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Chemical characterisation of dissolved organic matter in natural matrices

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Chemical Characterisation of Dissolved Organic Matter in Natural Matrices

A thesis submitted to the
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To Andy

He taught me English.

He taught me to measure twice and cut once.

He exemplified quinone toxicity.

Summary

A methodology was developed to analyse chemical and biochemical characteristics of DOM (Dissolved Organic Matter) on filtered natural water samples. The analysis was based upon simple assays that were matched to the reaction mechanisms occurring during humification. The chemical characteristics of DOM and the surrounding water matrix, pH, conductivity, temperature, TOC (Total Organic Carbon), complexing strength towards phenol, fluorescence, colour, UV absorbency at 200 and 240 nm, total, poly- and monophenolics, were condensed into a graphical 'fingerprint'. Assays to investigate the biochemical activity of DOM towards porcine pancreatic lipase activity and towards the precipitation of BSA (Bovine Serum Albumin) were developed.

The seasonal variation of DOM in six freshwater sites, three rivers and three wetlands, was investigated. Application of the 'fingerprinting' methodology showed a small effect of season and a large effect of site, substantiating the allochthonous origin of DOM. The application of multivariate statistics to the results, using principle component analysis, separated the assays into two groups relating to either chemical characteristics of DOM (polyphenolics, total phenolics, TOC, complexing strength towards phenol, colour and UV absorbance at 240 nm) or to the surrounding water matrix. The biochemical activity of DOM originated either from a physicochemical phenomenon, complexation, or the interaction of phenolics with protein. Humic and fulvic acid samples submitted to the same analysis showed similar fingerprints and responses in the protein precipitation assay, but inhibited lipase activity by 90 % compared to a maximum value of 30 % inhibition for the natural water samples at a similar TOC level.

A controlled decomposition study of cellulose, chitin and lignin in nutrient solution supplied with a broad-spectrum inoculant was performed. Two different treatments 'light' and 'dark' were given for each unit. The 'light' treatment induced algal growth and generated an additional microbially labile carbon source during the decomposition. The chemical characteristics of the DOM analysed after 15 months of degradation showed the 'fingerprint' to be dependent upon the chemical characteristics of the substrate supplied. The contribution of lignin to humification was substantiated by a high phenolic content of the lignin-containing units. Chitin, containing intramolecular nitrogen, was found to degrade rapidly, showing high values for fluorescence, and UV absorption at 200 nm after 15 months. The biochemical activity of the resulting DOM towards lipase activity was inhibitory for lignin 'dark', lignin & chitin 'light' and 'dark' and stimulating for chitin 'light'. The provision of cellulose & lignin as substrate had a neutral effect upon lipase activity.

The conclusions from the work presented in this thesis substantiate the importance of the chemical characteristics of the starting material for humification. Intramolecular nitrogen allowed for rapid degradation. Complexation and polymerisation with plant defence agents induce a random structure slowing enzymatic breakdown and thus leading to recalcitrance.

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Chapter 1: Introduction

1.1 Carbon-Cycle

Carbon is the main element for the building blocks of life. One of the characteristics of living organisms is their ordered structure. Decay, decomposition of dead matter, is described as the process of the organism's molecules returning to a random arrangement (Mauseth 1995). Dissolved Organic Matter (DOM), predominantly compounds leached from dead matter (Serrano 1992, Guggenberger and Zech 1994), encompasses a range of compounds of low molecular weight like monosaccharides, amino and carbocyclic acids to high molecular weight humic substances (Thurman 1985). The large contribution of dead organic matter to the overall cycling of carbon on the planet is illustrated in Figure 1.1, where the pools of carbon in air, soil and water and the associated fluxes are shown.

Fundamentally carbon associated with living or dead matter is termed organic and comprises more complex compounds than inorganic, by definition non-living carbon, which occurs in the form of carbonate, bicarbonate and carbon dioxide. The largest pool of carbon is inorganic with sediments and rocks containing 2×10^7 giga tonnes (Gt) C (Larson and Weber 1994), followed by dissolved inorganic carbon (DIC) with an estimated 1 to 4×10^5 Gt C (Larson and Weber 1994, Zepp and Sonntag 1995). The carbonate equilibrium induces the release of dissolved carbon dioxide into the atmosphere. Currently its pool is estimated to be 7×10^2 Gt C (Schlesinger 1991, Larson and Weber 1994, Zepp and Sonntag 1995).

The conversion from inorganic carbon into organic carbon occurs during photosynthesis where living matter is synthesised from carbon dioxide, water and energy, an overall reductive process leading to an estimated annual fixation of 7×10^2 Gt C in plants (Zepp and Sonntag 1995, Larson and Weber 1994). Decay describes the eventual oxidation of dead organic matter back to inorganic carbon, carbon dioxide.

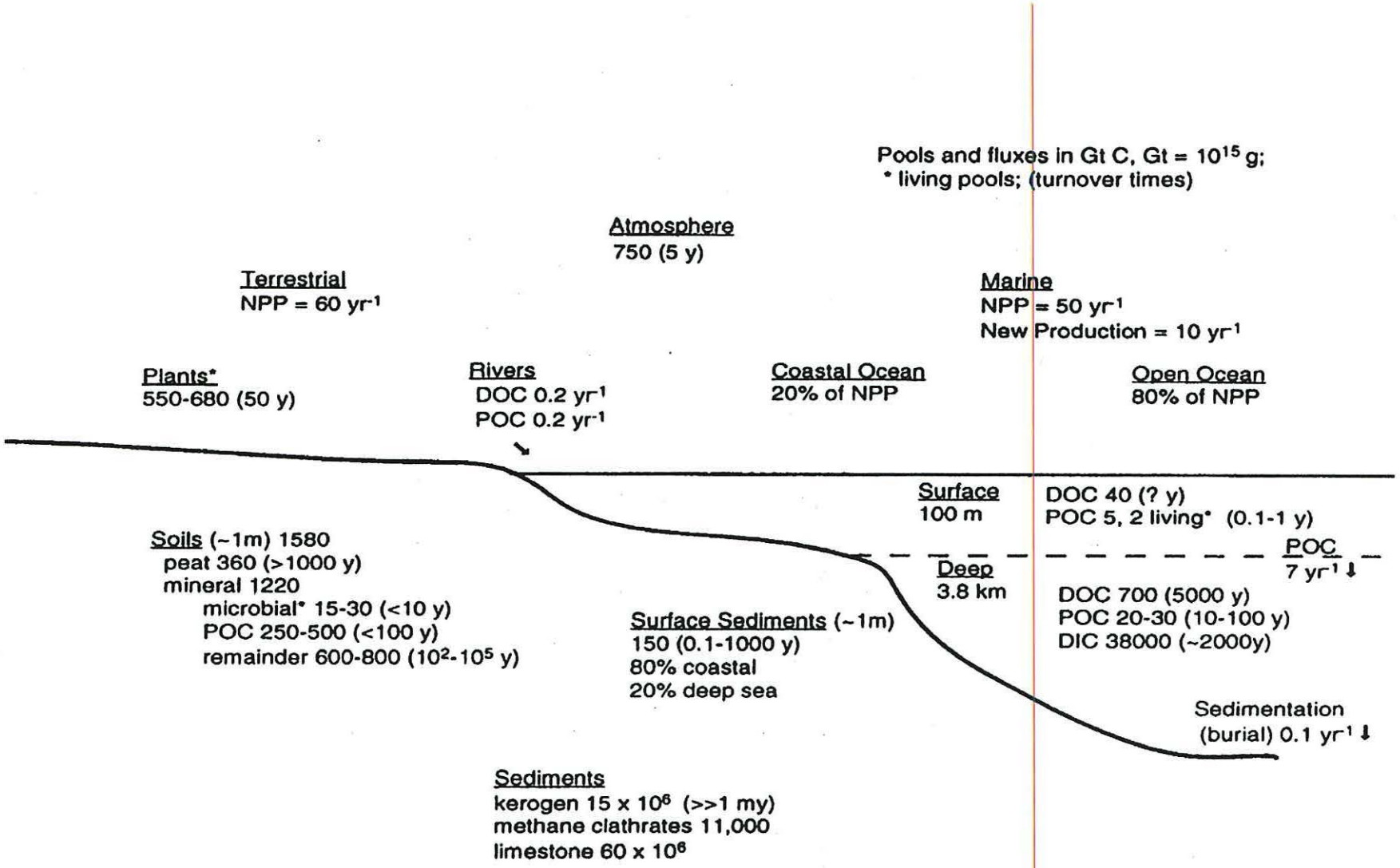

Figure 1.1: Pools, fluxes and residence times of carbon (Zepp and Sonntag 1995). NPP: Net Primary Production

The amount of dead organic matter on the planet is estimated to be at least three times that of living matter (Schlesinger 1991, Larson and Weber 1994, Zepp and Sonntag 1995). It occurs in the form of soil organic matter (SOM) or dissolved in rivers or oceans (DOC: Dissolved Organic Carbon and POC: Particulate Organic Carbon). The fossil fuels coal, oil and peat represent an ancient pool of dead organic matter. Regarding the fluxes of carbon from one pool to another it is estimated that SOM can take from more than 1000 years for peat to less than 10 years for microbially bound carbon to be turned over (Zepp and Sonntag 1995). Hayes (1997) gave a mean residence time of 32 years for carbon as part of SOM based upon the data provided by Schlesinger (1991).

This study is concerned with DOM in freshwater, DOM being carbon-containing compounds which pass through a 0.7 μm filter (Whatman GF/F). The carbon continuum (Figure 1.2) illustrates the size and weight range of compounds found in any given water sample. The pre-treatment of a water sample thus determines the analyte DOM. According to Thurman (1985) about 50 % of DOM is made up of humic substances, an operationally defined class of compounds (Thurman and Malcolm 1981).

Humic substances have been studied for nearly 200 years and are best described as high molecular weight compounds that contain acid groups, possess aromatic character, contain nitrogen, phenolic and quinone moieties and are joined via ether and ester bridges (Stevenson 1994, Hayes 1997, Davies and Ghabour 1999). The heterogeneous nature of humic substances gave rise to the suspicion that in any given water sample 'no two molecules' of the high molecular weigh fraction are likely to be the same (Stevenson 1994).

