1991; Benner *et al,* 1992). The samples were concentrated to about 2 L and were stored frozen at -20°C for transport to the laboratory. In the laboratory, the major part of the salts were removed from the concentrated samples by diafiltration with 20 L of distilled water. Although generally about 10% of the DOC is lost during diafiltration (Benner, 1991), it is essential to remove most of the salts before the analyses by analytical pyrolysis. Subsequently,

water was removed by evaporation *in vacuo* at 30°C using a rotary evaporator followed by freeze-drying of the samples.

DOC sampling

During the sample processing procedure 10-mL samples were taken in duplicate for measuring DOC. Samples were taken from the filtrates after prefiltration, ultrafiltration and diafiltration, from the retentates after ultrafiltration and diafiltration and from the distilled water used for diafiltration. The samples were stored in precombusted glass ampoules at 5°C after acidifying with 4 drops of 40% phosphoric acid.

DOC measurements

A High Temperature Catalytic Oxidation (HTCO) method was used for determination of DOC concentrations. The method is described in detail by Wiebinga and de Baar (1998). The HTCO is based on the method described by Sugimura and Suzuki (1988). A modified Ionics Model 555 carbon analyzer with a pure platinum gauze catalyst at 775°C was used for direct converting DOC to CO_2. Samples of 100 µL were injected into a quartz tube containing the catalyst. A stream of ultrapure oxygen gas carried the water vapor and combustion gases through a series of traps before entering a Li-Cor Model LI-6252 CO_2-analyzer. The signal generated by the non-dispersive infrared (NDIR) detection of CO_2 is quantified as peak area on a Shimadzu Model C-R6A integrator.

The concentration of DOC in each sample was determined by calibration against standard addition curves of potassium phthalate in MilliQ water (0 up to 1667 mM). The slope of the line was used as a conversion factor for assessing TOC in real samples. The data have not been corrected for any blank.

Solid-state ^{13}C NMR

Dried samples of ultrafiltered dissolved organic matter (UDOM) were subjected to solid-state ^{13}C NMR by placing them in a 9-mm diameter ceramic rotor packed mostly with Teflon tape as a filler. The small amount of material, generally 50 mg dry weight per sample (TOC of only 7 to 19%; Table 9.1), necessitated such an approach to optimize the weak signals. The rotor was spun at 3.5 kHz at the magic angle (54.7°) in the probe of a Chemagnetics, Inc., M-100 spectrometer operating at 25.2 MHz for carbon. The standard cross-polarization pulse sequence with 1 ms contact time and 0.7 s cycle time was used and approximately 10^5 acquisitions were accumulated to obtain the spectra. Exactly 512 data points were acquired and zero-filled to 4096 data points prior to filtering with 75 Hz linebroadening and Fourier transformation. The chemical shifts were referenced externally to hexamethylbenzene.

Determination of C_{org}, $\delta^{13}C$ and ^{14}a values (AMS)

Dried samples of UDOM were acidified with dilute hydrochloric acid to remove carbonates, dried over KOH and placed in quartz tubes together with oxidized copper wire. The tubes were evacuated and sealed and placed in a furnace at 800°C for 6 hours. The combustion gases were led over a copper oven at 600°C and a silver oven at 400°C to remove nitrogen oxide, sulfur oxides and halogens. Carbon dioxide was trapped in liquid air. The amount of CO_2 was determined by expanding the gas into a known volume and measuring its pressure.

$\delta^{13}C$ values were determined with a VG SIRA 9 isotope ratio mass spectrometer. The carbon dioxide was reduced to graphite with hydrogen in the presence of an iron catalyst at 600°C. The resulting graphite/catalyst mixture was pressed into a target for the Tandem-Accelerator Mass Spectrometer (AMS; HVEE BV, Amersfoort; Aerts-Bijma *et al.*, 1997; Wijma and van der Plicht, 1997).

From the measured ^{14}C:^{12}C ratio (^{14}R) of the sample and a known standard the ^{14}a value, expressed as percent of modern carbon (pMC), is calculated as: $^{14}a = (^{14}R_{sample}/^{14}R_{standard}) * \{(1- 25\ ‰)/(1 + \delta^{13}C)\}^2 * 100$ pMC. The standard to which ^{14}C data are referred to is oxalic acid from 1950. According to the official definition by Stuiver and Pollach (1977) a correction has to be made for the time of measurement after 1950, but the effect of this is negligible. The ^{14}a value is related to the age of the sample, since due to radioactive decay ($T_{1/2}$ = 5730) ^{14}C is disappearing from the sample over time. Because the ^{14}C concentration is also affected by isotopic fractionation, a correction is made with the factor $(1-25‰)/(1+\delta^{13}C)$.

When comparing marine and terrestrial samples, samples with the same age do not have the same ^{14}a value because the carbon source for organisms in the ocean usually has a lower ^{14}C:^{12}C ratio than CO_2 in the atmosphere, due to the so called reservoir effect. Therefore, and because the measured samples are probably mixtures of components with different ages, ^{14}a values are reported instead of ages.

Curie-point pyrolysis-gas chromatography (CuPy-GC)

Curie-point pyrolysis-gas chromatography (CuPy-GC) analyses were performed using a Hewlett-Packard 5890 gas chromatograph, equipped with a FOM-4LX pyrolysis unit (Boon *et al.*, 1987) and a cryogenic unit. A 25 m fused silica capillary column coated with chemically bound DB 1701 (0.253 mm i.d., film thickness 0.25 µm) was used with helium as a carrier gas. A flame ionization detector (FID) at 320 °C was used for detection. The temperature program was as follows: initial temperature 0°C (5 min); heating rate 3°C/min; final temperature 300°C (10 min). Samples were pressed onto flattened ferromagnetic wires (Curie temperature 610°C) and placed into the pyrolysis unit. The pyrolysis unit was connected to a FOM high frequency generator that heated

the wires inductively in 0.15 s to the Curie temperature. This temperature was maintained for 9 s.

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC/MS)

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC/MS) analyses were carried out in the same way as the CuPy-GC analyses. A mass spectrometer was used for detection instead of a FID. The Hewlett-Packard 5890 gas chromatograph was connected to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of *m/z* 700-35 and a cycle time of 1 s.

9.4 RESULTS AND DISCUSSION

DOM from the North Sea samples was concentrated and isolated using the method of tangential-flow ultrafiltration. Benner *et al.* (1992) and van Heemst *et al.* (2000a) have shown that tangential-flow ultrafiltration is a successful method used in recovering DOM from seawater and estuarine waters. It concentrates compounds on the basis of molecular size and shape. Ultrafiltration (using 1000 Dalton cut-off filters) has been shown to isolate a much larger fraction (up to ~30%) of seawater DOM (Benner *et al.*, 1992) than conventional DOM isolation methods. The 70% that is not isolated contains particles smaller than 1000 Dalton. Therefore, the isolated ultrafiltered DOM (UDOM) is not actually dissolved, but more of a colloidal nature. In Table 9.2 the amounts of dry weights of the recovered North Sea UDOM samples are listed together with the calculated ultrafiltered DOC concentrations from the percentages of organic carbon present in the UDOM samples.

Table 9.2 DOC, $\delta^{13}C$ and ^{14}C data.

sample	*UDOM powder (mg)*	*% Corg UDOM powder*	*UDOC (mg C)*	*$\delta^{13}C$ (‰)*	*^{14}C (%)*
1	609	6.3	39	-22.9	99
2	572	5.7	33	-21.0	103
3	493	5.0	25	-22.3	99
4	552	2.9	16	-22.2	96
5	859	1.6	14	-23.0	90
6	1199	1.4	17	-22.3	93

A net current along the Dutch, German and Danish coast transports vast amounts of material discharged from a number of large rivers like the Meuse, Rhine and Elbe through the latter area. This is reflected in the salinity (Table 9.1). Salinities for water samples 1-3 (Figure 9.1; Table 9.1) are much lower than those of the water samples 5-6 (Figure 9.1; Table 9.1), which suggests mixing of seawater with some freshwater. Furthermore, temperatures of water samples 1-3 are a few degrees lower than temperatures of water samples 5-6, which is probably an effect of the mixing of seawater with relatively colder river water. In November in Middle and Northern Europe, river water is much colder than the water in the North Sea. Samples 1-3 may therefore represent DOM with substantial terrestrial input and samples 4-6 may therefore represent DOM with only minor terrestrial input. Thus, discrimination may be made between a marine and a land-derived signal.

DOC recoveries

Typically, amounts of 500-1200 mg of UDOM powder were recovered from the North Sea water samples (Table 9.2). The organic carbon contents were ranging from 1.4 up to 6.34%. From the total amount of UDOM powder and its organic carbon content, the total amount of isolated organic carbon (UDOC) was calculated. The pure marine samples (4-6) have recoveries of 14-17 mg C from 160 L of seawater and the samples with a potential terrestrial influence (1-3) have recoveries of 25-50 mg C from 160 L of seawater. The UDOC recovery of sample 1 is 38.62 from 120 L seawater (Tables 9.1 and 9.2). Corrected to 160 L, this represents a recovery of 51.5 mg C.

The amounts of organic carbon were monitored during the UDOM isolation process (Table 9.3). Again, there was a clear distinction between samples 1-3 and 4-6. The amounts of DOC in samples 1-3 are higher than those in samples 4-6. For a further comparison, total amounts of organic carbon were calculated from the DOC concentration and the volume of the water sample (Table 9.3). Amounts of DOC in the original seawater were ranging from 143.2-213.7 mg C, which corresponds to DOC concentrations from 75-200 μM C.

The recovery after the first concentration step, reducing the volume of the water sample from 160 L (120 L for sample 1) to 2 L, is a measure for the total carbon balance. This recovery was calculated by simply adding all the organic carbon present after the concentration step (*i.e.* the carbon present in the filtrate and the retentate) and dividing it by the total amount of organic carbon in the original sample. Needless to say, this recovery should be 100%, assuming there is no loss or contamination. Taking into account the accuracy of the DOC values and especially the accuracy of measuring exact sample volumes, the values of the recovery of most of the samples were around 100% (Table 9.3). The recovery of samples 1 and 6 appear too high. It is assumed that the filtrate of sample 6 was contaminated.

Table 9.3 DOC recoveries.

sample	*SW (mg C)*	*F (mg C)*	*R (mg C)*	*Rec. (%)*	*UDOC rec. (%)*	*F(df) (mg C)*	*R(df) (mg C)*	*UDOC rec. (df) (%)*	*UDOC powder (mg C)*	*UDOC rec. (powder) (%)*
1	193.1	168.7	77.7	*128*	*40.2*	17.5	47.5	*24.6*	38.62	*20.0*
2	213.7	160.0	57.6	*101*	*27.0*	8.6	33.0	*15.4*	32.80	*15.3*
3	187.6	145.3	47.9	*103*	*25.5*	8.7	24.7	*13.2*	24.50	*13.1*
4	179.7	120.6	39.8	*89.3*	*49.7*	11.6	21.6	*12.0*	15.83	*8.81*
5	143.2	129.8	33.4	*114*	*23.3*	8.5	17.2	*12.0*	13.75	*9.60*
6	157.6	390.9	37.9	*272*	*24.0*	11.9	18.4	*11.7*	16.79	*10.7*

Key: SW = amount of DOC in the original seawater sample; F = amount of DOC in the filtrate after concentration; R = amount of DOC in the retentate after concentration; Rec. = recovery of all DOC (Rec. = 100% x (F + R)/SW); UDOC rec. = recovery of DOC after utrafiltration; F(df) = amount of DOC in the filtrate after diafiltration with 20 L distilled water; R(df) = amount of DOC in the retentate after diafiltration; UDOC rec. (df) = recovery of DOC after diafiltration; UDOC powder = amount of DOC in the UDOM powder (see Table 9.2); UDOC rec. (powder) = final recovery of DOC.

The recovery of the size-fraction from 1000 Dalton to 0.2 μm was in the range of 23.3-49.7% (Table 9.3). This value is usually reported in the literature (*e.g.* Benner *et al.,* 1992) as being the "DOC efficiency", a measure for the amount of UDOC.

Following the concentration of DOC using ultrafiltration, the samples were diafiltered using 20 L of distilled water. As shown in Table 9.3, there is a significant loss of organic carbon during the diafiltration process. The recovery of UDOC after ultrafiltration followed by diaflitration is about 50% of the recovery of UDOC after ultrafiltration. Rotary evaporation followed by freeze-drying of the sample after diafiltration only causes a minor loss of organic carbon (Table 9.3). UDOC of samples 1-3 represented 13.1-20.0% and UDOC of samples 4-6 represented 8.8-10.7% of total DOC, present in the water samples. Although a significant amount of DOC was isolated by the method used in this study, the major part of DOC could not be recovered and was therefore destined to remain uncharacterized.

Determining a final recovery of UDOC is much more realistic and provides more detailed information than only reporting a recovery during an intermediate stage (after the first concentration step) of UDOC reported in other studies (*e.g.* Benner *et al.,* 1992) ignoring dramatic losses during further processing of the sample.

Isotope data

All values of $\delta^{13}C$ of the North Sea UDOM revealed similar numbers (Table 9.2). The numbers are in the range of –23 to –21‰, which is in the range (-24 to -21‰) of typical $\delta^{13}C$ values for oceanic UDOM, reported in the literature (Williams and Gordon, 1970). Based on the $\delta^{13}C$ values, no significant terrestrial input is observed in any of the samples. The values for $\delta^{13}C$ are in the same range as the values determined for total DOC (from –24 to –19) of the same samples (le Clercq *et al.,* 1997).

The ^{14}a values of the North Sea UDOM were all in the same range as well (Table 9.2), *i.e.* from 90 to 103% pMC indicating a very recent origin, pointing to autochthonous marine origin. No specific trend is detected in these values.