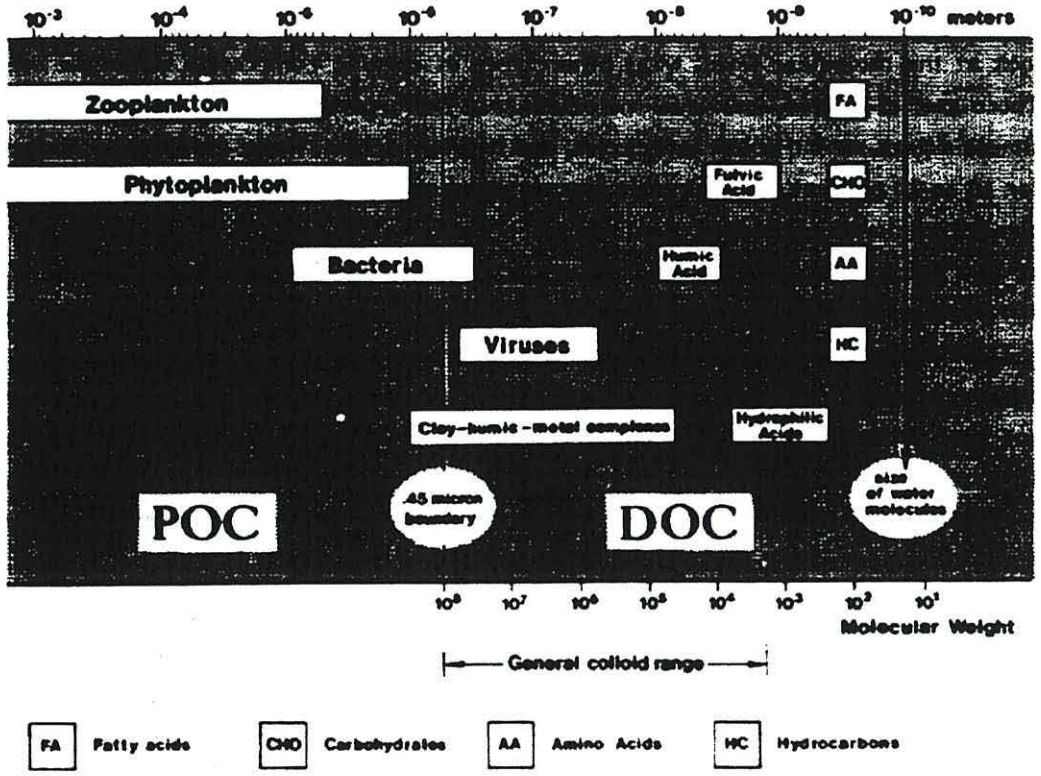

Figure 1.2: Organic carbon continuum in river waters (Thurman 1985).

1.2 Humification

Humification describes the conversion of plant debris via either environmental conditions or microbial transformations into high molecular weight humic substances. Despite their elusive nature, theories about humification have been developed over the years, whereby the different pathways relate to classes of compounds commonly encountered in plants or are microbially synthesised (Stevenson 1994).

Lignin corresponds to the remaining plant tissue after a series of extractions were performed with dichloromethane, hot water and warm sulphuric acid (Aber and Mellilo 1991). Lignin is the result of polymerisation reactions between p-hydroxycinnamyl, coniferyl and synapyl alcohols. Although the lignin precursors are known, no structural elucidation of lignin in its native state has so far been possible (Mann 1996). Tannins are vegetable polyphenols (Haslam 1998). Tannins are also called polyphenolics. The difference in name originated from polyphenols having been identified on a molecular level in contrast to polyphenolics which have no known molecular structure, but contain more than one phenolic moiety (Mole and Waterman 1994). Thus lignin and tannins are both polyphenolics.

Figure 1.3 shows the four possible pathways for humification according to Stevenson (1994):

1. **Sugar-Amine-Condensation:** Reducing sugars and amino acids, formed as by-products of microbial metabolism, undergo non-enzymatic polymerisations to form brown nitrogenous polymers. The underlying reaction mechanism is the Maillard reaction that occurs for example during the browning of bruised fruit.
2. **Polyphenols of non-lignin sources:** Microrganisms are thought to produce polyphenolics from non-lignin sources, like cellulose. These polyphenolics are oxidised, e. g. by the phenoloxidases, to quinones that polymerise with amino compounds to produce humic substances.

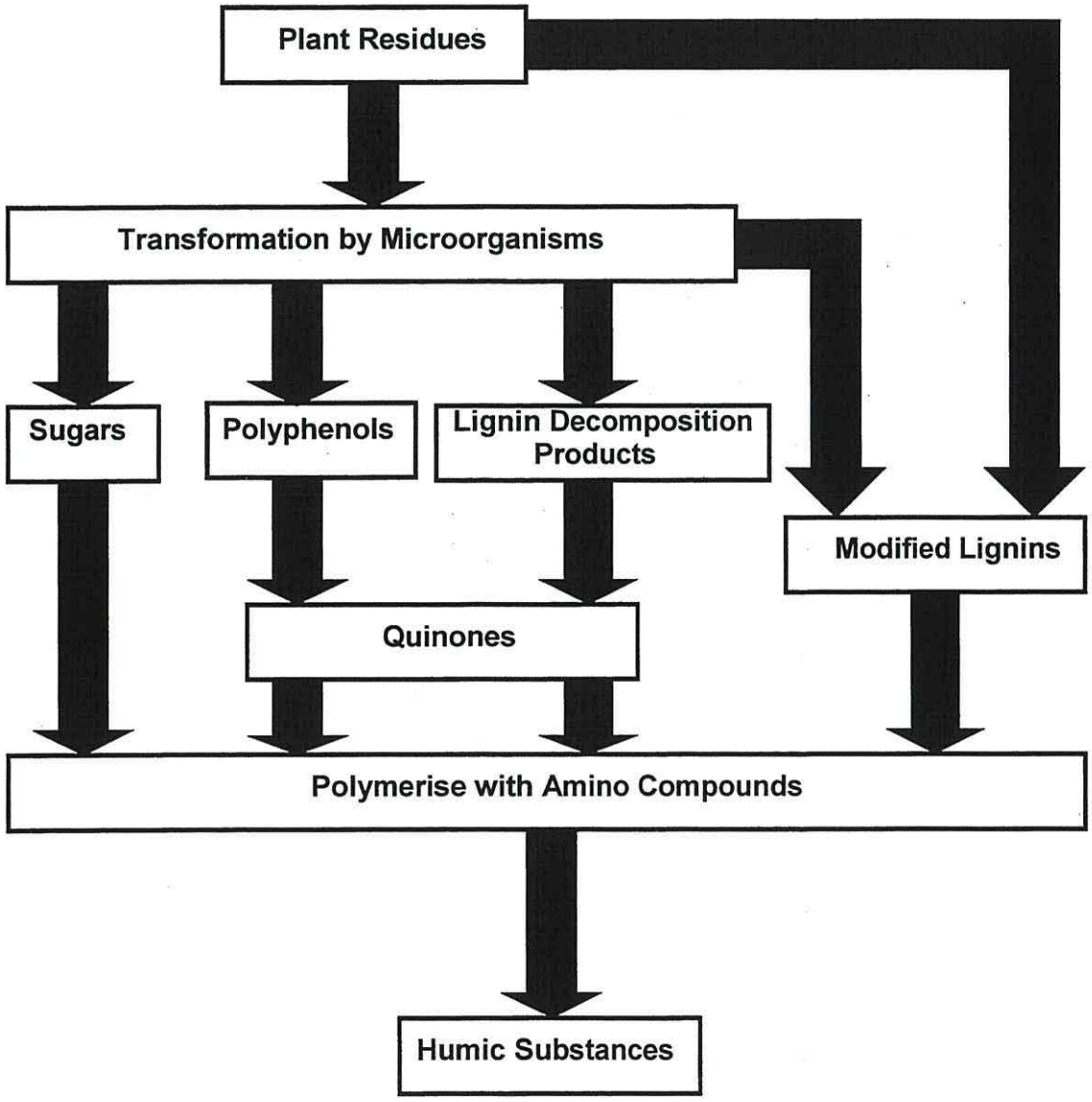

Figure 1.3: Pathways of humification (modified from Stevenson 1994)

3. **Lignin decomposition products:** Microorganisms release phenolic aldehydes and phenolic acids during the transformation of lignin. Phenoloxidases oxidise those lignin degradation products to quinones that polymerise with amino-containing compounds to form humic substances.

4. **Lignin theory:** The earliest theory of humification goes back to Waksman (1932) who postulated that humic substances are that part of lignin not utilised by microorganisms. According to Waksman, lignin was demethylated and oxidised before it condensed with amino compounds to form humic substances.

For pathways **2** and **3**, the oxidation of phenols to quinones is a key reaction. Decomposition eventually returns dead organic matter back into carbon dioxide, thus overall it is an oxidative process, compared to photosynthesis which overall is a reductive process, whereby living matter is formed from the highest oxidised form of carbon, carbon dioxide.

Polyphenolics are involved in three out of the four humification pathways, while the sugar amine condensation occurs in environments that lack a phenolic precursor. Examples of such environments are peat bogs with *sphagnum* moss as the main vegetation (Stevenson 1994). *Sphagnum* is an unusual plant insofar as it separated from the mainstream of evolution 220-280 million years ago. *Sphagnum* does not contain polyphenolics with tanning ability. However, *sphagnum*-dominated environments show preserving properties, thought to originate from the acidic conditions in peat bogs, a binding of bivalent cations and the presence of sphagnan. Sphagnan is a keto-sugar, 5-D-keto mannuronic acid. The keto groups in sphagnan in addition to the aldehyde and carboxyclic end groups allow extensive hydrogen bonding with amino groups. The tanning of sphagnan is thus an aldehyde tanning. The polymeric bridges that develop during the reaction between sphagnan and proteins make up a network, comparable to a spider's web (Painter 1995).

All four pathways include complexation of amino compounds with reducing sugars, quinones or modified lignin prior to their polymerisation. Complexation is used in this context as achieving the spatial proximity to allow polymerisation to occur. However, it is suspected that not every complexation is necessarily followed by a polymerisation reaction. The main reaction mechanisms involved in humification are thus oxidation, complexation and polymerisation. Similar chemical processes have been described as being of importance for DOM: adsorption, precipitation, oxidation/reduction and complexation (Hope et al. 1994).

Two closely related classes of naturally occurring polyphenolics can be distinguished, lignin and tannins (Stafford 1988). Lignin is the most abundantly produced aromatic compound on earth. Lignin's ecological role is to give vascular plants the rigidity they need to stand upright and to protect their structural polysaccharides. Historically tannins are classed as plant defence agents. Plant evolution has been traced through the development of biosynthetic pathways for polyphenolics (Mole and Waterman 1994, Gottlieb 1992). The definition of tannins according to Bate-Smith and Swain (1962) is: 'Water soluble phenolic compounds having molecular weights between 500 to 3000 (Daltons) and, besides giving the usual phenolic reactions, ... have special properties such as the ability to precipitate alkaloids, gelatin and other proteins.' (Haslam 1998).

The chemical structure of polyphenolics is important for humification. Microbial decomposition is driven by the activity of extracellular enzymes. Thus, the energetic cost to produce those enzymes has to be balanced with the energy gain from the products of decomposition. Generally, polyphenolics are classed as having 'low carbon quality' due to the presence of resonance stabilised double bonds (Aber and Mellilo 1991, Wetzel 1991). In addition they do not contain nitrogen, thus they have a low nutrient-to-energy content. Both properties of polyphenolics explain their slow decomposition and thus their accumulation as part of DOM.

The primary difference between lignin and tannins is their respective water solubility. Lignin is water insoluble while tannins are water soluble, and thus are more likely to form part of DOM (Serrano 1992). Forty years of research into plant polyphenolics, vegetable tannins, could aid the understanding of the ecological activity of DOM (Haslam 1998). The biochemical activity of polyphenolics can be explained by virtue of three distinctive characteristics which they all possess to a greater or lesser degree, and which derive in essence from the properties of the simple phenolic nucleus itself, namely:

1. Their ability to complex with metal ions.
2. Their antioxidant and radical scavenging activities.
3. Their ability to complex with other molecules including macromolecules such as proteins and polysaccharides (Haslam 1998).

Polyphenolics in plants give flavour and colour. The defence action of polyphenolics is thought to originate from their ability to bind to protein and thus inhibit the activity of enzymes (Goldstein and Swain 1965, Mole and Waterman 1987, Wetzel 1991 & 1993). This is primarily important in the context of fungal plant diseases (Scalbert 1992, Schultz et al. 1992). The mode of action of polyphenolics responsible for enzyme inhibition depends on the environmental conditions (Appel 1993). In acidic environments, phenolics act primarily via hydrogen bonding and hydrophobic interactions, mildly alkaline conditions favour ionic bonding, while under more highly alkaline conditions (pH 10) oxidation of phenolics to quinones occurs.

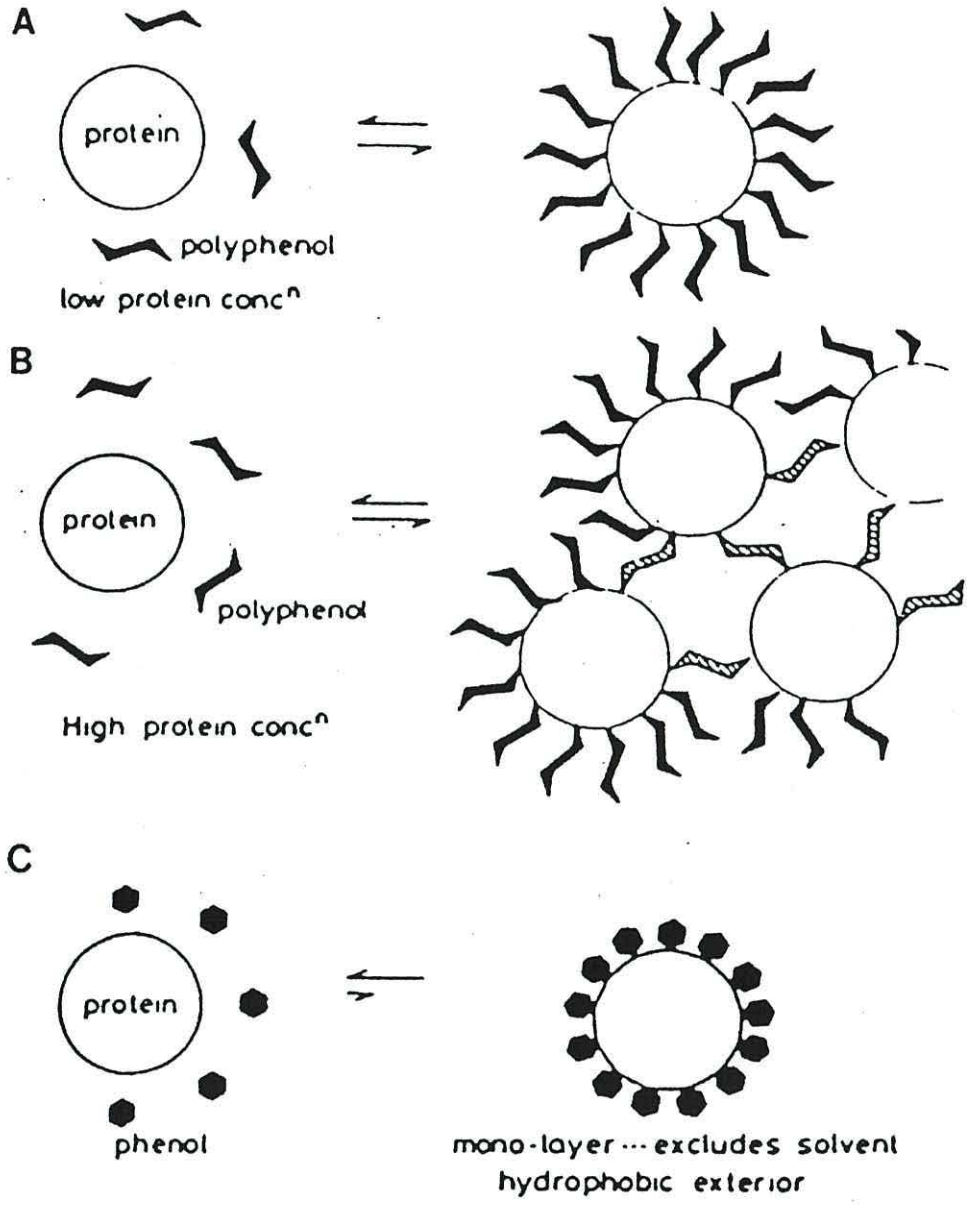

Figure 1.4: Interaction between monomeric and polymeric phenolics and protein (from Spencer et al 1988).

The interaction between polyphenolics and enzymes has been identified as the key mechanism in their defence action and subsequent regulatory functions (Figure 1.4) (Goldstein and Swain 1965, Spencer et al. 1988, Wetzel 1991, Field and Lettinga 1992, Haslam 1998). Gallic acid, a monomeric phenolic originating from hydrolysable tannic acid, possesses three hydroxy groups, that can hydrogen bond with carbonyl groups of proteins at low pH. Proanthocyanidins, a type of polyphenols, show a C6-3-C6 structure with hydroxy groups on each C6 moiety to undergo hydrogen bonding. Polyphenolics, starting with a nominal weight of 500 D, are at least dimers of, for example, proanthocyanidins, thus they can cross-bridge even more effectively. Research into the enzyme inhibiting activity of different polyphenolics showed a reduction in inhibition concentration with an increase in molecular weight of the polyphenolics studied (Field and Lettinga 1992). At neutral pH values and above, highly hydroxylated polyphenolics can autoxidise or are likely to be oxidised by enzymes such as peroxidases to quinones with subsequent polymerisation. Pillinger et al. (1994) studied the anti-algal acitivity of rotting barley straw and found quinones to be more potent inhibitors by two orders of magnitudes compared to phenolics. However, with regard to polyphenolics and polymerised quinones and their interaction with enzymes, the molecular weight of the compounds determines their activity. The higher the molecular weight, the more effective the compounds are at cross-bridging, however, in order to inhibit intracellular enzymes, the condensed or polymerised polyphenolics have to be able to cross cell membranes. The maximum molecular weight for tannins able to penetrate cells of methanogenic bacteria was estimated to be 3000 D (Field and Lettinga 1992), hence the definition of tannins giving a molecular weight range from 500 to 3000 D. A decrease in protein precipitation of extracellular enzymes by polymerised polyphenolics was found when the molecular weight exceeded 20,000 D. For the biodegradation of tannins, small molecular weight phenolics were found to be biodegraded, while condensed tannins tended to polymerise and resisted degradation (Field and Lettinga 1992).

1.3 Ecological Functions of DOM

The main ecological functions of DOM have been summarised by Williamson et al. (1999) as being overall an ecosystem regulator. The underlying reaction mechanisms for DOM functioning are the same as those occurring during humification. However, while polymerisation is the key to the synthesis of high molecular weight DOM, complexation is the key mechanism by which its regularity function is achieved.

DOM of riverine ecosystems is the base of food chains (Allan 1995). Heterotrophic microorganisms enzymatically degrade plant debris resulting in the production of DOM and thus retrieve the chemical energy stored in dead matter (Wetzel 1991). The degradation of leaves is the most researched area. It was found that initially soluble compounds, including polyphenolics (Serrano 1992), were leached from the plant debris (Allan 1995). A gradual reduction in size occurred during leaf degradation from coarse to fine particulate organic matter leading eventually to DOM. The main criterion for the food value of DOM was the measurement of change in microbial biomass and enzyme activity. To investigate DOM-microbial-interactions, fractionations based upon nominal molecular weight were employed. The low molecular weight fraction of less than 1000 D and the high molecular weight fraction of 10,000 D and above were found to sustain increased microbial growth, compared to DOM of intermediate molecular weight (Allan 1995, Transvik 1990). It is interesting to note the similarity between the biological activity of vegetable polyphenols, molecular weight range 500 to 3.000 D, and the medium size molecular weight range, 1,000 to 10,000 D sustaining the least microbial growth.

In another study, the effect of high molecular weight DOM (above 10,000 D) upon microbial metabolism was measured calorimetrically; Freeman and Lock (1992) found an inhibitory effect. They suspected this to be caused by a physico-chemical phenomenon, the occlusion of biofilms. Thus they proposed the high molecular weight fraction to be a regulator of ecosystem

metabolism.

A separation technique to isolate the high molecular weight fraction was introduced by Thurman and Malcolm (1981). The separation of humic and fulvic acids involved passing water samples through cation and anion exchange resins, after which low molecular weight compounds were separated using a neutral column. The aim behind the isolation of humic substances was to obtain a crystalline powder that was low in ash and allowed the application of spectroscopic techniques like ^{1}H-NMR and ^{13}C-NMR. In addition, the humic substances could serve as reference materials to compare work performed by different laboratories.

However, there are some disadvantages with the isolation procedure. The hydrophobic fraction of humic substances tended to be tightly bound to the column and did not elute (Lara and Thomas 1994). Additionally the isolation of humic substances from the mineral content of the water might alter its biological activity and thus information relevant for biogeochemical changes (De Haan 1992). Perdue and Gjessing (1990) also suspected a loss of information relevant for the ecological activity of humic substances and suggested the need for further research to be directed towards finding bulk characteristics of DOM.

Researching the interactions between enzymes and humic substances, Wetzel (1993) studied alkaline phosphatase activity in the presence of humic acid. He found the enzyme activity to be dependent upon the presence of monovalent or bivalent cations. Complexation between bivalent cations and humic acid was suspected to explain the increased productivity in hard water lakes and rivers, because the complexation of enzymes, and thus their inhibition by humic acids, was reduced due to them already being complexed by the bivalent cations (Ladd and Butler 1970). Dudley and Churchill (1995) studied the interaction between humic acids and two aquatic extracellular

proteases. While humic acids inhibited the serine protease at natural pH and concentrations, the metallo protease was not inhibited. Adding calcium ions or increasing pH reversed the inhibition of the serine protease. Lu and Huang (1994) determined 50 % inhibition of xanthine oxidase activity at humic acid concentrations between 25 to 75 mg l^{-1}. Jahnel and Frimmel (1994) used the proteolytic enzyme pronase E and determined that concentrations of humic and fulvic acids of 153 to 6,360 mg l^{-1} induced 50 % inhibition. These studies showed the interaction between humic substances and enzymes to be dependent upon the type of enzyme used, the pH and the presence of bivalent cations competing for complexing sites on the humic acids.