Solid state ^{13}C-NMR results

The North Sea UDOM samples all revealed similar ^{13}C-NMR spectra (Figure 9.2). The major signal in the NMR spectrum of all samples is observed at a chemical shift of 72 ppm. This signal can be attributed to hydroxylated aliphatic carbons, present in carbohydrates and alcohols. The signal, present at a chemical shift of 101 ppm can be partially attributed to carbohydrate moieties as well. This signal is assigned to both aromatic carbons and the anomeric carbon in carbohydrates. The extensive signal at 175 ppm is assigned to carboxyl and amide carbons. The signal with a chemical shift of 164 ppm reveales the presence of inorganic carbon in the form of carbonates. This signal was strong in the UDOM samples with a relatively low organic carbon content, such as samples 5 and 6. Furthermore, the NMR spectra of all UDOM samples revealed the presence of some aliphatic moieties (signals at 0-60 ppm).

Overall, not much difference was observed in the NMR spectra of the different UDOM samples. The spectra revealed the presence of vast amounts of carbohydrate moieties, in agreement with the analytical pyrolysis results, discussed hereafter. It is noteworthy that the ^{13}C-NMR spectra of UDOM from the North Sea presented in this study show a remarkable similarity to the ^{13}C-NMR spectra of UDOM from the Pacific Ocean (Benner *et al.,* 1992).

CuPy-GC/MS results

Chromatograms of the pyrolyzates of the North Sea UDOM samples are shown in Figure 9.3. The pyrolyzates are dominated by alkylphenols, alkylbenzenes, alkylpyrroles and pyrolysis products of carbohydrates (alkylcyclopentenones, alkylcyclopentanones, alkylfurans and alkylcyclopentadienes). Unaltered polysaccharides yield vast amounts of acetic acid, alkylfurans, alkylfuraldehydes and deanhydromonosaccharides like levoglucosenone, 1,4:3,6-dianhydro-α-D-glucopyranose and levoglucosan (Pouwels *et al.,* 1989; Pastorova *et al.,* 1994) upon pyrolysis. The presence of relatively large

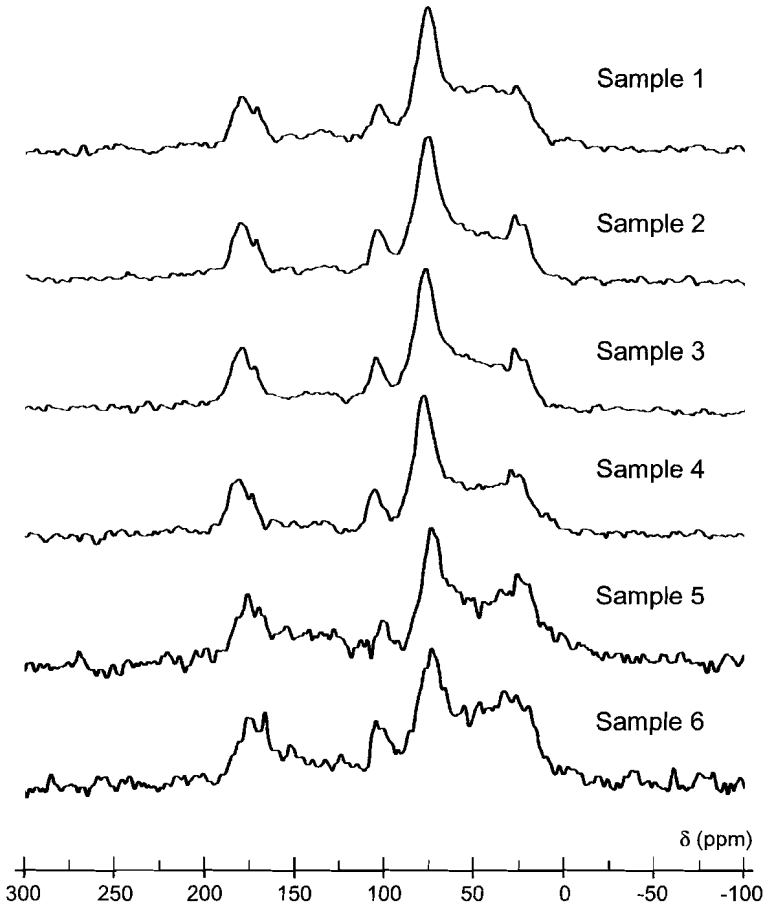

Figure 9.2 Solid-state ^{13}C NMR spectra.

amounts of salts in the UDOM samples may influence the formation of certain compounds upon pyrolysis, like the deanhydromonosaccharides.

Overall, no major differences between the chromatograms of the pyrolyzates of the samples are observed. The composition of the UDOM constituents in the North Sea does therefore not seem to be very different throughout the North Sea.

Aliphatic amino acid moieties in peptide and protein material show very characteristic pyrolysis products, which can be monitored by mass chromatography of masses *m/z* 195+209 (Boon and de Leeuw, 1987). These pyrolysis products are indicative for the presence of proteins and were encountered in the pyrolyzates of samples 1-4, but not in the pyrolyzates of samples 5 and 6. The pyrolyzates of samples 1-4 also reveal the presence of relatively large amounts of acetamide and aminosugars,

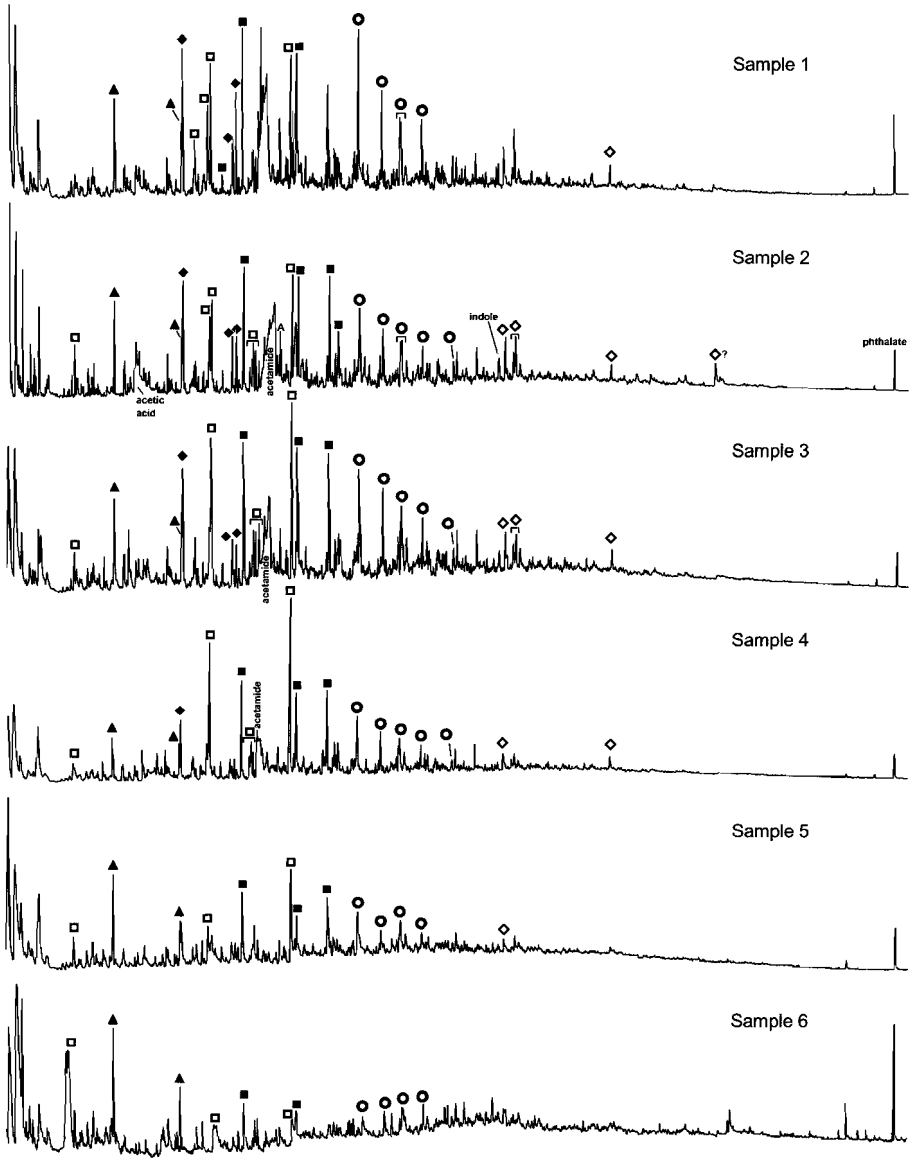

Figure 9.3 Chromatograms of the pyrolyzates (610°C) of the North Sea UDOM samples. Key: triangles = benzenes; solid diamonds = pyrroles; open squares = furaldehydes; solid squares = cyclopentanonens and cyclopentenones; open circles = phenols; open diamonds = chitin markers.

which are indicative for the presence of proteins and/or chitin. The presence of chitin in the UDOM samples is unlikely, since the preservation potential of chitin is fairly low (de Leeuw and Largeau, 1993; Poulicek and Jeuniaux, 1991). It is usually not observed in the refractory part of DOM, although chitin is the component of crustaceans that is most resistant to degradation (Baas *et al.,* 1995). Therefore, it is more likely that acetamide and the aminosugars have a different precursor, such as bacterial peptidoglycan (McCarthy *et al.,* 1998).

No large amounts of lipids such as fatty acids, phytadienes and sterols were encountered in any of the UDOM pyrolyzates. The chromatograms of the pyrolyzates show that UDOM of the three samples, that were taken from the current along the eastern coast of the North Sea are very similar. They also show a clear difference from the UDOM of the samples that were taken from the southward current from the Atlantic Ocean into the North Sea.

Mass chromatography was used to monitor specific chemical classes of compounds, *i.e.* alkylphenols (Figure 9.4), pyrolysis products of (partly) altered polysaccharides (Figure 9.5) and alkylpyrroles (Figure 9.5). The mass chromatograms, origins and geochemical importance of the different compound classes are discussed in the following paragraphs.

Alkylphenols

Alkylphenols have been encountered in many pyrolyzates of marine DOM and POM samples from different locations (van Heemst *et al.,* 1993 and 2000a; Sicre *et al.,* 1994; Peulvé *et al.,* 1996a). Mass chromatograms reflecting C_0-C_2 alkylphenols in the pyrolyzates of all six North Sea UDOM samples are shown in Figure 9.4. The pyrolyzates of the samples show very similar alkylphenol distribution patterns. Their distribution patterns are very similar to those of alkylphenols in the pyrolyzates of oceanic DOM like the Pacific Ocean DOM (van Heemst *et al.,* 1993; 1996), suggesting similar precursors. Alkylphenols in pyrolyzates are generally formed upon pyrolysis of lignins (Meuzelaar *et al.,* 1982; Saiz-Jimenez and de Leeuw, 1986b) or degraded lignins (*e.g.* Saiz-Jimenez and de Leeuw, 1984a). In pyrolyzates of lignins and degraded lignins, methoxyalkylphenols and dihydroxyalkylbenzenes are encountered in addition to alkylphenols. However, in the pyrolyzates of the 4000 m-sample from the Pacific Ocean, neither methoxyalkylphenols nor dihydroxyalkylbenzenes were observed. Therefore lignin was excluded as major precursor of the alkylphenols present in the UDOM pyrolyzates studied. In pyrolyzates of proteins, alkylphenols have been encountered (Tsuge and Matsubara, 1985; van Heemst *et al.,* 1999). It has been shown recently that these phenols can originate from macromolecules rich in tyrosine moieties resulting from degradation of proteins (van Heemst *et al.,* 1999). Hence, the precursors of the alkylphenols encountered are likely to represent heavily degraded proteins either brought

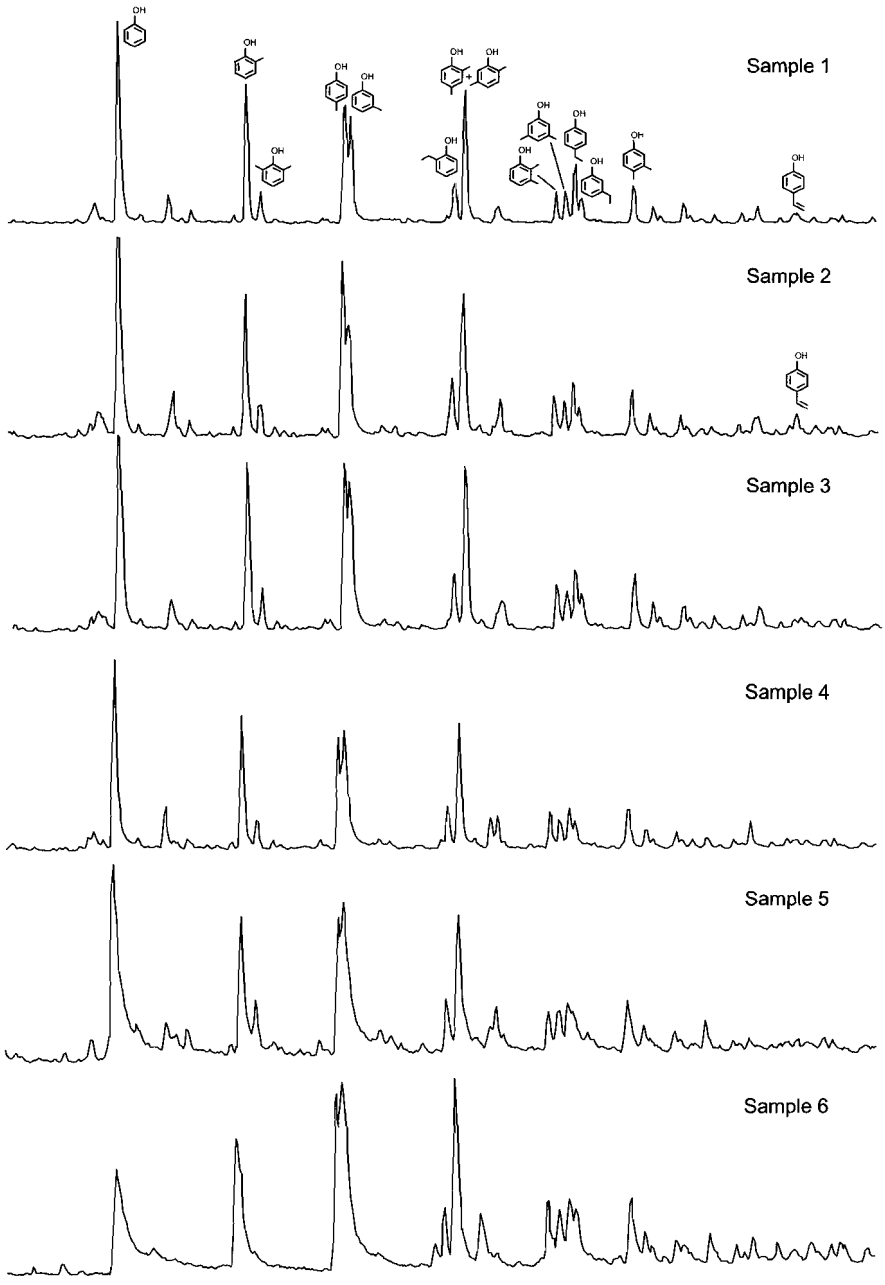

Figure 9.4 Mass chromatograms (*m/z* 94+107+108+121+122) of alkylphenols.