During the preceding paragraphs the complexing ability of DOM or humic substances was introduced. Each pathway during humification included the complexing of polyphenolics or quinones with amino containing compounds. Similarly the inhibition of enzymes by humic substances was thought to originate from such a complexation. Furthermore this complexation could stabilise the enzyme (Dudley and Churchill 1995), or be the reason for inhibition due to introducing a change in enzyme folding. The reduction in inhibition on addition of calcium ions as measured by Dudley and Churchill (1995) and Wetzel (1993), was also thought to originate from complexation between humic substances and calcium ions, reducing the number of possible sites for complexing the enzyme. Overall the complexing ability of DOM or humic substances forms a key aspect of their ecological activity.

DOM or humic substances are considered to alter contaminant behavior and nutrient availability in natural waters (Williamson et al. 1999). The distribution of metals or anthropogenic compounds is facilitated by the complexing ability of DOM or humic substances. The stability constants of metal humic complexes have been determined using fluorescence quenching or ion selective electrodes (Saar and Weber 1980, Ryan and Weber 1982, Ryan et al 1983). For organic solutes, humic substances increased their water

solubility (Chlou et al. 1987). Gauthier et al. (1987) studied the partitioning of pyrene between water and octanol and found the partition coefficient to change upon the addition of humic substances. Traina et al. (1996) studied the association between linear alkylbenzenesulfonates and humic substances using fluorescence quenching. The stability constants thus obtained were compared with the bioavailability of the linear alkylbenzenesulfonates for the fathead minnow, *Pimephales promelas*. They found that the substrate was less available when associated with humic substances.

In conclusion the ecological functions of DOM and humic substances are closely associated with their complexing properties. As already illustrated during the humification process, complexation with amino containing compounds was identified as a key step. Enzymes, the driving forces of degradation, are amino containing compounds and are thus equally submitted to complexation leading to stabilisation or inhibition. The function of DOM and humics to alter contaminant toxicity and nutrient availability is equally based upon complexation.

DOM also forms the base of aquatic food chains. In defining food, the supply of organic fuel molecules to an organism is referred to; The oxidation of these using oxygen provides the energy for essential processes (Ritter 1996).

Overall, for the ecological functions of DOM, the same two reaction mechanisms occurred that have already been discussed for humification: oxidation and complexation (Williamson et al. 1999).

1.4 Conclusions

Dead organic matter represents a major pool of carbon in soil and natural waters, estimated to be three times that of the carbon stored in living matter. The transformations of plant debris follow a succession with the removal of the high carbon quality substrates being parallel to a reduction in molecular

size eventually leading to DOM. The low carbon quality substrates of aromatic nature, especially phenolics, are accumulated during the formation of DOM. These phenolics undergo reactions, described as humification, of oxidation and complexation to polymerise to high molecular weight recalcitrant humic substances. The same reaction mechanisms explain the ecological functioning of DOM in being an ecosystem regulator, complexing toxicants and nutrients and being the base of food chains.

1.5 Outlook

The ecological importance of DOM for aquatic ecosystems derives from the same chemical reaction mechanisms that also lead to the formation of humic substances. The work presented in this study uses those reaction mechanisms for the development of a suite of assays to analyse DOM, because a need for assays to be applied directly onto filtered natural water samples has been identified. The assays developed concentrate on phenolics as a target, because of their recalcitrance, complexing ability and oxidation potential. As shown in the preceding sections, phenolics are implicated in humification and the ecological functioning of DOM. The suite of assays developed was separated into chemical characteristics (called 'fingerprint') and biochemical activity of DOM. Complexation is the most important reaction mechanism for the formation of DOM and its ecological function, however, no assay for its evaluation was found.

The methodology is applied onto three rivers and three different wetland sites, whereby samples have been taken throughout the year to investigate seasonal variations of DOM. Additionally, a controlled decomposition study using terrestrial and marine bulk carbon sources is conducted to study the dependency of humification on the chemical characteristics of the starting material. The results of the field and controlled decomposition study are combined to gain further insight into decomposition and evaluate the contribution of the different pathways for humification.

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Chapter 2: Analysis

Synopsis: The aim of this study was to chemically and biochemically analyse DOM (Dissolved Organic Matter) in an unmodified matrix using quick and simple analysis. The assays were matched onto the different pathways during humification. Thus the main reaction mechanisms upon which the analysis is based are oxidation, complexation and polymerisation.

The analysis to chemically characterise DOM concentrated on three distinct aspects: environmental parameters, bulk carbon characteristics and phenolic content. Difficulties in the interpretation of each assay were anticipated due to DOM being a heterogeneous analyte (Appendix 1). As a result, each chemical characteristic was covered with two assays, were possible. The biochemical assays determining the influence of DOM upon lipase activity and BSA (Bovine Serum Albumin) precipitation were also developed.

This broad approach to DOM characterisation led to the accumulation of a large data set. Therefore multivariate statistical techniques, PCA (Principle Component Analysis) and cluster analysis, were applied that assumed independence of the data. The graphical display of the results was presented as a 'fingerprint', collating all 12 assays that chemically describe DOM within one graph.

The analyses were developed to provide a suite of methods to characterise DOM in unmodified matrices. After the initial work on standard solutions it was necessary to confirm the suitability of the assays by working with real samples. The samples used were either freshwater samples (further details in Chapter 3 and Appendix) or samples obtained from the controlled decomposition study (further details in Chapter 4). The results and discussion sections in this chapter concentrate on the evaluation of the assays for the purpose of the analysis. Interpretation of data gained from samples regarding their chemical or biochemical characteristics can be found in Chapters 3 and 4.

2.1 The Matrix

2.1.1 Introduction

The matrix is the phase that surrounds the analyte, the target for an analysis. The analyte was Dissolved Organic Matter (DOM). Water was the solvent that leached carbon containing compounds from plants and other organic matter debris (Serrano 1992), whose degradation was influenced by the water chemistry (Jenkins & Suberkropp 1995).

Temperature, pH and conductivity were identified as environmental parameter of relevance for decomposition reactions. There were other components of the matrix such as dissolved minerals or gases. These minerals could act as nutrients or be heavy metals. Dissolved gases included oxygen, carbon dioxide and/or methane. Overall their concentration determined conductivity and pH, parameters which influence biological activity in freshwater and thus the types of decomposition processes. Conductivity indicates the quantities of ions present in the water and pH is relevant for most biochemical reactions, in particular enzyme activity and complexation based on hydrogen bonding (Ladd and Butler 1970, Serrano and Boon 1991, Wetzel 1991,1993, Dudley and Churchill 1995). Temperature is an equally important parameter influencing chemical and biological rates of reaction. The change in standard free energy ($\triangle G^{o}$) of chemical reactions is dependent upon the molar gas constant R, temperature T and equilibrium constant K (equation 1) (Streitweiser and Heathcock 1981).

(1) $$\triangle G^{o} = - R T \ln K$$

In order to maintain the matrix in as un-disturbed a state as possible, the aim of the assays was to design the analyses to be chemically 'mild'. For all measurements it was necessary to have water that did not contain dissolved minerals or dissolved organic matter and could serve as a blank: pure water.

2.1.2 Materials and Methods

Environmental Parameters

Conductivity, pH and temperature were measured on-site using the Hanna Water Tester. The water tester was calibrated for conductivity using a 0.01 M KCl solution (Analar from BDH), whose conductivity was 1413 μS at 25 °C. Buffer solutions of pH 4, 7 and 10 (from BDH) were used to calibrate pH. A two-point calibration, pH 4 and 7, was performed and the buffer pH 10 measured to assure linear calibration. Temperature was compared with a mercury thermometer.

Preparation of 'Organic Matter Free Water' using Double Distillation

Double distillation was used with the first still being continually fed with tap water, suspected to be of high organic but low inorganic load. Alkaline potassium permanganate was dissolved in the first still to oxidise organic carbon to carbon dioxide. To remove dissolved carbon dioxide and to prevent other volatile carbon containing compounds from entering the still, it was kept under positive nitrogen pressure. The second still received the distillate from the first still and re-distilled it. For maintenance, the first still was soaked in sodium meta-bisulphate to remove manganese dioxide that precipitated on the heating element.

2.1.3 Results and Discussion

In order to verify the suitability of the doubly distilled water as a blank to be used in this study, an analysis for 15 metals using Inductive Coupled Plasma Optical Emission Spectroscopy (ICP-OES) was conducted on MilliQ, doubly distilled and commercial HPLC grade water. Apart from phosphorus with 0.04 mg l^{-1} all the other elements, As, Se, Cr, Zn, Pb, Co, Cd, Ni, Si, Mn, Fe, Pt, Mg, Ca, Cu, Al, Na and K, showed concentrations below 0.01 mg l^{-1}. After pre-concentration of MilliQ and doubly distilled water by a factor of 200 using solid phase extraction, the pre-concentrate was analysed using ICP-OES for

14 metals (Sn, Cr, Zn, Pb, Ni, Si, Mn, Fe, Mg, Cu, Ca, Al, Na, and K) and concentrations of 0.01 mg l^{-1} (Calcium in Milli Q) or less were detected. In an analysis for dissolved carbon it was found that MilliQ water contained 0.5 mg C l^{-1} and Elga water contained 1 mg C l^{-1} more than the doubly distilled water.

Considering the environmental parameters, the doubly distilled water tended to be slightly alkaline, pH between 7 and 9, due to alkaline $KMnO_4$ being used in the first distillation. Conductivity < 1 μS was determined.

Overall, the doubly distilled water was found suitable as a blank, having less dissolved carbon and similar levels of dissolved metals when compared with alternative sources of available de-ionised water.

Acknowledgements

G. C. Connolly developed the method and performed the metal analysis for the comparison of MilliQ, doubly distilled and commercial HPLC grade water. S. Garcia-Ortega pre-concentrated MilliQ and doubly distilled water and performed the metal analysis.

2.2 Dissolved Organic Matter (DOM)

2.2.1 Introduction

DOM is operationally defined as organic matter, which passes through a Whatman GF/F filter with a nominal pore size of 0.7 μm, according to the manufacturer. DOM consists of a continuum of compounds ranging from small molecular weight substances such as hydrocarbons, fatty acids, carbohydrates and carboxylic acids to high molecular weight substances, in particular humic and fulvic acids (Figure 1.2, Thurman 1985). Ten to twenty-five percent of DOM is usually identifiable, comprising mostly carbohydrates and a small fraction of monomeric sugars, disaccharides and amino acids (Thurman 1985, Allan 1995, Larson & Weber 1994). Humic and fulvic acids are also operationally defined and comprise approximately fifty percent of DOM (Thurman 1985). They are obtained by processing a water sample over XAD resin to remove associated minerals and small molecular weight neutral compounds. Dissolution of the freeze-dried eluent and acidifying the solution to pH 1 separates humic acids from fulvic acids. While Humic acids precipitate at pH 1 fulvic acids stay in solution (Thurman and Malcolm 1981). The aim of separating humic and fulvic acids from water was to produce a crystalline powder low in ash which could then be further analysed, e. g. using spectroscopic techniques such as ^{1}H or ^{13}C-NMR.