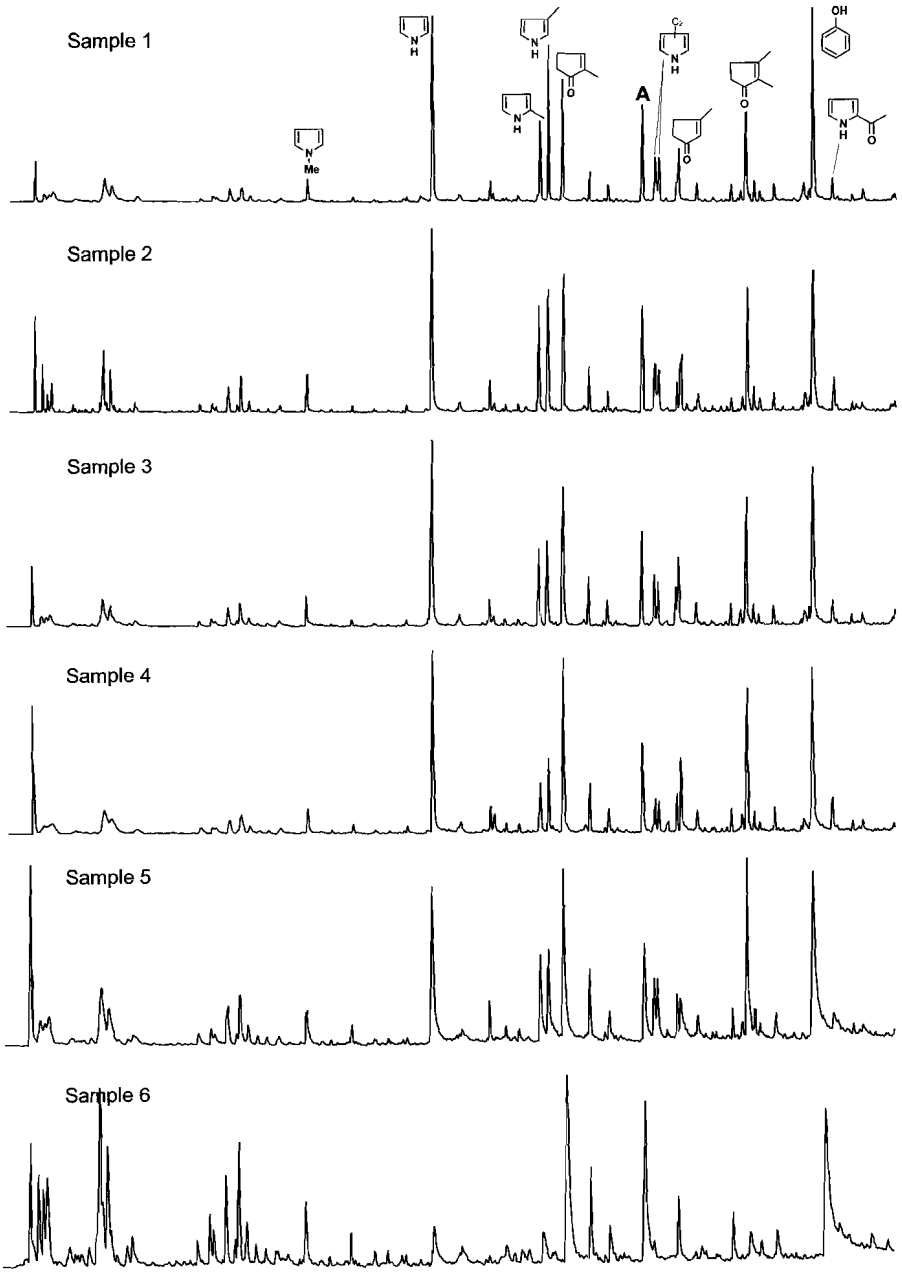

Figure 9.5 Mass chromatograms (*m/z* 67+80+94) of alkylcyclopentenones, alkylpyrroles and alkylcyclopentenes.

in as river transported material or resulting from degradation of autochthonous organic matter.

Alkylbenzenes

All pyrolyzates contain relatively large amounts of alkylbenzenes. Mass chromatography (not shown) reveals similar distribution patterns of C_0-C_3 alkylbenzenes in the pyrolyzates of the North Sea UDOM samples. The exact precursors of these alkylbenzenes are unknown. These compounds have been reported to occur also in pyrolyzates of other DOM and POM samples, *e.g.* in pyrolyzates of DOM from the Pacific Ocean (van Heemst *et al.,* 1993), of sediment trap material from the Mediterranean (Peulvé *et al.,* 1996a) and of organic matter present in algae (van Heemst *et al.,* 1996). In ocean water DOM, and thus probably also in North Sea UDOM, the precursor of the alkylbenzenes is part of the refractory DOM. This is based on the observed relative increase of alkylbenzenes in the pyrolyzates with increasing depth (van Heemst *et al.,* 1993), suggesting selective preservation of the precursors of these alkylbenzenes. However, their source(s) is (are) presently unknown. They may be related to some extent to aromatic moieties present in the macromolecular structure of organic matter (Hartgers *et al.,* 1994).

Nitrogen-containing compounds

Nitrogen-containing compounds are encountered in pyrolyzates of a number of macromolecules, like chitin, proteins and macromolecules present in bacterial cell walls. The mass chromatograms shown in Figure 9.5 reveal distribution patterns of C_0-C_2 alkylpyrroles in the North Sea UDOM pyrolyzates. The precursors of the alkylpyrroles are yet unknown nitrogen-containing compounds. These compounds are not likely to be derived from the degradation products of pigment molecules produced by bacteria and plants (*e.g.* chlorophyll-a) based on comparison of the alkylpyrrole distributions in the UDOM samples with those of the appropriate standards (Sinninghe Damsté *et al.,* 1992).

A specific pyrolysis product of chitin is 2-(N-acetylamino)-levoglucosan (van der Kaaden *et al.,* 1984; Baas *et al.,* 1995). This compound was not encountered in the pyrolyzates of the UDOM samples. However, other specific chitin pyrolysis products have been encountered in the North Sea UDOM samples 1-4, especially in sample 2.

No unaltered proteins are present in the North Sea UDOM samples because of the absence of the specific protein pyrolysis products mentioned before. However, in pyrolyzates of albumin bovine serum, pyrrole and methyl-pyrroles have been encountered (van Heemst *et al.,* 1999). Therefore, it can be speculated that some moieties present in proteins have been preserved during condensation reactions catalyzed by polysaccharide moieties (van Heemst *et al.,* 1999).

9.5 CONCLUSIONS

UDOM was isolated with initial recoveries (before diafiltration) between 23 and 50%. However, the recovery of UDOM after diafiltration and rotary evaporation was between 9 and 20% of total DOC.

No major differences could be detected in the different UDOM samples. However, it was determined that the UDOM mainly consists of refractory organic matter, with a large contribution of carbohydrate moieties.

The $\delta^{13}C$ isotope values of the North Sea UDOM were in the range of –23 to –21‰, which is in the range of typical $\delta^{13}C$ values for oceanic UDOM. Based on these values, a significant terrestrial input may be excluded.

The ^{14}a values of the UDOM samples were in the range from 90 to 103% pMC, indicating a very recent origin, pointing to an autochthonous marine origin.

Pyrolysis results indicated the presence of bacterial peptidoglycan in a number of the UDOM samples.

9.6 ACKNOWLEDGEMENTS

The authors wish to thank the Netherlands Organisation for Scientific Research (NWO) for providing a studentship to J. D. H. van Heemst. Also, the authors wish to thank the captain and the crew of the RV *Pelagia* for assistance during sampling, L. Megens for performing the isotope data, S. Gonzales and C. J. Wiebinga for analyzing the DOC samples and Jackie Bortiatynski and Patrick Hatcher for the ^{13}C-NMR analyses.

CHAPTER 10

Molecular and Carbon Isotope Characterization of North Sea Macromolecular Dissolved Organic Matter (Part 2)

10.1 ABSTRACT

Water samples from the North Sea were selected for a study to characterize ultrafiltered dissolved organic matter (UDOM). Eight different samples were taken throughout the whole area of the North Sea in May 1996. The UDOM in these samples was concentrated utilizing tangential-flow ultrafiltration and it represented the size fraction between 1000 Dalton and 0.2 μm. The samples were analyzed by analytical pyrolysis, stable and radiocarbon isotopes measurements and solid-state ^{13}C-NMR. UDOM was isolated with recoveries between 23 and 50% and final recoveries between 18 and 15% of total DOC. The $\delta^{13}C$ isotope values were in the range of –23 to –19‰, excluding a significant terrestrial contribution. The ^{14}a values of the UDOM samples were in the range from 77 to 121% pMC. Some UDOM samples were compared with UDOM samples isolated in November 1995 at the same locations to access seasonal influences. UDOM from May 1996 contains slightly more fresh and more recent organic matter than UDOM from November 1995. All samples revealed a dominant presence of altered polysaccharides moieties. Overall, the UDOM analyzed in this study seemed to be mainly refractory in nature, although the UDOM from one sample contained major amounts of fresh material.

10.2 INTRODUCTION

During a previous cruise in November 1995 (chapter 9), six surface water samples were collected at various locations throughout the North Sea. The water samples were prefiltered using 0.2-μm filters to separate dissolved organic matter (DOM) and particulate organic matter (POM). The high-molecular weight fraction of DOM was isolated using tangential-flow ultrafiltration. A filter with a nominal cut-off value of 1000 Dalton was used. Therefore, the high-molecular weight fraction contained particles with a diameter between 1 nm and 0.2 μm, referred to as ultrafiltered DOM (UDOM).

In this study, water samples were collected during a cruise in May 1996 from different parts of the North Sea, as well as different depths. A number of samples were taken at the same location where water samples were taken during the November 1995

cruise (chapter 9). Since these samples were taken during two different seasons (November *vs.* May), they could be used to study seasonal variations of UDOM at these locations.

10.3 EXPERIMENTAL

Site and sample description

The site, *i.e.* the North Sea, and its characteristics have been described in detail elsewhere (chapter 9). During a cruise with the RV *Pelagia* in May 1996, eight 120-L water samples were taken and processed on-board (Figure 10.1; Table 10.1). Samples J1, J2, J3, J7 and J8 were taken from the surface water layer. The water column in some parts of the North Sea was stratified, therefore sample J6 was taken from the deep water and sample J9a was taken from the chlorophyll maximum layer. Furthermore, samples J1, J2 and J3 were taken at the same location and at the same depth as in a previous sampling cruise in November 1995 (chapter 9).

Table 10.1 Hydrographic CTD data.

sample	*location*	*depth (m)*	*salinity (‰)*	*temperature (°C)*
J1	56°23'N; 07°51'E	3.0	32.69	6.60
J2	56°40'N; 04°30'E	4.1	34.78	6.23
J3	53°43'N; 04°30'E	3.5	33.90	8.19
J5	60°30'N; 00°00'E	4.2	35.24	7.86
J6	58°00'N; 02°00'E	49.0	35.02	5.59
J7	53°40'N; 01°33'E	2.2	34.67	7.82
J8	52°22'N; 04°30'E	3.5	34.28	7.92
J9a	56°00'N; 02°00'E	34.5	34.81	6.04

Sample collection and processing

Water samples were collected using a CTD equipped with temperature, conductivity, dissolved oxygen, fluorescence and pressure sensors. An aluminum rosette containing 24 12-L Niskin bottles was used to obtain water samples from predetermined depths. The CTD and rosette were deployed on a 3-conductor cable allowing for the real-time display of data and acquisition, and for tripping the bottles in areas of interest in the water column. Immediately after sampling, the water samples were poured through a 63-μm mesh net in order to remove large particles and prefiltered through 0.2-μm pore sized Sartorius regenerated cellulose filters using 0.5 bar nitrogen pressure in order to

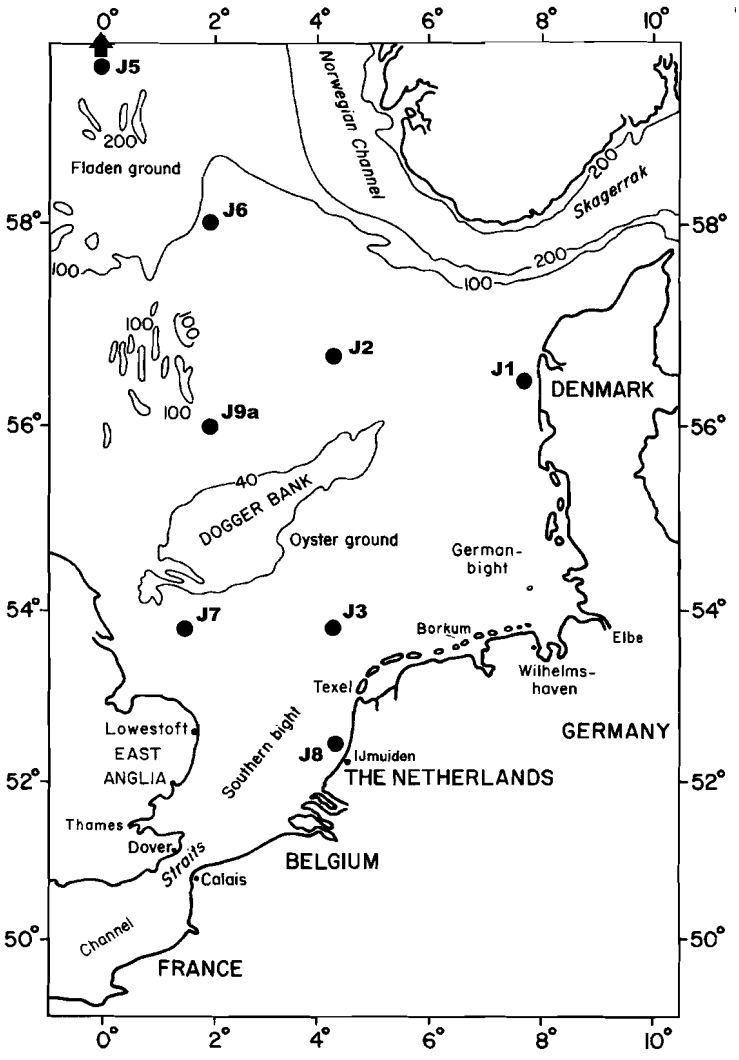

Figure 10.1 Map of the sampling area.

separate DOM and POM. For each sample, 120 L of prefiltered sample was collected. The prefiltered samples were subsequently concentrated to about 2 L using a custom-made Amicon SP-60 tangential-flow ultrafiltration system equipped with Amicon spiral-wound polysulfone 1000 Dalton cut-off filters. Most of the salts were removed from the concentrated samples by diafiltration with 20 L of distilled water. The final samples were stored frozen at -20°C for transport to the laboratory, where water was

removed by evaporation *in vacuo* at 30°C using a rotary evaporator followed by freeze-drying of the samples.

DOC sampling

During the sample processing procedure 10-mL samples were taken in duplicate for measuring DOC. Samples were taken from the filtrates after prefiltration and diafiltration and from the retentate after diafiltration. The samples were stored in precombusted glass ampoules at 5°C after acidifying with 4 drops of 40% phosphoric acid.

DOC measurements

A High Temperature Catalytic Oxidation (HTCO) method was used to determine DOC concentrations. The method is described in detail by Wiebinga and de Baar (1998). The HTCO is based on the method described by Sugimura and Suzuki (1988). A modified Ionics Model 555 carbon analyzer with a pure platinum gauze catalyst at 775°C was used for direct converting DOC to CO_2. Samples of 100 µL were injected into a quartz tube containing the catalyst. A stream of ultrapure oxygen gas carried the water vapor and combustion gases through a series of traps before entering a Li-Cor Model LI-6252 CO_2-analyzer. The signal generated by the non-dispersive infrared (NDIR) detection of CO_2 is quantified as peak area on a Shimadzu Model C-R6A integrator.

The concentration of DOC in each sample was determined by calibration against standard addition curves of potassium phthalate in MilliQ water (0 up to 1667 mM). The slope of the line was used as a conversion factor for assessing TOC in real samples. The data have not been corrected for any blank.

Solid-state ^{13}C NMR

Dried samples of ultrafiltered dissolved organic matter (UDOM) were subjected to solid-state ^{13}C nuclear magnetic resonance (NMR) spectrometry, using cross polarization magic angle spinning (CP-MAS), a ramp-CP procedure, and TPPM (two pulse phase modulation). Samples were weighed and placed in 7-mm diameter ceramic rotors. The rotors were generally packed with 100 mg dry weight per sample (TOC of only 0.82 to 5.30%; Table 10.1). They were spun at 13 kHz at the magic angle (54.7°) in the probe of a Bruker DMX-300 MHz NMR operating at 75.5 MHz for carbon. A cross-polarization pulse sequence with 2 ms contact time and 1 s cycle time was used and approximately 10^5 acquisitions were accumulated to obtain the spectra. Exactly 2048 data points were acquired and zero-filled to 4096 data points prior to filtering with 100 Hz linebroadening and Fourier transformation. The chemical shifts were plotted using the carboxyl signal of glycine as external standard (176.03 ppm).

Determination of C_{org}, $\delta^{13}C$ and ^{14}a values (AMS)

Dried samples of UDOM were acidified with dilute hydrochloric acid to remove carbonates, dried over KOH and placed in quartz tubes together with oxidized copper wire. The tubes were evacuated and sealed and placed in a furnace at 800°C for 6 hours. The combustion gases were led over a copper oven at 600°C and a silver oven at 400°C to remove nitrogen oxide, sulfur oxides and halogens. Carbon dioxide was trapped in liquid air. The amount of CO_2 was determined by expanding the gas into a known volume and measuring its pressure.

$\delta^{13}C$ values were determined with a VG SIRA 9 isotope ratio mass spectrometer. Subsequently the carbon dioxide was reduced to graphite with hydrogen in the presence of an iron catalyst at 600°C. The resulting graphite/catalyst mixture was pressed into a target for the Tandem-Accelerator Mass Spectrometer (AMS; HVEE BV, Amersfoort; Aerts-Bijma *et al.,* 1997; Wijma and van der Plicht, 1997).

From the measured ^{14}C:^{12}C ratio (^{14}R) of the sample and a known standard the ^{14}a value, expressed as percent of modern carbon (pMC), is calculated as: $^{14}a = (^{14}R_{sample}/^{14}R_{standard}) * \{(1- 25\ ‰)/(1 + \delta^{13}C)\}^2 * 100$ pMC. The standard to which ^{14}C data are referred to is oxalic acid from 1950. According to the official definition by Stuiver and Pollach (1977) a correction has to be made for the time of measurement after 1950, but the effect of this is negligible. The ^{14}a value is related to the age of the sample, since due to radioactive decay ($T_{1/2}$ = 5730) ^{14}C is disappearing from the sample over time. Because the ^{14}C concentration is also affected by isotopic fractionation, a correction for this is made using the factor $(1-25‰)/(1+\delta^{13}C)$.

When comparing marine and terrestrial samples, samples with the same age do not have the same ^{14}a value because the carbon source for organisms in the ocean usually has a lower ^{14}C:^{12}C ratio than CO_2 in the atmosphere, due to the so called reservoir effect. Therefore, and because the measured samples are probably mixtures of components with different ages, ^{14}a values are reported instead of ages.

Curie-point pyrolysis-gas chromatography (CuPy-GC)

Curie-point pyrolysis-gas chromatography (CuPy-GC) analyses were performed using a Hewlett-Packard 5890 gas chromatograph, equipped with a FOM-4LX pyrolysis unit (Boon *et al.,* 1987) and a cryogenic unit. A 25 m fused silica capillary column coated with chemically bound DB 1701 (0.253 mm i.d., film thickness 0.25 μm) was used with helium as a carrier gas. A flame ionization detector (FID) at 320 °C was used for detection. The temperature program was as follows: initial temperature 0°C (5 min); heating rate 3°C/min; final temperature 300°C (10 min). Samples were pressed onto flattened ferromagnetic wires (Curie temperatures of 610°C) and placed into the pyrolysis unit. The pyrolysis unit was connected to a FOM high frequency generator

that heated the wires inductively in 0.15 s to the Curie temperature. This temperature was maintained for 9 s.

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC/MS)

Curie-point pyrolysis-gas chromatography/mass spectrometry (CuPy-GC/MS) analyses were carried out in the same way as the CuPy-GC analyses. A mass spectrometer was used for detection instead of a FID. The Hewlett-Packard 5890 gas chromatograph was connected to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of *m/z* 700-35 and a cycle time of 1 s.

10.4 RESULTS AND DISCUSSION

DOM from the North Sea was concentrated and isolated using the method of tangential-flow ultrafiltration. Several studies (*e.g.* Benner *et al.,* 1992; Guo and Santschi, 1996; Benner *et al.,* 1997; van Heemst *et al.,* 2000a; Chapter 9) have used tangential-flow ultrafiltration to recover DOM from seawater and estuarine waters. Tangential-flow ultrafiltration concentrates compounds on the basis of molecular size and shape. Ultrafiltration (using 1000 Dalton cut-off filters) has been reported to isolate a much larger fraction (up to ~30%) of seawater DOM (Benner *et al.,* 1992) than conventional DOM isolation methods. The 70% that could not be isolated exists of particles smaller than 1000 Dalton. Therefore, isolated ultrafiltered DOM (UDOM) is not truly dissolved, but more of a colloidal nature. In Table 10.2 the amounts of ultrafiltered DOC (UDOC) of the water samples from the North Sea UDOM samples are listed.

Table 10.2 UDOM data.

sample	*UDOM powder (mg)*	C_{org} *(%)*	*UDOC (mg C)*	$\delta^{13}C$ *(‰)*	^{14}C *(%)*
J1	1246.4	2.78	34.65	-20.72	101.8
J2	1038.4	1.54	15.99	-20.25	n.a.
J3	1017.3	3.35	34.08	-19.25	121.2
J5	727.9	1.01	7.35	-21.64	77.5
J6	871.6	1.48	12.90	-21.50	79.9
J7	1021.2	1.56	15.93	-21.37	95.3
J8	771.1	5.30	40.87	-22.47	111.3
J9a	1716.3	0.82	14.07	-22.50	82.0

n.a. = not available

DOC recoveries

Typically, amounts of 700-1700 mg of UDOM powder were recovered from 120 L of North Sea water sample (Table 10.2). The organic carbon contents were ranging from 0.82 up to 5.30%. From the total amount of UDOM powder and its organic carbon content, the total amount of isolated organic carbon (or UDOC) could be calculated.

The amounts of organic carbon were determined before and after the UDOM isolation process (Table 10.3). For comparison, total amounts of organic carbon are presented in Table 10.2. These were calculated from the DOC concentration and the volume of the water sample. DOC concentrations in the original seawater were ranging from 79.9-166 µM C. The DOC seawater concentration of sample J6 was measured as being 3307, a rather unrealistic value. Unfortunately, the duplicate of this sample got lost.

Table 10.3 DOC recovery data.

sample	*seawater DOC (µmol C/L)*	*seawater DOC (mg C)*	*filtrate DOC (mg C)*	*retentate DOC (mg C)*	*UDOC rec.(%)*	*total rec. (%)*
J1	166	239.04	164.05	50.91	21.30	89.9
J2	84.8	122.12	119.44	21.96	17.98	115.8
J3	124.4	179.14	165.05	40.09	22.38	114.5
J5	67.6	97.34	4061.54	22.60	23.21	4195.7
J6	3307	4762.08	107.67	16.89	0.35	2.6
J7	86.8	124.99	109.47	27.52	22.01	109.6
J8	139.6	201.02	141.94	50.82	25.28	95.9
J9a	79.9	115.06	181.93	22.76	19.78	177.9

The total recovery is a measure for the carbon balance. This recovery was calculated by simply adding all the organic carbon present after the concentration step (*i.e.* the carbon present in the filtrate and the retentate) and dividing it by the total amount of organic carbon in the original sample. Needless to say, this recovery should be 100%, assuming there is no loss or contamination. Taking into account the accuracy of the DOC values and especially the accuracy of measuring exact sample volumes, the values of the recovery of most of the samples were around 100% (Table 10.3). The total recovery of sample J9a seems to be too high. Also, the total recovery of sample J5 seems much too high and the total recovery of sample J6 much too low. From its high DOC value, it looks like the filtrate of sample J5 was contaminated. The unrealistic values of sample J6 were mentioned above.

The recovery of the size-fraction from 1000 Dalton to 0.2 µm was in the range of 18-25% (Table 10.3), not taking into account sample J6. This value is a measure for the percentage of UDOC with regard to total DOC. Although a significant amount of

DOC was isolated by the method used in this study, the larger part of DOC was not be recovered and was therefore destined to remain uncharacterized.

Isotope data

All values of $\delta^{13}C$ of the North Sea UDOM are similar and are in the range of –22.50 to –19.25‰ (Table 10.2). These values are slightly higher than typical $\delta^{13}C$ values for oceanic UDOM, reported in the literature (-24 to –21‰; Williams and Gordon, 1970). The UDOM analyzed in this study was isolated in May 1996. North Sea UDOM isolated in November 1995 (Chapter 9) revealed relatively lower $\delta^{13}C$ values, ranging from –22.94 to –20.95‰. The lower $\delta^{13}C$ values of UDOM isolated in May compared to those of UDOM isolated in November may indicate a higher contribution of phyto- and zooplankton biomass (with lower $\delta^{13}C$ values) to the UDOM of the North Sea.

The ^{14}a values of the North Sea UDOM are ranging from 77.5 to 121.2 pMC (Table 10.2). It is not clear if these values represent any particular trend. However, there was a larger difference between ^{14}a values of the different UDOM samples than there was in the UDOM isolated in November 1996 (Chapter 9). The ^{14}a values in that study were in the range of 90 to 103 pMC.