Although humic substances represent approximately fifty percent of the carbon pool in freshwater, a disadvantage of the processing necessary to gain humic substances is that DOM has to be submitted to strong reaction conditions. Shifts in pH between pH 2 while loading the column and pH 12 while eluting the column succeed in obtaining the recalcitrant pool of DOM, but might induce changes in the chemical composition of high molecular weight DOM. De Haan (1992) and Perdue and Gjessing (1990) suspected that the processing of water samples to gain humic substances might introduce artefacts, and thus recommended research into methods for bulk carbon characteristics to study DOM in an un-modified matrix, an approach

adapted in this study.

The concentrations of DOM encountered in natural water samples are low. According to Allan (1995) the highest mean annual concentration of TOC encountered in streams and rivers in the USA is 28 mg C l^{-1} with common values from 1 to 4 mg C l^{-1}. Larson and Weber (1992) reviewed DOC in freshwater and found levels at 5 to 6 mg C l^{-1}. Thurman (1985) reported concentrations of DOC up to 40 mg C l^{-1} in wetlands, for which Larson and Weber (1992) quoted a range from 20 to 50 mg C l^{-1}. These concentrations are too low to apply most spectroscopic techniques apart from UV-VIS absorption.

The heterogeneous nature of DOM was confirmed during the pre-concentration experiments (Appendix 1). Additionally it was suspected that a pre-concentration technique would remove only part of DOM. Because of this, it was decided to omit pre-concentration and instead to use a number of quick and simple assays to determine bulk characteristics of DOM. TOC was measured via oxidation of DOM to carbon dioxide with infrared detection. Fluorescence at excitation 350 and emission 445 nm, thought to be derived from coumarin derivatives, was used as an indicator of humic substances (Larson and Weber 1994). An assay to measure the quenching of phenol fluorescence added at naturally occurring levels was developed (Eftink 1991). Colour in Grade Hazen was determined, as routinely performed during drinking water analysis (APHA). The last assays measured UV absorption at 200 and 240 nm. The reaction mechanisms employed for the bulk carbon characteristics are oxidation for TOC, complexation originating from the quenching of phenol fluorescence and UV absorption being indicative of polymerisation

Six, partly novel, assays of bulk parameters of DOM were performed on Whatman GF/F filtered natural water samples. For humic substances it was thought that 'no two molecules' of the high molecular weight fraction were the same (Stevenson 1994), thus determining a variety of bulk

characteristics seemed a promising way for the analysis of DOM.

2.2.2 Materials and Methods

TOC (Total Organic Carbon)

TOC was measured on a Shimadzu Total Organic Carbon Analyser. Potassium hydrogenphthalate (0 to 25 mg C l^{-1}, Analar from BDH) was used as a standard. The standards and samples were acidified with 1/1000 of their volume conc. nitric acid (Analar from BDH). The standards and samples were purged with ultra pure nitrogen for 15 min prior to analysis.

Fluorescence

The fluorescence of natural water samples was measured at excitation 370 nm, slit width 15 nm and emission 445 nm, slit width 15 nm on a Perkin Elmer Luminescence Spectrometer LS 50.

Assay to Measure the Complexation of Phenol by DOM: Complexed Phenol Assay

Phenol is fluorescent at excitation 270 nm, slit width 15 nm, and emission 300 nm, slit width 10 nm, (Daharu and Sporns 1984). A calibration of 0 to 1 mg phenol l^{-1} was obtained. The fluorescence of samples and samples spiked with 0.1 and 0.2 mg phenol l^{-1} was measured. A linear regression was performed for the calibration and the sample and spiked samples. The difference between the slope of the calibration and the slope of the sample spiked with phenol was thought to have originated from phenol being complexed by DOM. Thus the free phenol in the spiked samples is fluorescent, while the complexed phenol is non-fluorescent.

% Phenol complexed = (1 - slope spiked sample / slope calibration) x 100

UV spectroscopy

The sample was placed in a quartz cuvette and the absorbance at 200 and 240 nm was measured in a Unicam UV/VIS spectrophotometer. The spectrophotometer was blanked with doubly distilled water.

Colour in Hazen Units

K_2PtCl_6 (125 mg, from Sigma) and $CoCl_2 \times 6\ H_2O$ (100 mg, Analar from BDH) were dissolved in doubly distilled water; conc. HCl (10 ml, Analar from BDH) was added and the total volume adjusted to 100 ml. The stock solution represents 500 Hazen units (APHA). The stock solution was diluted to 0, 25, 50, 75, 100 125, 150, 175 and 200 Hazen units. The absorbance of standards and samples was measured at 350 nm (APHA).

2.2.3 Results and Discussion

Measuring TOC via oxidation of dissolved carbon containing compounds is a common assay in ecological studies (Allan 1995, Thurman 1978) and requires no further discussion.

Fluorescence of DOM has been summarised by Larson and Weber (1994). They stated that the nature of the fluorophore was unconfirmed; however, caffeic acid, a degradation product of lignin, was converted into the coumarin derivative esculetin upon exposure to light and/or oxygen. Esculetin has an excitation maximum of 363 nm and emission maximum of 447 nm which were close to the excitation and emission wavelength commonly employed for humic substances. Additionally, as the application of the assays during the seasonal variation study will illustrate, fluorescence did not vary strongly for each individual site but did vary between sites, substantiating the allochthonous origin, derived from the vegetation on the watershed.

The complexed phenol assay, developed during this study, illustrated the interaction between phenol and DOM under site specific conditions. The only change induced in the sample was the spiking with naturally occurring levels

of phenol (0.1 and 0.2 mg l^{-1}, as determined during this study). At the pH of most natural water samples, 4 to 8, phenol was not appreciably ionised (Appel 1993), thus no buffer was necessary. The complexed phenol assay measured the complexing strength of DOM using quick and simple analysis.

DOM was known to complex hydrophobic organic pollutants like DDT (Chlou et al. 1987) and pyrene (Gauthier et al. 1987, Kenworthy and Hayes 1997). The strength of humic-metal (Saar and Weber 1980, Ryan et al. 1983, Ryan and Weber 1982) and humic-linear alkylbenzenesulfonates complexes (Traina et al. 1996) was measured using fluorescence quenching. As discussed in the description of the fluorescence assay, the fluorescence of DOM was thought to derive from esculetin rather than bulk DOM; thus a more worthwhile approach seemed to be to utilise a fluorescent ligand, phenol (Daharu and Sporns 1984), to assess the complexing strength of DOM (Eftink 1993) via the quenching of phenol fluorescence.

The last three assays used the UV/VIS absorption of DOM as bulk characteristics. The UV absorbance at 254 nm (Perdue and Gjessing 1990) and the 465 to 665 nm ratio (Sparks 1996) had been used for the study of humic substances. The colour of natural water determined in grade Hazen was similarly employed. For the colour assay a standard was used, potassium hexachloroplatinate, to allow comparison between different laboratories (APHA).

UV spectra of DOM commonly show a gradual decline with increasing wavelength (Haider et al 1975, Sparks 1996). The absence of peaks in UV spectra of natural waters illustrated the heterogeneous nature of the DOM. However, UV absorbance was considered important because it was suspected that it originated primarily from aromatic moieties and increased with the extension of aromatic systems (Streitweiser and Heathcock 1981). Measuring UV absorbance on three different wavelength, 200, 240 and 350 nm, gave an estimate of the slope of the decline and was thought indicative of the degree of polymerisation.

During the application of the assays, for the controlled decomposition and for the seasonal variation (Chapters 3 and 4), it was noted that samples providing conditions for high biological activity showed an absorption maximum between 200 and 240 nm.

Overall, six assays to measure bulk carbon characteristics of DOM were researched and developed. The suite encompassed TOC, representing the pool of carbon on the site and illustrating the oxidative potential of DOM, fluorescence was thought to indicate lignin origin, complexation of phenol under site specific conditions and UV/VIS absorbance measurements indicative of the extent of polymerisation.

2.3 Phenolics

2.3.1 Introduction

Phenolics are a class of compounds containing an alcohol group attached to an aromatic ring. During plant evolution higher plants synthesise phenolics, in particular lignin and tannins, as defence agents (Mole and Waterman 1994, Haslam 1998). Monomeric phenolics, such as vanillin and syringealdehyde, have been used as biomarkers to illustrate the terrestrial origin of DOM in ocean matter (Ertel et al. 1984). The connection between phenolics and humic substances in the humification process was shown by Stevenson (1994), with polyphenolics as the starting compounds for three out of the four pathways of humification (Figure 1.3). These polyphenolics are either modified via environmental conditions or microbially transformed to monophenolics which after oxidation to quinones, polymerise with amino containing compounds to humic substances.

Conceptually, degradation is the gradual reduction in size. The decomposition of polymeric phenolics is expected to produce monomeric phenolics. Thus as a route into the analysis of phenolics it was thought worthwhile to find assays that distinguished between mono- and polyphenolics as part of the phenolic pool in freshwater bodies. The term total phenolics referred to the total concentration of phenolic hydroxy groups in tannin analysis of plant matter (Mole and Waterman 1994). Based on that accepted definition the term monophenolics in this study is used to describe compounds with one phenolic hydroxy group and the term polyphenolics is used for compounds with two or more phenolic hydroxy groups. Phenol as the parent compound was chosen to serve as a standard for monophenolics, thus the concentration of monophenolics found for the water sample is given as PE (Phenol Equivalents). Tannic acid was chosen as a standard for polyphenolics and the concentration found was given as TAE (Tannic Acid Equivalents).

Colorimetric analysis of phenolic compounds was chosen because of simplicity, the filtered water sample simply being mixed with one or more reagents. Previous research had used colorimetric analysis primarily for the determination of phenolics in plants and phenol in industrial effluents (Gibbs 1926, Mohler and Jacob 1957, Cheeseman and Wilson 1972, Mole and Waterman 1994). For the present colorimetry-based assays, microplates were used, which had the advantage of small reagent volumes, replication and speed of measurement - 96 wells could be determined in 15 seconds.

The following assays were thus investigated for their applicability for natural waters: Folin-Ciocalteau (Box 1983), Prussian blue (Price and Butler 1977), iron complexation (Mole and Waterman 1994, Serrano 1992), Gibbs (Gibbs 1927, Quitana et al. 1997, Daharu and Sporns 1984) and 4-aminoantipyrine (Daharu and Sporns 1984, APHA).

The assays had already been used singly in analysis of natural waters, where, e.g., a relationship between phenolic content of the high molecular weight fraction (> 1000 D) DOM and the inhibition of biofilm metabolisms was suspected (Freeman et al 1990). Serrano (1992) measured polyphenolics of vegetation leachates and used the iron complexation assay because of interference by non-phenolic compounds in the Folin-Ciocalteau assay. Pollution of marine environments by phenol using the 4-aminoantipyrine assay was investigated by Dahab (1996) and Kadam and Bhangale (1996). However, this present study sought to evaluate the simultaneous application of complementary assays to investigate multiple aspects of the phenolic pool of natural waters.

2.3.2 Materials and Methods

Preparation of Standards

Phenol (Analar from BDH) was used as a standard for monophenolics. A hydrolysable tannic acid (Tannic Acid BP 73 from Roy Wilson Dickson, Chester, England) was used as a standard for polyphenolics. All standards

were prepared fresh in double-distilled water. The assays were carried out in microplates (96 flat well, from Phillip Harris) and the absorbance was read on a Labsystem Multiskan MS (Life Sciences International).

Procedure

Initially all assays were carried out with both standards in a range of 0 to 5 mg l^{-1}. To investigate the sensitivity of the assays for either mono- or polyphenolics, the CoD (Criterion of Detection according to Caulcutt and Boddy 1983) for each was determined. To determine specificity of the Prussian blue and iron complexation assays, 5 mg phenol l^{-1} was included in the standard to detect interference by monophenolics. For the Gibbs and 4-aminoantipyrene assays, 50 mg l^{-1} tannic acid was included in the standards to detect interference by polyphenolics.

All samples were filtered (Whatman GF/F) prior to usage. For the work on the rivers the samples were spiked independently with 0.1 and 0.2 mg phenol l^{-1} and 1 and 2 mg tannic acid l^{-1}. A table of site characteristics for the rivers used can be found in the appendix. The specificity of the assays towards mono- and polyphenolics was tested with the spiked samples. Recovery rates of the spike were measured and the 95 % confidence interval for the concentration of mono- and/or polyphenolics in the samples was calculated according to Caulcutt and Boddy (1983).

Assay to Measure Total Phenolics: Folin-Ciocalteau Assay

For the Folin-Ciocalteau assay 1 ml of the water sample, 0.15 ml Na_2CO_3 (200 g l^{-1} AnalaR from BDH) solution and 0.065 ml Folin-Ciocalteau reagent (from BDH) were pipetted into a microtube and inverted twice. Two microtubes were run per sample. After two hours two wells were filled with 0.35 ml from each microtube. The absorbance was read at 750 nm. Tannic acid (0 to 5 mg l^{-1}) was used for calibration.

Assay to Measure Polyphenolics: Prussian Blue

The method was based on Price and Butler (1977), but the concentration of the reagents was altered to accommodate the smaller levels of phenolics found in natural water samples. A 0.25 ml sample, 0.025 ml $K_3[Fe(CN)_6]$ solution (Analar from BDH, 0.0025 M solution prepared on the day) and 0.025 ml $FeCl_3$ solution (Analar from BDH, 0.1 M $FeCl_3$ in 0.1 M HCl) were mixed. The microplate was left at room temperature for 30 min and the absorbance was read at 750 nm. Tannic acid (0 to 5 mg l^{-1}) was used for calibration.

Assay to Measure Monophenolics: Gibbs Assay

This assay was based on the work of Daharu and Sporns (1984). For the Gibbs assay, 1 ml of the water sample and 0.1 ml buffer (60 g Na_2CO_3 and 40 g $NaHCO_3$ per litre both Analar from BDH, pH 9.8) were placed in a microtube. Gibbs reagent (0.1 ml of 2,6-dichlorobenzoquinone-N-chloroimine 0.04 % (w/v) in 95 % ethanol, from Prolabo, made fresh on the day and stored in the fridge) was added while submerging the pipette tip and the microtube was inverted twice. The microtubes were placed in a water bath at 40 °C for 30 min. The tubes were left to cool to room temperature. From each tube, two wells were filled with 0.35 ml and the absorbance was read at 620 nm. Phenol (0 to 1 mg l^{-1}) was used for calibration.

Evaluation of the Gibbs assay regarding its response to different monophenolics

The compounds studied were lignin degradation products, expected to occur in natural water samples. Eighteen monophenolics (*isoeugenol* (2-methoxy-4-*trans*-propen(1)ylphenol), *p-hydroxy-acetophenone* (4-hydroxyacetophenone), *acetovanillone* (4-hydroxy-3-methoxy-acetophenone), *p-coumaric acid* (4-hydroxycinnamic acid), *ferulic acid* (4-hydroxy-3-methoxycinnamic acid), *4-vinylguaiacol* (2-methoxy-4-vinylphenol), *4-ethylguaiacol* (4-ethyl-2-

meth-oxyphenol), *syringaldehyde* (4-hydroxy-3,5-dimethoxybenzaldehyde), *isovanillic acid* (3-hydroxy-4-methoxybenzoic acid), *vanillic acid* (4-hydroxy-3-methoxybenzoic acid), *syringic acid* (4-hydroxy-3,5-dimethoxybenzoic acid), *p-cresol* (4-methylphenol), *vanillin* (4-hydroxy-3-methoxybenzaldehyde) all from Lancaster, Morecambe, England, *protocatechuic acid* (3-4-dihydroxy-benzoic acid), *acetosyringone* (3-5-dimethoxy-4-hydroxyacetophenone), *p*-hydroxy-benzoic acid, *p*-hydroxybenzaldehyde all from Aldrich, Dorset, England, *eugenol* (2-methoxy-4-propen(2)ylphenol) from Hopkins and Williams Ltd, Essex, England, were used to evaluate the Gibbs assay. Stock solutions of the compounds were prepared in a buffer. In order to compare the molar extinction coefficients of the compounds with phenol, the same molarity, 0.001 M, was used in each stock solution. Initially 1 : 100 dilutions of the stock were prepared and their spectra were measured (200 - 800 nm) on a Unicam UV 4 Spectrophotometer. A sample of the diluted solution (5 ml) was mixed with 0.5 ml buffer and 0.5 ml reagent, heated at 40 °C for 30 min and the spectrum taken again. The compounds that gave a positive response, they showed absorbance at 620 nm, were then used as standards between 0 to 10 μM to determine their molar extinction coefficients. The procedure given above for the Gibbs assay was followed.

2.3.3 Results and Discussion

The aim of this study was to distinguish between mono- and polyphenolics. The investigation went in three stages. Initially five assays were chosen from the literature and applied to standard solutions containing phenol as a standard for monophenolics and tannic acid as a standard for polyphenolics to determine the Criterion of Detection (CoD). The experiment was repeated with one standard solution in excess of the other standard to evaluate specificity. Naturally occurring levels of monophenolics were estimated to be 1 mg l^{-1}. A level of 5 mg phenol l^{-1} was chosen as a suitable excess for this study. For polyphenolics naturally occurring levels were estimated to be 10 mg tannic acid l^{-1} thus 50 mg tannic acid l^{-1} was chosen as excess for this

study.

Secondly, the Gibbs assay was found to be specific and sensitive for monophenolics at naturally occurring concentrations. Thus the response of the Gibbs assay towards monophenolic lignin degradation products was studied to evaluate its use for the purpose of the analysis.

Thirdly, the assays were applied to natural water samples to investigate whether they were able to distinguish between mono- and polyphenolics. The samples were spiked with standards to obtain recovery rates and where poor recovery was obtained the assay was improved.

Investigation into the Sensitivity and Specificity of the Colorimetric Assays to Distinguish between Mono- and Polyphenolics

Although the Folin-Ciocalteau, Prussian blue, iron complexation, Gibbs and 4-aminoantipyrine assays were in themselves not novel, no study was found that had used them together to distinguish between mono- and polyphenolics in natural water samples. Initially concentration ranges up to 50 mg l^{-1} tannic acid for polyphenolics and up to 5 mg l^{-1} phenol for monophenolics were chosen to determine sensitivity. These concentrations were chosen because they covered the range of phenolics determined in earlier studies of leaf leachates, plant material and river water (Serrano 1992, Herrera-Silvera and Ramirez-Ramirez 1996, Freeman et al. 1990). The Folin-Ciocalteau assay was responsive to both standards. The Prussian blue and the iron complexation assays (Hagerman and Butler 1978) gave positive responses to tannic acid at the 50 mg l^{-1} level and did not detect up to 5 mg l^{-1} phenol. The 4-aminoantipyrine (Daharu and Sporns 1984) and the Gibbs assays gave positive responses to 5 mg l^{-1} phenol but did not detect up to 50 mg l^{-1} tannic acid. However, natural water samples contained both mono- and polyphenolics, thus any assay that was specific for monophenolics should not be subject to interference by polyphenolics and vice versa. Thus calibration curves for up to 50 mg l^{-1} tannic acid in the presence of phenol (5 mg l^{-1}) in the Prussian blue and iron complexation assays and up to 5 mg l^{-1}

phenol in the presence of an excess of tannic acid (50 mg l^{-1}) in the 4-aminoantipyrine and Gibbs assays were taken. To assess the performance of the assays, the CoD, which is the equivalent concentration of 3 standard deviations of the blank from six independent analyses (Caulcutt and Boddy 1983), was calculated. The CoD gives an estimate of the concentration required to give a significantly different reading from the blank (p=0.05). For the Folin-Ciocalteau assay a CoD of 0.081 mg l^{-1} for phenol and 0.025 mg l^{-1} for tannic acid was obtained. The Prussian blue and the iron complexation assays had CoDs of 0.169 mg l^{-1} and 3.0 mg l^{-1} for tannic acid, respectively. Polyphenolics interfered in the 4-aminoantipyrine assay; 50 mg l^{-1} tannic acid gave the same response as 2 mg l^{-1} of phenol. In the absence of tannic acid a CoD of 0.076 mg l^{-1} was obtained for the 4-aminoantipyrine assay. The Gibbs assay had a CoD of 0.027 mg l^{-1} for phenol.

The preliminary work above showed that three of the five assays investigated had the potential for quick and simple analysis of natural water samples, the Folin-Ciocalteau assay for total phenolics, the Prussian blue assay for polyphenolics and the Gibbs assay for monophenolics. Table 2.1 gives the analytical characteristics of the three assays. The reaction mechanisms underlying these assays are oxidation for the Folin-Ciocalteau and Prussian blue assay and polymerisation for the Gibbs assay. The iron complexation assay was excluded because it had too high a CoD to be used on most natural water samples. The low sensitivity of the iron-complexation assay was noted by Mole and Waterman (1987b) in comparison with the Folin-Ciocalteau assay. Where iron-complexation had been employed (Serrano 1992, Herrera-Silvera and Ramirez-Ramirez 1996), the concentration of either leaf leachates or tannic acid itself was investigated, but no application to natural water samples was found.

Table 2.1:. Analytical characteristics for the Gibbs, Prussian Blue and Folin-Ciocalteau assay.