Solid-state ^{13}C-NMR results

The North Sea UDOM samples all revealed similar ^{13}C-NMR spectra (Figure 10.2). The major signal in the NMR spectra was observed at a chemical shift of 72 ppm. This signal may be attributed to hydroxylated aliphatic carbons, present in carbohydrates and alcohols. The signal, present at a chemical shift of 101 may partially be attributed to carbohydrate moieties too. This signal is assigned to both aromatic carbons and the anomeric carbon in carbohydrates. The NMR spectra of all UDOM samples revealed an extensive signal at a chemical shift of 175 ppm, which was assigned to carboxyl and amide carbons. The signal at a chemical shift of 164 revealed the presence of inorganic carbon in the form of carbonates (Figure 10.3). This signal was strong in the UDOM samples with a relatively low organic carbon content, such as samples J5, J6 and J9a. Furthermore, the NMR spectra of all UDOM samples revealed the presence of some aliphatic moieties (signals at 0-60 ppm).

Overall, not much difference could be observed in the NMR spectra of the different UDOM samples. The spectra revealed the presence of vast amounts of carbohydrate moieties, in agreement with results of analytical pyrolysis, as described hereafter. It is remarkable that spectra with a good signal to noise ratio were obtained for samples with such extremely low organic carbon contents (as low as 0.82%). This demonstrates the usefulness of applying high-field ramp-CPMAS ^{13}C-NMR to analyze samples that have a low organic carbon content, such as isolated marine UDOM samples.

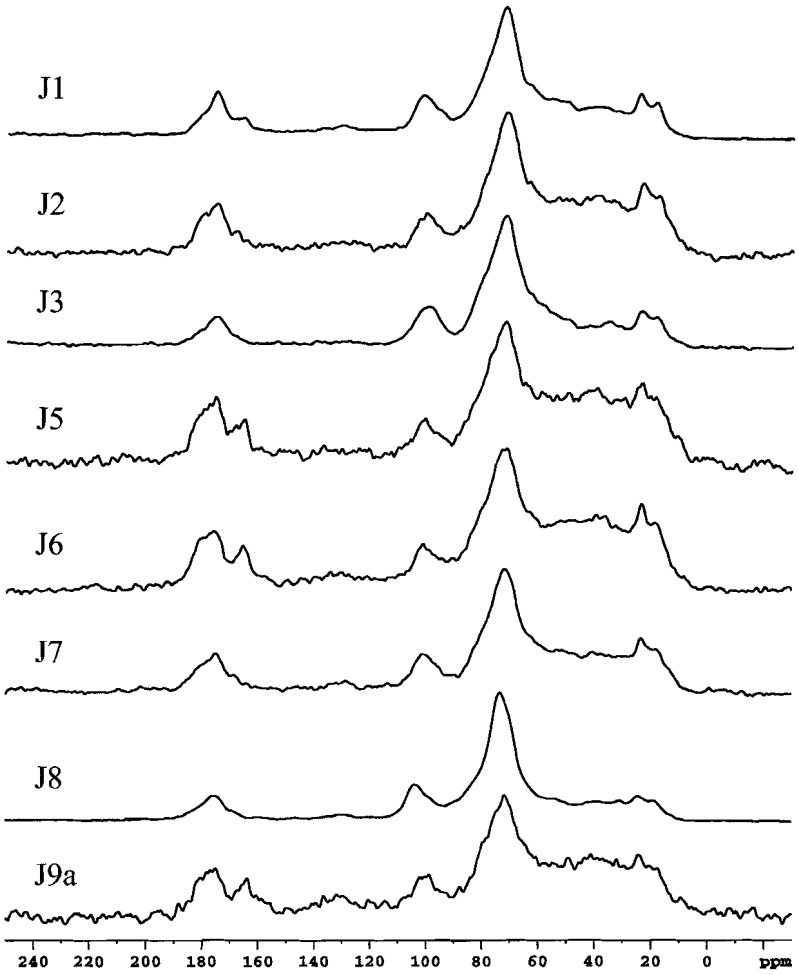

Figure 10.2 Solid-state ^{13}C NMR spectra of the North Sea UDOM samples.

CuPy-GC/MS results

Chromatograms of the pyrolyzates of the North Sea UDOM samples are shown in Figure 10.4. The pyrolyzates are dominated by alkylphenols, alkylbenzenes, alkylpyrroles, pyrolysis products of carbohydrates (alkylcyclopentenones, alkylcyclopentanones, alkylfurans and alkylcyclopentadienes) and siloxanes. Alkylphenols as well as alkypyrroles may have many different precursors, but in the UDOM they are probably of extensive altered proteinaceous origin (van Heemst *et al.,* 1999). The siloxanes represent

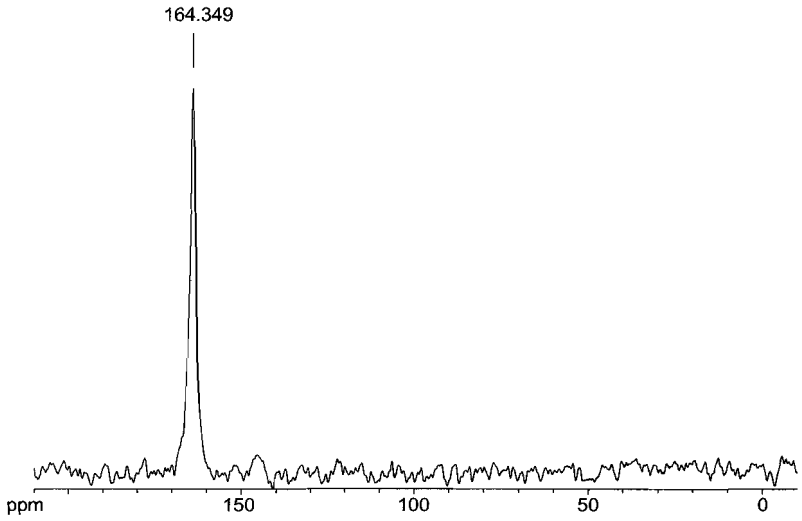

Figure 10.3 Solid-state ^{13}C-NMR spectra of sodium bicarbonate.

contaminants. Some samples (J5, J6, J7 and J9a) contained relatively large amounts of these contaminants, as was also observed from the DOC recoveries mentioned before.

Pyrolysis products of polysaccharides are acetic acid, alkylfurans and alkylfuraldehydes (Pouwels *et al.,* 1989; Pastorova *et al.,* 1994). In addition, unaltered polysaccharides yield relatively large amounts of deanhydromonosaccharides like levoglucosenone, 1,4:3,6-dianhydro-α-D-glucopyranose and levoglucosan upon pyrolysis. The presence of relatively large amounts of salts in the UDOM samples may influence the formation of certain compounds upon pyrolysis, like the deanhydromonosaccharides. In none of the pyrolyzates of the UDOM samples these deanhydromonosaccharides were detected, except in the pyrolyzate of UDOM sample J8. This means that (except for sample J8) the contribution of fresh, unaltered polysaccharide to the UDOM is of minor importance. It appears that sample J8 has been taken from an area with a lot of fresh organic material. This may indicate that an algal bloom occurred during May 1996 in the North Sea were sample J8 was taken.

Furaldehydes were detected in relatively large amounts in the pyrolyzates of the UDOM samples J1, J2, J3, J8 and J9a, indicating the presence of polysaccharide in the samples. The pyrolyzates of all UDOM samples contain varying relative amounts of cyclopentenones and cyclopentanones. Cyclopentenones have been encountered in pyrolyzates of soil organic matter (Saiz-Jimenez and de Leeuw, 1984b and 1986a) and

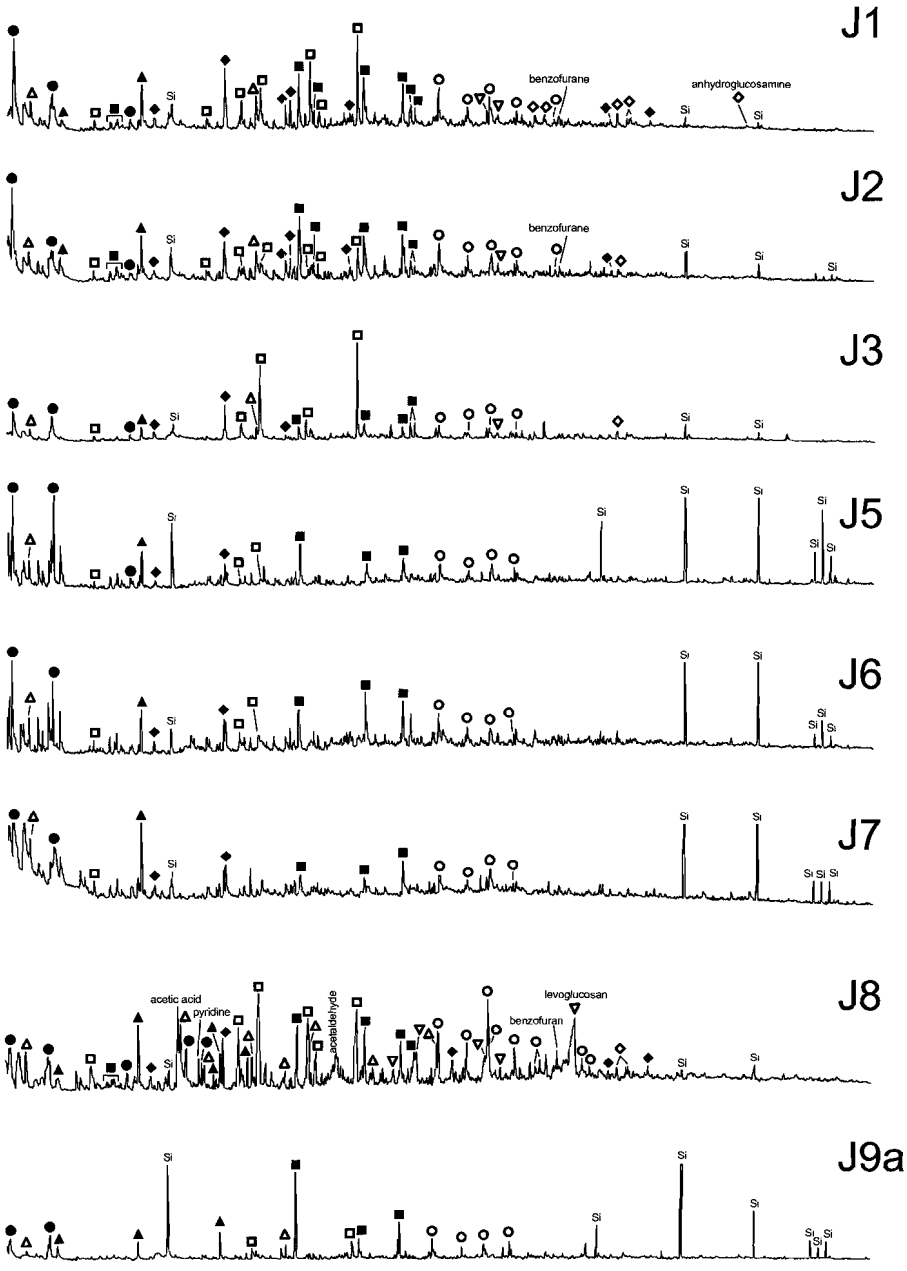

Figure 10.4 Chromatograms of the pyrolyzates (610°C) of the North Sea UDOM samples. Key: triangles = benzenes; solid diamonds = pyrroles and indoles; open squares = furaldehydes; solid squares = cyclopentanonens and cyclopentenones; open circles = phenols; open diamonds = chitin and/or bacterial lipopolysaccharide markers; open triangles = furans and furanones; open upside-down triangles = dianhydrosugars; closed circles = alkylketones and aldehydes.

amylose (van der Kaaden *et al.,* 1983) and as burned sugar aroma components (Mills and Hodge, 1976). It is believed that the precursors of the alkylcyclopentanones in the UDOM samples are related to refractory organic matter, possibly originating from altered polysaccharides. No significant amounts of furaldehydes were detected in the pyrolyzates of UDOM samples J5, J6, J7 and J9a. However, these pyrolyzates contain relatively large amounts of cyclopentanones and cyclopentenones. This indicates that UDOM from the western part of the North Sea contains relatively large amounts of altered (refractory) polysaccharides and no significant relative amounts of unaltered (fresh) polysaccharides. The UDOM from the other parts of the North Sea contains a mixture of these types of polysaccharides. The polysaccharides in UDOM sample J3 almost exclusively consist of unaltered polysaccharides.

The pyrolyzates of most of the UDOM samples reveal the presence of relatively small amounts of pyrolysis products, containing an N-acetylamino group, which may be indicative for the presence of chitin and/or bacterial lipopolysaccharides (McCarthy *et al.,* 1998).

Aliphatic amino acid moieties in peptide and protein material show very characteristic pyrolysis products, which can be monitored by mass chromatography of masses *m/z* 195+209 (Boon and de Leeuw, 1987; not shown). These pyrolysis products are indicative for the presence of proteins and were encountered in the pyrolyzates of the UDOM of samples J1, J7 and J8, but not in the pyrolyzates of the other samples.

No large amounts of lipids like fatty acids, phytadienes and sterols were encountered in any of the UDOM pyrolyzates either. Therefore, the UDOM of the North Sea seems to be refractory in nature.

Seasonal differences in the composition of North Sea UDOM

The UDOM samples J1, J2 and J3, isolated from water samples were collected in May 1996. At the same sample locations, water samples from which UDOM was isolated were collected in November 1995 (samples 2, 4 and 6 respectively; chapter 9).