	Standard	Syst. error	Linear cal.	r^2	CoD
Gibbs	Phenol	0.321 mg l^{-1}	0 to 1 mg l^{-1}	0.999	0.027 mg l^{-1}
Prussian Blue	Tannic acid	0.361 mg l^{-1}	0 to 5 mg l^{-1}	0.997	0.169 mg l^{-1}
Folin-Ciocalteau	Tannic acid	0.019 mg l^{-1}	0 to 5 mg l^{-1}	0.996	0.025 mg l^{-1}

The Response of the Gibbs Assay towards Monophenolic Lignin Degradation Products

To be relevant for ecological studies of humification, the non-specificity for phenol in the Gibbs assay (Dacre 1971, Josephy and van Damme 1984) was thought advantageous. Thus the behaviour of the Gibbs assay with a variety of monophenolics, the products of lignin degradation (Chen and Chang 1985, Camarero et al. 1994, Ertel et al. 1984), was studied. Eighteen model compounds were examined regarding the wavelength of maximum absorption and relative extinction coefficient of the responsive compounds, because the λ_{max} in the Gibbs assay could vary with the substitution pattern on the aromatic ring. Spectra were taken at a concentration of 10 mg l^{-1} for phenol and the same molar concentration for the other compounds prior to and after completion of the Gibbs assay. Phenol, isovanillic acid, vanillic acid, syringic acid and ferulic acid had a λ_{max} between 588 nm to 614 nm and showed an absorbance between 0.28 (ferulic acid) and 1.00 (phenol) for 100 μM. Protocatechuic acid showed an absorbance of 0.06 and a λ_{max} of 540 nm. Neither was the absorbance strong nor was the λ_{max} close to the measurement wavelength of 620 nm. Non-responsive were: syringaldehyde, vanillin, p-hydroxybenzoic acid, 4-vinylguaiacol, acetosyringone, p-hydroxybenzaldehyde, p-coumaric acid, p-cresol, eugenol, isoeugenol, 4-ethylguiacol, acetovanillone and p-hydroxyacetophenone. The compounds that showed a similar maximum absorbance to phenol were then used as standards to determine their relative response (Figure 2.1) in relation to phenol in the relevant concentration range (0 to 10 μM). The indophenol produced after the Gibbs reaction between phenol and the Gibbs reagent had a molar extinction coefficient of 16300 l mol^{-1} cm^{-1}. Molar extinction coefficients for the other reactive compounds are not given, because UV evidence suggested incomplete reaction and the reaction was known not to follow a fixed stoichiometry (Pallagi et al. 1994). The compounds not giving indophenols after the Gibbs reaction showed strong absorbance for 100 μM at 366 nm (syringaldehyde A=1.8), 350 nm (vanillin A=2.1), 334 nm (p-

hydroxy benzaldehyde A=2.0), 358 nm (acetosyringone A=1.7) and 330 nm (p-coumaric acid A=1.5). Those peaks were shifted to longer wavelength when compared with the scans of the unreacted compound, suggesting an extension of the conjugated system. Pillinger et al (1994) reported an increase in λ_{max} for 2,6-dimethoxyphenol in an alkaline solution with time (up to two days later), that was dissimilar to 2,6-dimethoxy-p-benzoquinone. Overall the assay measured at 620 nm clearly gave a response representing the sum of those for each responsive monophenolics present in a natural water sample. The concentration of PE, phenol equivalents, measured could be an underestimation due to the non-responsive compounds and due to phenol itself having the highest extinction coefficient of all responsive compounds investigated (Figure 2.1).

The underlying reaction mechanism of the Gibbs assay is an oxidative coupling in para position. The indophenol formed during the coupling is thus an example of a polymerisation reaction between a quinone, the oxidised phenol, and an amino containing compound, the reagent 2,6-dichloro-benzoquinone-N-chloroimine. As opposed to complexation reactions where no covalent bonds are formed, the Gibbs assay exemplifies the beginning of polymerisation reactions.

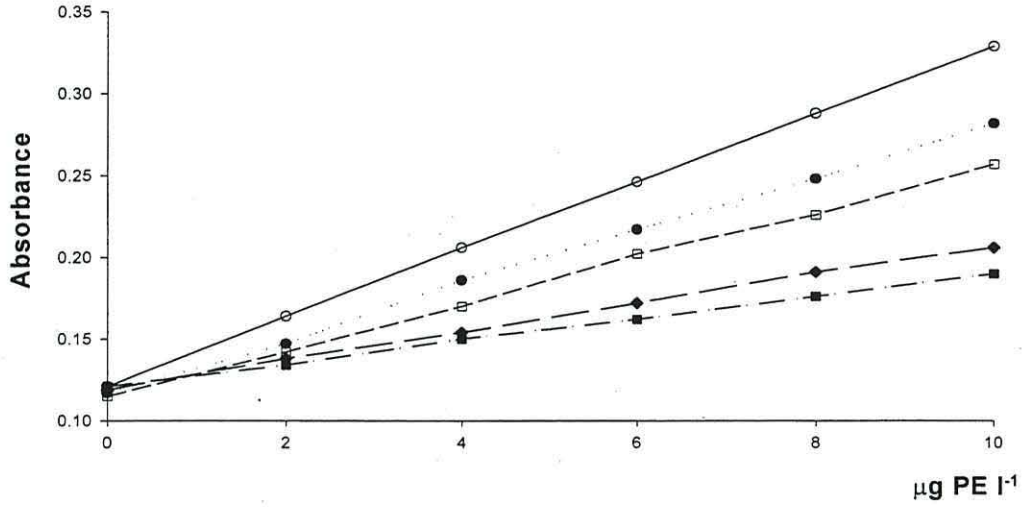

Figure 2.1: Absorbance in the Gibbs assay at 620 nm versus concentration (µM) PE (Phenol Equivalents) of various phenols: ○ phenol, ● isovanillic acid, □ vanillic acid, ■ ferulic acid and ◆ syringic acid.

Application of the Three Assays to River Water Samples

Using the protocol recommended above, the Folin-Ciocalteau, Prussian blue and Gibbs assays were applied to river water samples. A standard addition was included (Caulcutt and Boddy 1983), because on determining the percentage recovery of a spike the performance of the assays could be assessed. In order to achieve repeatable results the samples had to be spiked prior to performing the assay. Additionally it was found that the Folin-Ciocalteau and the Gibbs assays led to precipitates with samples of high conductivity because of the high carbonate concentration in the buffers. They were thus performed in microtubes to let possible precipitates settle out, and pipetting the solution into microplates after a fixed reaction time. The assays were performed twice and were found to be repeatable.

To establish whether a distinction between monophenolics and polyphenolics was achieved, three river samples, Aber, Clywedog and Conwy (table of site characteristics in appendix), were spiked independently with 0.1 and 0.2 mg l^{-1} of phenol and 1 and 2 mg l^{-1} of tannic acid. Tannic acid did not interfere in any site with the Gibbs assay, confirming its specificity for monophenolics. For the Prussian blue assay, 0.1 and 0.2 mg l^{-1} phenol spike were found significantly different ($p=0.05$, t-test) in the Aber, 0.2 mg l^{-1} phenol spike was found significantly different ($p=0.05$, t-test) in the Clywedog and no significant difference at the 5% level was detected for the phenol spike in the Conwy. Thus, whether phenol is detected with the Prussian blue assay was dependent on the matrix. The origin of the matric dependency for the Prussian blue assay was thought to be complexation of phenol. The results for the complexing strength towards phenol in the seasonal variation gave on average 20 % for the Aber, 38 % for the Clywedog and 73 % for the Conwy. It was confirmed that phenol was being detected with the Folin-Ciocalteau assay (Box 1983).

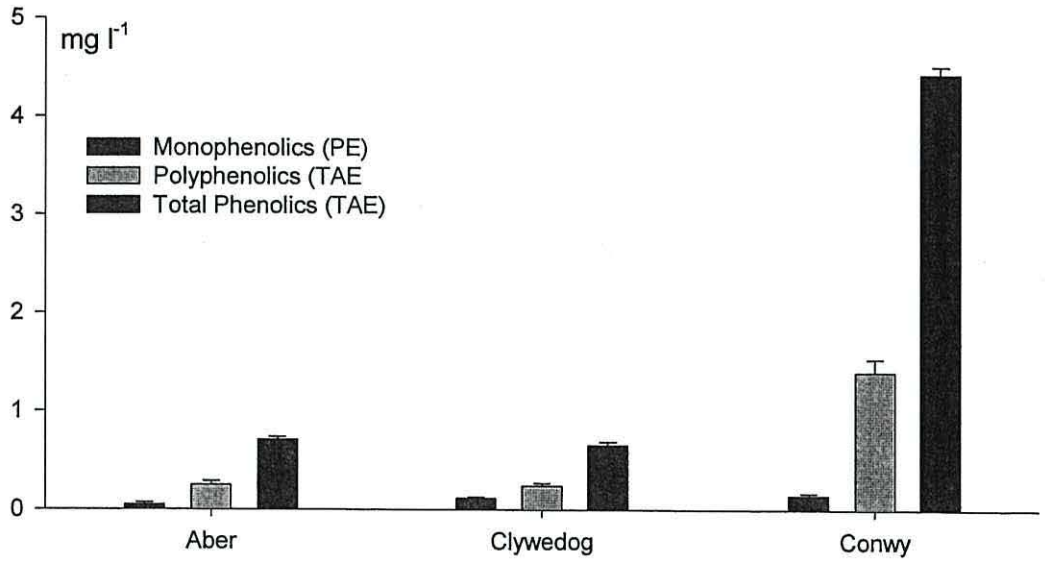

Figure 2.2: Concentration in mg/l PE (Phenol Equivalents) of monophenolics (Gibbs, measured on-site), in mg/l TAE (Tannic acid equivalents) polyphenolics (Prussian Blue) and total phenolics (Folin-Ciocalteau) as found at the three sites (sampled 27.4.1998). Error bars are ± 1 standard error.