The DOC concentrations at these locations in May 1996 (85-124 μmol C/L; Table 10.3) are similar to those in November 1995 (82-111 μmol C/L; chapter 9). However, the recoveries of UDOC, isolated in the May samples are slightly higher (18-22%), than the recoveries of UDOC from the November samples (9-15%). This may be due to the different isolation procedure used in the different studies. The UDOM isolated in May 1996 was diafiltered on-board, immediately after the concentration step, whereas the UDOM isolated in November 1995 was diafiltered in the laboratory after freezing, transporting and subsequent thawing of the frozen concentrated UDOM prior to the diafiltration prcedure. This has probably induced some loss of organic material.

The $\delta^{13}C$ values of the May 1996 UDOM samples (-20.7 to -19.25‰; Table 10.2) are slightly higher than those of the November 1995 UDOM samples (-22.9 to -21.0‰; chapter 9). The ^{14}a values of the May 1996 UDOM samples (102-121%) are higher than

the ^{14}a values of the November 1995 UDOM samples (93-103%). This indicates that the UDOM collected in May 1996 has more recent organic matter than the November 1995 UDOM.

This trend is also confirmed by the CuPy-GC/MS data of the UDOM samples from the two different seasons. The pyrolyzates of the UDOM samples J1 and J3 from May 1996 (Figure 10.4) contain relatively more furaldehydes compared to cyclopentenones than the samples 2 and 6 from November 1995 (chapter 9), indicating a relatively larger contribution of more fresh than refractory polysaccharides to the UDOM. The pyrolyzates of UDOM samples J2 (May 1996) and sample 4 (November 1995) do not reveal any major differences.

The solid-state ^{13}C-NMR data does not reveal any noticeable differences between the UDOM from May 1996 (Figure 10.2) and November 1995 (chapter 9). This is due to the fact that solid-state ^{13}C-NMR is too insensitive to detect subtle differences in organic matter on the molecular level.

10.5 Conclusions

UDOM from the North Sea mainly consists of refractory organic matter, with relatively large contributions of carbohydrate, aromatic and phenolic moieties and some contribution of bacterial peptidoglycan in a number of the UDOM samples. However, the UDOM of one of the samples (J8) contained a relatively large amount of fresh or labile organic matter as was revealed by the presence of specific polysaccharide pyrolysis products in the pyrolyzate.

UDOM from the North Sea was isolated with recoveries between 18 and 25% of total DOC. The δ^{13}C isotope values of these UDOM samples are in the range of –23 to –19‰, which is slightly higher than typical δ^{13}C values for oceanic UDOM. Based on these values, a significant terrestrial input may be excluded. The ^{14}a values of the UDOM samples are in the range from 77 to 121% pMC,

Some seasonal influence in UDOM from the North Sea exists. UDOM isolated in May 1996 contains slightly more fresh and more recent material than UDOM isolated in November 1995.

10.6 Acknowledgements

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the isotope data, S. Gonzales for analyzing the DOC samples and K. J. Dria for conducting the ^{13}C-NMR analyses.

CHAPTER 11

Characterization of Estuarine and Fluvial Dissolved Organic Matter by Thermochemolysis using Tetramethylammonium Hydroxide

11.1 SUMMARY

Selected samples of dissolved organic matter (DOM) isolated by ultrafiltration (UDOM) have been analyzed by thermochemolysis in the presence of tetramethylammonium hydroxide (TMAH). This technique cleaves ester and ether bonds of bio- and geological macromolecules and releases monomer subunits and methylates them *in situ* as their methyl ethers and methyl esters. Compared with conventional pyrolysis, TMAH thermochemolysis avoids decarboxylation of preexisting carboxylic moieties and produces aromatic acids as their methyl esters. Various phenolic derivatives, which might originate from incorporated lignin-derived structures, from the highly aliphatic and resistant biopolymer cutan and also from proteinaceous materials, were identified among the products produced from UDOM upon thermochemolysis. The presence of lignin derivatives in UDOM indicates input of organic matter derived from terrestrial sources. Various aromatic acids, perhaps representing the final steps in the oxidation of the side-chain during microbial oxidation of lignin, were released upon TMAH thermochemolysis, suggesting they are structural constituents of the UDOM. Different ratios of lignin-derived materials, commonly determined using the CuO oxidation method, such as the Λ value, indicative of the amount of lignin present, the acid/aldehyde ratio $(Ad/Al)_G$, indicative of the extent of oxidative degradation of the lignin component, and the syringyl/guaiacyl (S/G) and *p*-hydroxyphenyl/guaiacyl (P/G) ratios, indicative of the contribution for the different types of lignin, were determined.

11.2 INTRODUCTION

Structural characterization of complex organic matter such as dissolved organic matter (DOM) in natural water is a challenging task. The bulk of DOM *in toto* can nondestructively be examined by spectroscopic methods such as UV (Ludwig *et al.*, 1997), IR and NMR. Solid-state ^{13}C NMR of isolated, macromolecular fractions of DOM makes it feasible to identify relative contributions of specific carbon types (Benner *et al.*, 1992; Hedges *et al.*, 1992; Malcolm, 1990). Studies to date have shown

that XAD resin isolated material from oceanic DOM is very aliphatic in nature, while similar isolates of DOM from rivers is more aromatic in nature (Hedges *et al.,* 1992; Meyers-Schulte and Hedges, 1986). This represents the added contribution of terrestrial organic matter to river water DOM, which indeed tends to have a more aromatic character (Malcolm, 1990). This type of organic matter also appears to have a high proportion of oxygen-containing functional groups such as carboxyl, alkoxyl and carbonyl groups. This high functionality probably contributes greatly to its solubility characteristics and to its affinity to adsorption by resins.

However, degradative methods need to be applied for more detailed information on the structure of DOM at a molecular level (Norwood, 1988). Wet chemical degradation techniques, such as the alkaline CuO method are able to identify components derived from lignin moieties (Hedges and Ertel, 1982), but these structures are present in only minor amounts in DOM (Meyers-Schulte and Hedges, 1986). Pyrolytic degradation coupled to gas chromatography/mass spectrometry has also provided important information regarding the chemical structure of humic fractions of DOM (Abbt-Braun *et al.,* 1989) and other fractions of DOM, isolated using tangential-flow ultrafiltration (van Heemst *et al.,* 1993 and 1996). However, the major fraction of the DOM, probably much larger than 50%, still remains poorly characterized.

Recently, a new technique involving thermochemolysis in the presence of tetramethylammonium hydroxide (TMAH) has been introduced by Challinor (1989) for the characterization of polar macromolecules. This technique has also been applied in many other studies to characterize geo- and biopolymers (de Leeuw and Baas, 1993; Challinor, 1995; Clifford *et al.,* 1995; Martin *et al.,* 1994; McKinney *et al.,* 1995; McKinney and Hatcher, 1996; del Rio *et al.,* 1994 and 1998) and humic materials (Hatcher and Clifford, 1994; del Rio *et al.,* 1995 and 1996; del Rio and Hatcher, 1996). The technique yields the methyl esters of carboxylic acids and methylates compounds containing hydroxyl groups, yielding products volatile enough for gas chromatographic analyses. TMAH thermochemolysis has also been applied for structural characterization of lignins (Challinor, 1995; Clifford *et al.,* 1995; Martin *et al.,* 1995a and1995b). β-O-4 ether bond cleavage of the lignin macromolecule by TMAH produces lignin unit derivatives, that subsequently become methylated as was shown by Morrison and Mulder (1994) and Mulder *et al.* (1992). Use of this thermochemolysis technique for the analysis of polar macromolecules rich in lignin moieties, such as humic substances and coals, has greatly enhanced product yields and produces methyl esters of carboxylic acids, not observed by conventional pyrolysis (Martin *et al.,* 1994; del Rio *et al.,* 1994 and 1996; Hatcher and Clifford, 1994). This technique has recently been used to examine a DOM sample obtained from a leachate of *Juncus effusus* L. (Wetzel *et al.,* 1995) yielding primarily methylated derivatives of lignin-derived phenols.

A similar suite of products have been encountered by Hatcher and Clifford (1994) and Clifford *et al.* (1995) at both sub-pyrolysis reaction temperatures (300 and

310°C) and pyrolysis temperatures (610 and 700°C) indicating that the lower temperature is sufficient to yield product monomers with TMAH. In a recent paper by McKinney *et al.* (1995) an off-line procedure is described for the TMAH thermochemolysis of the lignin macromolecule at 300°C using sealed glass ampoules. TMAH thermochemolysis at sub-pyrolysis temperatures causes ether bond cleavage in lignin producing lignin-derived compounds in a more gentle way than the alkaline CuO oxidation procedure, which also involves carbon-carbon bond cleavage (Hatcher *et al.,* 1995).

The focus of this paper centers on demonstrating the value of the TMAH thermochemolysis as a rapid, low-cost, and easily implemented technique to detect and characterize traces of lignin and/or lignin moieties in some estuarine and fluvial DOM samples from three different regions in the world. TMAH thermochemolysis has been applied to DOM samples in previous studies by del Rio *et al.* (1998) and del Rio and Hatcher (1996). However, only qualitative data were shown in these studies. In the present study we provide quantitative data for various monomer constituents of DOM derived from lignin. Furthermore, the present study contains ultrafiltered DOM samples (UDOM) taken throughout a salinity gradient in an estuary. This allows us to examine the difference between pure riverine UDOM and riverine/seawater UDOM.

11.3 EXPERIMENTAL

Table 11.1 reflects a description of UDOM samples selected for this study. Samples were obtained from Galveston Bay (Texas, USA) and the Ems-Dollart Estuary (at the border of The Netherlands and Germany) by tangential-flow ultrafiltration (Benner *et al.,* 1992; Benner, 1991; Dai *et al.,* 1998; Guo and Santschi, 1996). DOM from the Suwannee River (Georgia, USA) was kindly provided by E. M. Perdue. UDOM samples (1-2 mg) were weighed and placed in glass ampoules with 100 µL of TMAH (25% in methanol) added. Methanol was evaporated under vacuum. Ampoules were sealed under vacuum and subsequently placed in an oven at 250°C for 30 minutes. After cooling, the tubes were cracked open and inside surfaces were extracted using methylene chloride (3 x 1 mL). The combined extracts were reduced to dryness under a stream of nitrogen and dissolved in 25 µL of methylene chloride, which contained an internal standard (575 ng *n*-icosane).

1 µL of the sample was analyzed by capillary gas chromatography. Gas chromatographic analyses were performed using a Hewlett-Packard 5890A gas chromatograph, equipped with a 30 m fused silica capillary column coated with chemically bound DB-5 (0.25 mm i.d., film thickness 0.25 µm). Helium was used as a carrier gas with a flow rate of about 1 mL/min. A flame ionization detector (FID) at 300°C was used for detection. The GC was temperature programmed as follows:

injection temperature 60°C; first heating rate 15°C/min to 150°C; second heating rate 4°C/min to a final temperature of 280°C.

Table 11.1 List and description of the UDOM samples selected for this study.

Sample name	*Description*
Suwannee River	UDOM isolated from the Suwannee River
Galveston Bay	UDOM isolated from Galveston Bay
Ems-Dollart Estuary (E1)	UDOM isolated from the Ems-Dollart Estuary (at salinity 0.43‰)
Ems-Dollart Estuary (E2)	UDOM isolated from the Ems-Dollart Estuary (at salinity 1.5‰)
Ems-Dollart Estuary (E3)	UDOM isolated from the Ems-Dollart Estuary (at salinity 20‰)

Gas chromatography/mass spectrometry (GC/MS) was carried out in the same manner as the GC analyses to allow for peak identification. A Kratos MS-80 RFA high-resolution gas chromatograph/mass spectrometer was used for separation and detection. Mass spectra were obtained at a scan rate of 0.6 s/decade of mass with a 0.2 s magnet settling time added. Compounds were identified comparing their mass spectra and relative retention times. Most of the peaks were identified by comparison with the Wiley/NBS library. However, some were confirmed by comparison with authentic standards. Mass spectra of many of the lignin derivatives having methoxylated side-chains were not included in the library. In these cases, identification is tentative and based only on analysis of the fragmentation patterns.

A single response factor was used for all lignin-derived compounds. This response factor was calculated by taking the average of the response factors of the commercial standards di- and trimethoxybenzaldehyde and di- and trimethoxybenzoic acid methyl ester relative to the standard.

The Λ parameter, described by Hedges and Mann (1979a) for the alkaline CuO oxidation procedure is used to calculate the quantity of lignin monomers released from a sample in order to estimate the total amount of lignin present. This parameter was calculated by normalizing the sum of the total amount of lignin-derived compounds per 100 mg of organic carbon in the sample. Ratios of carboxylic acid-containing guaiacyl phenols to aldehyde-containing guaiacyl phenols, $(Ad/Al)_G$, were calculated in a manner similar to that described by Ertel *et al.* (1984). These ratios were obtained by dividing the amount of 3,4-dimethoxybenzoic acid methyl ester by the amount of 3,4-dimethoxybenzaldehyde in the different samples. A similar ratio could be calculated for syringyl phenols, but low amounts of syringyl-type compounds precluded calculation of a useful $(Ad/Al)_S$. Syringyl/guaiacyl (S/G) ratios were calculated from the sum of amounts of compounds containing a syringyl structure divided by the sum of amounts of compounds containing a guaiacyl structure. Finally, *p*-hydroxyphenyl/guaiacyl (P/G) ratios were calculated from the sum of amounts of

compounds containing a *p*-hydroxyphenyl structure relative to amounts of compounds containing a guaiacyl structure.

11.4 Results and Discussion

Chromatograms of the products released by TMAH thermochemolysis of the UDOM samples are shown in Figures 11.1 and 11.2. Many lignin-derived phenolic compounds are encountered among the thermochemolysis reaction products. These compounds mainly originate from the cleavage of the β-O-4 bonds from incorporated lignin-derived moieties. These reaction products are similar to those produced upon on-line TMAH thermochemolysis of pure lignin or wood samples (Clifford *et al.,* 1995; Martin *et al.,* 1995b). The compounds, identified in the thermochemolyzates of the different samples, that have lignin-related structures, are listed in Table 11.2. Methylated derivatives of the three different lignin phenol subunits (*p*-hydroxyphenyl, guaiacyl and syringyl) were present in the thermochemolyzates. Likewise, several other di- and trimethoxybenzenes, which are not lignin-derived structures were also encountered in the thermochemolyzates (Table 11.3).

In comparison to conventional pyrolysis, TMAH thermochemolysis avoids decarboxylation of pre-existing carboxylic moieties by methylating them, thus forming methyl esters. The major components identified in all selected UDOM samples were benzoic acid derivatives of guaiacyl (3,4-dimethoxybenzoic acid methyl ester, **G6**) and syringyl (3,4,5-trimethoxybenzoic acid methyl ester, **S6**) derivatives. The latter product could also be derived from hydrolyzable tannin derivatives. 4-Methoxybenzoic acid methyl ester (**P6**) was also encountered in several of the samples albeit in minor amounts. In DOM from decomposing *Juncus effusus* upon on-line TMAH thermochemolysis, benzoic acid derivatives have also been identified as major compounds (Wetzel *et al.,* 1995). The presence of methyl esters of these methoxybenzoic acids in the thermochemolyzates of the studied UDOM samples suggests that these samples may contain lignin-derived moieties, which have been oxidized at the α-carbon of the side chain of the lignin subunit. Lignin polymers may be attacked by microbial enzymes which selectively oxidize the α-carbon in the side-chain (Crawford, 1981). Several other aromatic acid derivatives, having the carboxyl group at the β- and γ-carbons of the side chain of the lignin subunit, such as the phenylacetic acid derivatives, 4-methoxyphenyleacetic acid methyl ester (**P24**), 3,4-dimethoxyphenylacetic acid methyl ester (**G24**), 3,4,5-trimethoxyphenylacetic acid methyl ester (**S24**) and 3,4-dimethoxyphenylpropanoic acid methyl ester (**G12**), were also detected in some of the studied UDOM samples. Propenoic acid derivatives (from cinnamyl phenols) such as 4-methoxyphenylpropenoic acid methyl ester (***P18***) and the 3,4-dimethoxyphenylpropanoic acid methyl ester (**G18**), indicative of the presence of non-woody vascular plants, were also encountered.

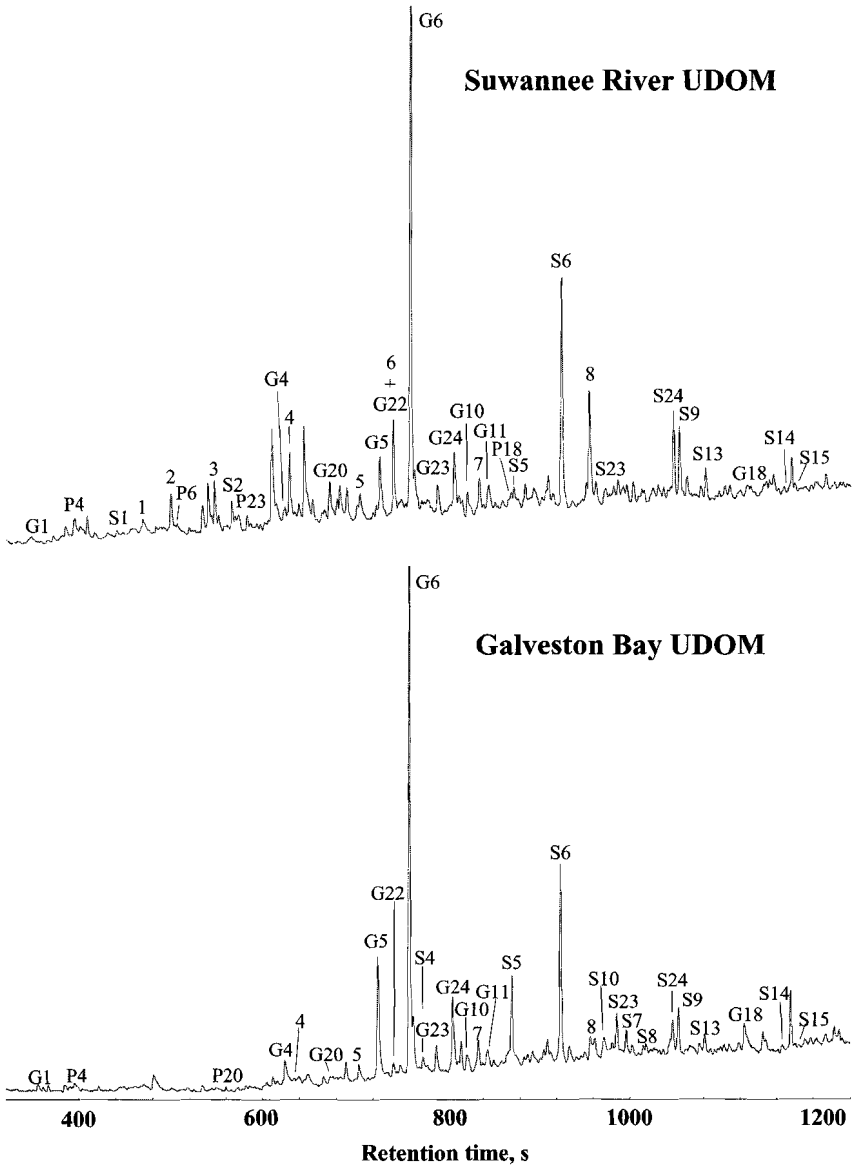

Figure 11.1 Gas chromatograms of the TMAH thermochemolysis products released from UDOM from the Suwannee River and Galveston Bay. For peak identifications refer to Tables 11.2 and 11.3.

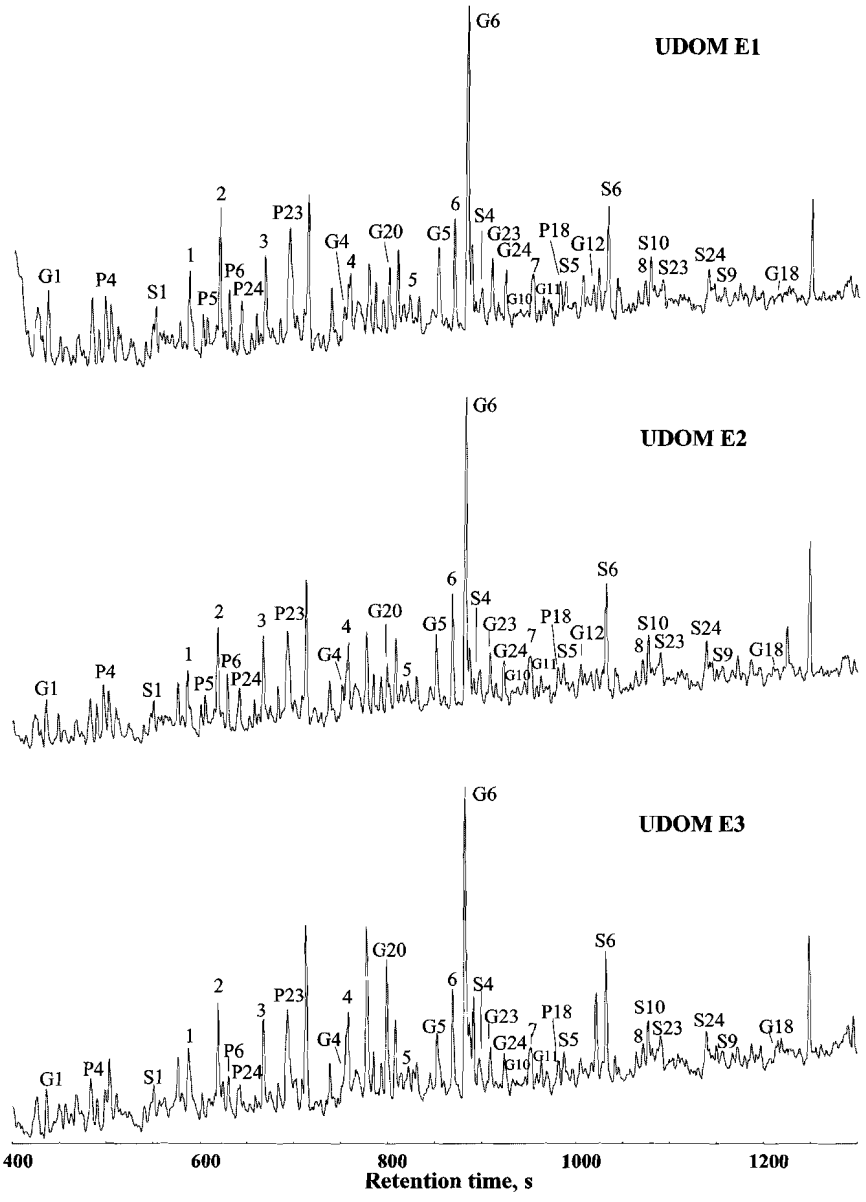

Figure 112 Gas chromatograms of the TMAH thermochemolysis products released from UDOM from the Ems-Dollart estuary (E1, E2, E3). For peak identifications refer to Tables 11.2 and 11.3.

Table 11.2 Compounds identified in the thermochemyzates of the UDOM samples.

	p-*hydroxyphenyl derived compounds*
P4	4-methoxybenzaldehyde
P5	4-methoxyacetophenone
P6	4-methoxybenzoic acid methyl ester
P18	4-methoxyphenylpropenoic acid methyl ester
P20	4-methoxyphenylmethanol methyl ether
P23	1-(4-methoxyphenyl)-2-methoxy-propane
P24	4-methoxyphenylacetic acid methyl ester
	guaiacyl derived compounds
G1	1,2-dimethoxybenzene
G4	3,4-dimethoxybenzaldehyde
G5	3,4-dimethoxyacetophenone
G6	3,4-dimethoxybenzoic acid methyl ester
G10	*cis* 1-(3,4-dimethoxyphenyl)-methoxyprop-1-ene
G11	*trans* 1-(3,4-dimethoxyphenyl)-methoxyprop-1-ene
G12	3,4-dimethoxyphenylpropanoic acid methyl ester
G18	3,4-dimethoxyphenylpropenoic acid methyl ester
G20	3,4-dimethoxyphenylmethanol methyl ether
G22	1-(3,4-dimethoxyphenyl)-2-propanone
G23	1-(3,4-dimethoxyphenyl)-2-methoxypropane
G24	3,4-dimethoxyphenylacetic acid methyl ester
	syringyl derived compounds
S1	1,2,3-trimethoxybenzene
S2	3,4,5-trimethoxytoluene
S4	3,4,5-trimethoxybenzaldehyde
S5	3,4,5-trimethoxyacetophenone
S6	3,4,5-trimethoxybenzoic acid methyl ester
S7	*cis* 1-(3,4,5-trimethoxyphenyl)-2-methoxyethylene
S8	*trans* 1-(3,4,5-trimethoxyphenyl)-2-methoxyethylene
S9	*cis* 1-(3,4,5-trimethoxyphenyl)-3-methoxyprop-1-ene
S10	*cis* 1-(3,4,5-trimethoxyphenyl)-methoxyprop-1-ene
S11	*trans* 1-(3,4,5-trimethoxyphenyl)-methoxyprop-1-ene
S13	*trans* 1-(3,4,5-trimethoxyphenyl)-3-methoxyprop-1-ene
S14	*threo/erythro* 1-(3,4,5-trimethoxy-phenyl)-1,2,3-trimethoxypropane
S15	*threo/erythro* 1-(3,4,5-trimethox-yphenyl)-1,2,3-trimethoxypropane
S22	1-(3,4,5-trimethoxyphenyl)-2-propanone
S23	1-(3,4,5-trimethoxyphenyl)-2-methoxypropane
S24	3,4,5-trimethoxyphenylacetic acid methyl ester

Table 11.3 Other phenolic compounds identified in the thermochemyzates of the UDOM samples.

no.	*identified compound*
1	3-methoxybenzoic acid methyl ester (isomer of **P6**)
2	1,2,4-trimethoxybenzene (isomer of **S1**)
3	1,3,5-trimethoxybenzene (derived from cutan)
4	2,4,6-trimethoxytoluene (derived from cutan)
5	1-(dimethoxyphenyl)-methoxypropane (isomer of **G23**)
6	3,5-dimethoxybenzoic acid methyl ester (derived from cutan)
7	methyl, dimethoxybenzoic acid methyl ester
8	methoxybenzenedicarboxylic acid dimethyl ester

Pyrolysis-GC and pyrolysis-GC/MS studies on the same UDOM samples from the Ems-Dollart Estuary as used in this study did not reveal the presence of methoxyphenols or dihydroxybenzenes, characteristic compounds from lignins and degraded lignins (van Heemst *et al.,* 2000a). Instead, a series of alkylphenols, which have been related to some extent to the presence of algal polyphenolic macromolecules (van Heemst *et al.,* 1996) and proteinaceous organic matter (van Heemst *et al.,* 1999), were encountered in the pyrolyzates of these UDOM samples. It is therefore likely that the contribution of polyphenolic macromolecules from non-lignin moieties to UDOM in the Ems-Dollart Estuary is far more important than from lignin moieties derived of vascular plants. It is likely that compounds arising from the pyrolysis of lignin moieties remain practically undetected by conventional pyrolysis due to their low concentration. TMAH thermochemolysis on the other hand, exclusively cleaves hydrolyzable ester bonds and some labile ether bonds, such as the β-O-4 bonds in lignin macromolecules. Therefore, it probably does not chemically attack the other polyphenolic macromolecules and releases predominantly the different lignin monomers.

In Table 11.4, a variety of parameters is listed that allow a quantitative description of the lignin content in the samples used in this study. Yields of TMAH thermochemolysis products derived from lignin moieties were used to determine values of Λ. Degraded wood samples may yield Λ values that are representative for pure lignin. McKinney and Hatcher (1996) have determined Λ values of 19 and 24 for Holocene samples of a degraded Douglas fir and a degraded *Taxonium* stem, respectively. If we assume that these samples were composed of pure lignin, the Λ value for pure lignin may be the average of these two values, *i.e.* 22. Therefore, the Λ value for the UDOM sample from the Suwannee River of 0.20 may represent a lignin content of 0.91% (w/w) (100% * 0.20 / 22). The range in Λ values for the selected UDOM samples (Table 11.4) of 0.13-0.94 may thus represent a range of lignin contents of 0.59-4.3% (w/w), suggesting that lignin moieties, although present in the

selected UDOM samples, are not major contributors to the bulk of the UDOM. This may explain why these lignin moieties are difficult to detect by conventional spectroscopic or pyrolytic methods (van Heemst *et al.*, 2000a).

Table 11.4 Lignin parameters for the selected UDOM samples.

parameter	*Suwannee River*	*Galveston Bay*	*E1*	*E2*	*E3*
Λ	0.20 ±0.01	0.13 ±0.01	0.46 ±0.02	0.94 ±0.01	0.46 ±0.01
$(Ad/Al)_G$	13.80 ±0.70	12.00 ±1.00	8.40 ±1.00	10.50 ±0.70	10.40 ±0.70
S/G	0.56 ±0.04	0.49 ±0.03	0.33 ±0.03	0.45 ±0.05	0.41 ±0.05
P/G	0.05 ±0.01	0.04 ±0.01	0.06 ±0.01	0.06 ±0.01	0.05 ±0.01

Λ values determined for XAD DOM isolates from lakes, using the alkaline CuO oxidation method, are between 2.5 and 3.4, for DOM from the Amazon River between 0.9 and 2.1 and for DOM from seawater between 0.11 and 0.19 (Meyers-Schulte and Hedges, 1986; Ertel *et al.*, 1984). The Λ values for the samples of UDOM used in this study and calculated from TMAH thermochemolysis are between 0.13 and 0.94. The Λ values for UDOM from Suwannee River (0.20) and the Ems-Dollart (0.46, 0.94 and 0.46) fall well within the range of Λ values observed in other studies for XAD isolates of DOM from seawater and Amazon River water. The Λ value for Galveston Bay UDOM (0.13) corresponds to the Λ of DOM from seawater.

These low Λ values (0.13-0.94) and the estimated values for lignin contents (0.59-4.3%) for the selected UDOM samples are in agreement with the conclusions of Opsahl and Benner (1997), who have shown recently that the percentage of terrigenous organic matter is only 0.7-2.4% of total DOM in the ocean. These values were calculated from yields of phenolic products using the alkaline CuO oxidation of UDOM samples concentrated by tangential-flow ultrafiltration. McCarthy *et al.* (1996) concluded that the major part of UDOM consists of polysaccharide material from marine origin.

Ratios of vanillic acid/vanillin $(Ad/Al)_G$ are indicative of the amount of oxidative degradation of the lignin component (Ertel *et al.*, 1984; Ertel and Hedges, 1985). A linear correlation exists between $(Ad/Al)_G$ ratios calculated using alkaline CuO oxidation and ratios calculated using TMAH thermochemolysis, but the TMAH thermochemolysis technique is possibly a more sensitive indicator of lignin degradation due to its larger dynamic range for $(Ad/Al)_G$ ratios (Hatcher *et al.*, 1995). For samples of fresh, undegraded vascular plant tissues (wood), $(Ad/Al)_G$ ratios calculated from TMAH thermochemolysis are typically less than 1. Characteristic $(Ad/Al)_G$ ratios of samples of microbially degraded woods, however, are higher than 1,

sometimes even higher than 10. In UDOM samples studied here, the $(Ad/Al)_G$ ratios are between 8.4 and 13.8 (Table 11.4), suggesting a high extent of oxidative degradation of lignin moieties present in DOM. These data are consistent with the alkaline CuO oxidation data on DOM samples examined by Meyers-Schulte and Hedges (1986) and Ertel *et al.* (1984), who also calculated relatively high $(Ad/Al)_G$ ratios for DOM. Del Rio *et al.* (1998) calculated a $(Ad/Al)_G$ ratio of 4.1 for a DOM sample from the North Pacific Ocean by TMAH thermochemolysis. They found a general trend in $(Ad/Al)_G$ ratios in the Gulf of Mexico DOM samples, from a relatively low $(Ad/Al)_G$ ratio for two off-shore DOM samples (5.1 in both cases) and a relatively high $(Ad/Al)_G$ ratio for a near-shore sample (9.5). A similar trend is observed for samples from the Ems-Dollart estuary, which is also in agreement with the diagenetic nature of Ems-Dollart UDOM (van Heemst *et al.*, 2000a).

Gymnosperm lignin is a dehydrogenation polymer of coniferyl alcohol monomer units (the precursor of guaiacyl (G) units in lignin). Angiosperm lignin is a mixed dehydrogenation polymer of coniferyl and sinapyl (the precursor of syringyl (S) units in lignin) alcohols and grass lignin is composed of a mixed dehydrogenation polymer of coniferyl, sinapyl and *p*-coumaryl (the precursor of *p*-hydroxyphenyl (P) units in lignin) alcohols (Crawford, 1981). Therefore, low syringyl/guaiacyl ratios (S/G) for all UDOM samples indicate the contribution to the UDOM samples studied of either both angiosperm and gymnosperm lignin, or of a highly degraded form of angiosperm lignin. Low *p*-hydroxyphenyl/guaiacyl (P/G) ratios for all UDOM samples indicate that the contribution of lignin from non-woody plants to the UDOM samples studied has been minor. The ranges found for these parameters are also in the range of the values reported for various DOM samples using the CuO oxidation method (Meyers-Schulte and Hedges, 1986).

Phenol derivatives which have an attached methoxylated C_3 side chain (*i.e.* compounds **P20**, **P23**, **G10/11**, **G20**, **G23**, **S7/8**, **S9/13**, **S10/11**, **S14/15**, **S23**), present in the thermochemolyzates of the studied UDOM samples may be related to lignin moieties preserved in the UDOM. TMAH thermochemolysis induces cleavage of ether bonds and methylation of hydroxyl groups attached to the side chain of the lignin moieties. This means that the backbone of the side chain units is preserved during thermochemolysis, in contrast to the alkaline CuO oxidation procedure, which causes cleavage of these side chains from the lignin units. Two isomers (*threo/erythro*) of 1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane (**S14/15**) have been identified in the thermochemolyzates of Suwannee River and Galveston Bay UDOM. The C_3 side chains of these compounds are fully methoxylated and completely preserved. Thus, these compounds are unambiguously related to relatively unaltered lignin moieties, present in the UDOM.

Some of the phenol-derivatives that have been assigned to lignin moieties may also have alternative origins, such as proteins, tannins and cutans. Such methylated phenolic derivatives, that were encountered after TMAH thermochemolysis of the

studied UDOM samples, are 1,3,5-trimethoxybenzene (**3**) and 2,4,6-trimethoxytoluene (**4**). These compounds are probably not derived from lignin. They have recently been identified as major components in TMAH thermochemolyzates of cutan (McKinney *et al.,* 1996), a highly aliphatic, resistant biopolymer present in leaf cuticles from certain plants (Tegelaar *et al.,* 1989b). 3,5-Dimethoxybenzoic acid methyl ester (**6**), identified in some of the studied UDOM samples, has also been identified in the TMAH thermochemolyzates of cutan. Therefore, the presence of these characteristic compounds in the thermochemolyzates of the studied UDOM samples may be related to the presence of degradation products from this other higher plant biopolymer (*i.e.* cutan).

11.5 Conclusions

The procedure described herein provides a simple method to evaluate the lignin content of UDOM that can be easily implemented in any laboratory having standard gas chromatographic facilities. A significant feature of this method is that it may be able to trace lignin inputs where extensive degradation has occurred and resulted in sufficient alteration of lignin. Compared to conventional pyrolysis methods for organic matter characterization, TMAH thermochemolysis avoids decarboxylation of pre-existing carboxylic moieties and releases aromatic acids as methyl esters. High amounts of aromatic acids were released from all the UDOM samples analyzed here. The aromatic acids released from UDOM after TMAH thermochemolysis probably represent compounds produced in the final steps of lignin side-chain oxidation during microbial degradation and are thought to be pristine components of the macromolecular structure of UDOM. Although it is not known at this point whether these aromatic acids are present in ester linkages or free, it is very likely that any lignin moiety that is entrained within UDOM would have a significant amount of free carboxyl functionality which would account for its solubility. Characteristic compounds arising from the highly aliphatic and resistant biopolymer cutan, present in the leaf cuticle of some plants, were also detected. This indicates that portions of this higher plant biopolymer are a part of the UDOM structure.

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PUBLICATIONS

Parts of this thesis have appeared or will appear in the following publications:

CHAPTER 2 – van Heemst J. D. H., Baas M., de Leeuw J. W. and Benner R. (1993) Molecular characterization of marine dissolved organic matter (DOM). In *Organic Geochemistry* (Edited by Øygard K.). Falch Hurtigtrykk, Oslo, Norway, 694-698.

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CHAPTER 5 – van Heemst J. D. H., Peulvé S., de Leeuw J. W., Sicre M.-A. and Saliot A. (1995) Algal polyphenolic resistant macromolecules in marine dissolved and particulate organic matter. In *Organic Geochemistry* (Edited by Grimalt J. O. and Dorronsoro C.). A.I.G.O.A., San Sebastian, Spain, 940-942.

CHAPTER 6 – van Heemst J. D. H., Peulvé S. and de Leeuw J. W. (1996) Novel algal polyphenolic biomacromolecules as significant contributors to resistant fractions of marine dissolved and particulate organic matter. *Org. Geochem.* **24,** 629-640.

CHAPTER 7 – van Heemst J. D. H., van Bergen P. F., Stankiewicz B. A. and de Leeuw J. W. (1999) Multiple sources of alkylphenols produced upon pyrolysis of DOM, POM and recent sediments. *J. Anal. Appl. Pyrolysis* **52,** 239-256.

CHAPTER 8 – Baas M., Briggs D. E. G., van Heemst J. D. H., Kear A. J. and de Leeuw J. W. (1995) Selective preservation of chitin during the decay of shrimps. *Geochim. Cosmochim. Acta* **59**, 945-951.

CHAPTER 11 – van Heemst J. D. H., del Rio J. C., Hatcher P. G. and de Leeuw J. W. (1999) Characterization of Estuarine and Fluvial Dissolved Organic Matter by Thermochemolysis using Tetramethylammonium Hydroxide. *Acta Hydrochim. Hydrobiol.* **28,** 69-76.

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CURRICULUM VITAE

Jasper D. H. van Heemst werd op 22 april 1967 te Wageningen geboren. Na het behalen van zijn OVWO-diploma aan de Rijksscholengemeenschap "Het Wagenings Lyceum" begon hij in 1985 aan zijn studie Moleculaire Wetenschappen aan de Landbouwhogeschool Wageningen. Na het behalen van het propaedeutisch examen werd de studie aan de – nu Landbouwuniversiteit Wageningen geheten – voortgezet.

Als eerste onderdeel van zijn doctoraal werd een afstudeervak bij de afdeling Organische Chemie gedaan. Dit afstudeervak (een nieuwe spectrofotometriche assaymethode voor lipase, gebruik makend van 2,4-dinitrofenylesters, en: synthetische surfactants, hun synthese en theoretische toepassing in de vorming van vesicles) werd uitgevoerd bij prof. dr. J. F. J. Engbersen, onder leiding van ir. E. W. J. Mosmuller.
Als tweede werd een afstudeervak bij de afdeling Levensmiddelentechnologie, sectie Proceskunde, gedaan. Dit afstudeervak (de invloed van de temperatuur op de continue productie van baculovirus door *Sp. frugiperda*, en modellering van de infectie van *Sp. frugiperda* cellen door baculovirus) werd uitgevoerd bij prof. dr. ir. J. Tramper, onder leiding van ir. F. F. J. van Lier.

Als derde werd vijf maanden stage bij de *School of Engineering,* onderdeel van de *University of Guelph,* te Guelph (Canada) gelopen. Deze stage (een simulatiemodel van de aërobische fermentatie in een CSTR) werd uitgevoerd bij en onder leiding van dr. G. L. Hayward.

Na het behalen van het doctoraalexamen in maart 1991 begon hij in september 1992 zijn promotieonderzoek (moleculaire karakterisering van opgelost organisch materiaal (DOM) in zeewater) aan de Technische Universiteit Delft bij de afdeling Organische Geochemie, waarvan dit proefschrift het eindresultaat is. In juni 1993 werd de gehele afdeling – omgedoopt in Mariene Biogeochemie – overgeplaatst naar het Nederlands Instituut voor Onderzoek der Zee, alwaar het promotieonderzoek voortgezet werd.

Nog voordat de promotie met een proefschrift afgerond was, verhuisde hij in juli 1997 naar de Verenigde Staten, waar hij als *Project Associate* aan de slag ging bij het *Center for Environmental Chemistry and Geochemistry* van de *Pennsylvania State University* te University Park (Pennsylvania, VS). Ook deze afdeling werd tijdens zijn verblijf verhuisd naar een andere universiteit, zodat hij van juli 1998 tot en met april 2000 als Post-doc bij de afdeling *Chemistry* van de *Ohio State University* te Columbus (Ohio, VS) werkzaam was.