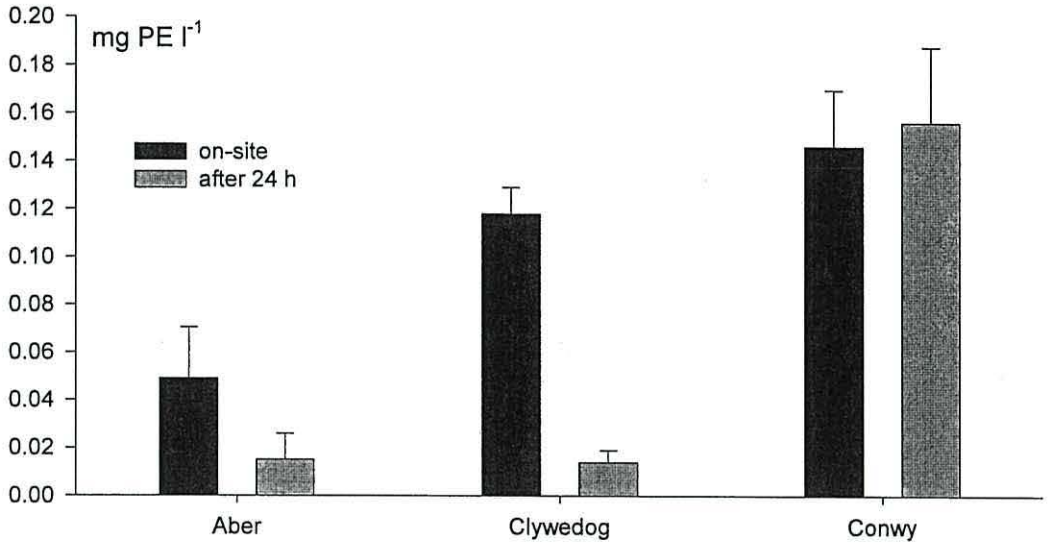

Figure 2.3: Concentration of monophenolics in mg l^{-1} PE (Phenol equivalents) measured with the Gibbs assay on-site and after 24 h (sampled 27.4.1998). Errors bars are ± 1 standard error.

The results of the spiking were analysed by performing a linear regression through all points. The slope indicated the recovery rate of the spike and was found to be 1.00 ± 0.14 for all three methods. The recommended protocol showed a correlation coefficient for the standard addition of above 95 %. Figure 2.2 shows the mono-, poly- and total phenolics content of the three rivers analysed.

During the evaluation of the Gibbs assay it was noted that the colour of some stock solutions of monophenolics changed rapidly; this was particularly marked for vanillin. It was thus suspected that the monophenolic fraction of natural water samples was unstable. This could be due to microbial degradation (Haider et al. 1975), complexation (Ohno and First 1998) or oxidation and polymerisation (Appel 1994). The Gibbs assay was thus performed on-site and also on the day after sample collection (Figure 2.3) to investigate whether any changes in the PE concentration occurred between sampling and measurement. The Clywedog sample showed a significant ($p=0.05$, t-test) reduction from 0.12 +/- 0.04 mg l^{-1} to 0.02 +/- 0.01 mg l^{-1} PE in less than 24 h. The other two sites did not show a significant reduction. Thus, depending on the matrix, changes in the PE concentration were found.

The analytical characteristics of each method are summarised in Table 2.1. All methods showed linear calibration for the levels found in the three sites and an acceptable CoD. Two methods, Prussian blue and Gibbs, had a high blank reading (Table 2.1: systematic error). The Gibbs assay developed a golden background during the analysis. The reason for this was thought to be polymerisation of unused oxidised reagent. In the procedure of Daharu and Sporns (1984), the same reagent concentration was used. Their calibration was found to be linear up to 30 mg phenol l^{-1}. Quitana et al. (1997) used the Gibbs reaction in a microplate-based assay to measure oxygenated aromatic biotransformation products. The Limit of Detection quoted was 0.5 mg phenol l^{-1} with a fiftyfold increase in reagent concentration. According to the levels of monophenolics determined in the rivers, a linear calibration up to 1 mg l^{-1} was deemed sufficient. Reduction of

the reagent concentration to 0.002 and 0.001 % (w/v) was thus investigated, but sensitivity was reduced and the calibration needed a polynomial regression fit. Overall the Gibbs assay complemented the Folin-Ciocalteau and the Prussian blue assay due to the difference in reaction mechanism employed. The specificity of the Gibbs assay for monophenolics was established, although no further conclusions regarding the individual compounds could be made. Cheeseman and Wilson (1972) thought the long reaction time of the Gibbs assay (up to 24h) a disadvantage. For the measurement of monophenolics on-site the long reaction time is considered advantageous, because the reagent were added on-site and travelling back from the sampling site was thus not time restricted.

2.3.4 Conclusions

The initial aim of the phenolics analysis, a distinction between mono- and polyphenolics, was achieved. The redox-based assays, Folin-Ciocalteau and Prussian blue, were responsive to both mono- and polyphenolics. The Folin-Ciocalteau assay determined total phenolics while Prussian blue was found more specific for polyphenolics, showing a dependency on the matrix. The Gibbs assay was specific for monophenolic lignin degradation products. All assays were tested using natural water samples and were shown to be applicable.

Acknowledgements

P. V. Courty performed the work regarding the specificity of the Gibbs assay towards monophenolic lignin degradation products as part of an MSc in Environmental Chemistry under the supervision of V. Thoss. Funding was provided by the European Social Fund.

2.4 Lipase Assay

2.4.1 Introduction

DOM contains phenolics and humic substances, both of which have been implicated in the interaction with enzymes (Goldstein and Swain 1965, Mole and Waterman 1987a, Freeman et al. 1990, Serrano and Boon 1991, Wetzel 1991, Freeman and Lock 1992, Wetzel 1993, Jahnel and Frimmel 1994, Dudley and Churchill 1995, Herreira-Silveira and Ramirez-Ramirez 1996). This section is concerned with a biochemical aspect of DOM - enzyme interaction, the chemical characteristics having already been covered in Sections 2.2 and 2.3.

It has been shown that degradation processes are driven by enzymatic alterations of DOM (Allan 1995, Wetzel 1991). Research into polyphenolics-enzyme interactions (Serrano and Boon 1991, Goldstein and Swain 1965) or humic acids-enzyme interactions (Dudley and Churchill 1995) found complexation occurring that led to a stabilisation of the enzyme. As additional factors, polyphenolics-substrate interaction (Mole and Waterman 1987a) and the influence of inorganic cations (Ladd and Butler 1970, Wetzel 1993) were established.

Common methods to investigate humic-enzyme interactions in natural water samples add a substrate, e.g methyl umbelliferyl phosphate, specific for a group of enzymes present in the sample, e.g. phosphatases, and measure the processing rate (Wetzel 1993, Freeman et al. 1990). A disadvantage of these assays was thought to be the lack of knowledge regarding the actual concentration of enzymes in the sample. An alternative approach is to study the effect of humic acid solutions of varying concentrations on the activity of enzyme solutions, e.g. Protease E (Jahnel and Frimmel 1994) or xanthine oxidase (Lu and Huang 1994). The concentration of humic acid necessary to induce 50% enzyme inhibition was evaluated. This concentration ranged from 153 to 6360 mg HA l^{-1} for the system employed by Jahnel and Frimmel

(1994) and 21 to 75 mg HA l^{-1} for Lu and Huang (1994).

In order to produce a standard assay for enzyme inhibition by DOM, a model enzyme - substrate system was chosen based upon the relatively low cost porcine pancreatic lipase and fluorescein diacetate. The enzyme had maximum activity at pH 7.0 and fluorescein showed maximum fluorescence at pH 8.0 (Guilbault and Kramer 1964), these being reasonable pH values with regard to the naturally occurring rivers and wetlands. The phosphate buffer (pH 7.0) employed did not contain divalent cations, which were known to complex polyphenolics and thus reduce enzyme inhibition (Wetzel 1993).

A fixed-time assay was chosen (Boyer 1993) for which a kinetic study was performed to determine K_M, the Michealis constant. The substrate concentration had to be higher than K_M to assure substrate saturation. The initial velocity v_0 was taken and compared to the buffer blank to measure inhibition or stimulation.

2.4.2 Materials and Methods

Determination of Kinetic Constants for Porcine Pancreatic Lipase

A 0.1 M phosphate buffer was prepared from 0.1 M KH_2PO_4 (50 ml) and 0.1 M NaOH (29.1 ml) made up to 100 ml; pH was adjusted to 7 if necessary. An enzyme solution of 1 g porcine pancreatic lipase l^{-1} (crude Type II from Sigma) was prepared. Stock solutions (1 mM) of fluorescein and fluorescein diacetate (both from Sigma) were made in warm acetone and diluted with buffer to final concentrations. Substrate concentrations of 100, 200, 400, 800, 1500, 2000, 4000 and 8000 nM were used in triplicate to determine K_M and v_{max}. The lipase solution was kept in the fridge, all other solutions were kept at 25 °C. The cuvette holder in the fluorimeter (Perkin Elmer Luminescence Spectrometer LS 50) was kept at 25 °C. For the kinetic study (equation (1)) sample (1.5 ml), fluorescein diacetate (0.75 ml) and lipase solution (0.75 ml) were pipetted into the cuvette, inverted and the fluorescence measured at excitation 490 nm, slit width 5 nm, and emission

520 nm, slit width 10 nm, every 5 s for 60 s. For the calibration, (0 - 500 nM) fluorescein was used to determine the relationship between fluorescence and the fluorescein concentration (2). The data was imported into Minitab 11 and converted according to (3) into v_0. A Lineweaver-Burk plot (4) was used to calculate K_M and v_{max} (Boyer 1993).

(1) $\text{Fluorescence} = C + m_{kin} \times [\text{fluorescein}]$

(2) $\text{Fluorescence} = C + m_{cal} \times [\text{fluorescein}]$

(3) $m_{cal} \times (m_{kin})^{-1} \times (\text{nM s}^{-1}) = v_0$

m_{cal} = slope from calibration

m_{kin} = slope from kinetic study

(4) $v_0^{-1} = K_M^{-1} + K_M \times v_{max}^{-1} \times [S]$

K_M = Michaelis constant

Initial Evaluation of lipase assay

The buffer concentration was reduced to 0.01 M KH_2PO_4 and pH was found to be 7.00 ± 0.10 when applied to filtered natural water samples. In Sept. 1997 samples from the controlled decomposition study (Chapter 4) were used to evaluate the lipase assay. 'Light' samples were used twice to check for repeatability. v_0 was measured with [S] = 10 µM fluorescein diacetate for triplicate samples and a t-test was performed to determine significance when compared to the blank, doubly distilled water.

2.4.3 Results and Discussion

Figure 2.4 shows the Lineweaver-Burk plot for the kinetic study of lipase. The linear regression gave v_{max} = 4.5 nM s^{-1} and K_M = 4.8 µM. The regression coefficient was 0.992. A substrate concentration of 10 µM was chosen for a fixed time assay to assure [S] >> K_M. The lipase concentration was 1 g l^{-1}.

The repeatability of the lipase assay was investigated using controlled decomposition 'light' samples. The null hypothesis was that DOM of any sample was not significantly different at the 5 % level from doubly distilled water. The assay was performed on 5.9.1997 and 11.9.1997. Figure 2.5 shows the different initial velocities determined while table 2.2 lists the ratio of the initial velocities of the sample in comparison to the blank.

Figure 2.5 shows an increasing trend in starting velocity for cellulose and a decrease for the lignin and lignin & chitin columns. Although a significant reduction of starting velocity for the chitin containing column was measured on 5.9.1997, it was not repeated on 11.9.1997. Overall, a similar trend was observed for the controlled decomposition light columns. Cellulose had no effect or stimulated lipase, while chitin had no effect or inhibited lipase. In the columns containing lignin & cellulose or lignin & chitin the same effect was repeated.

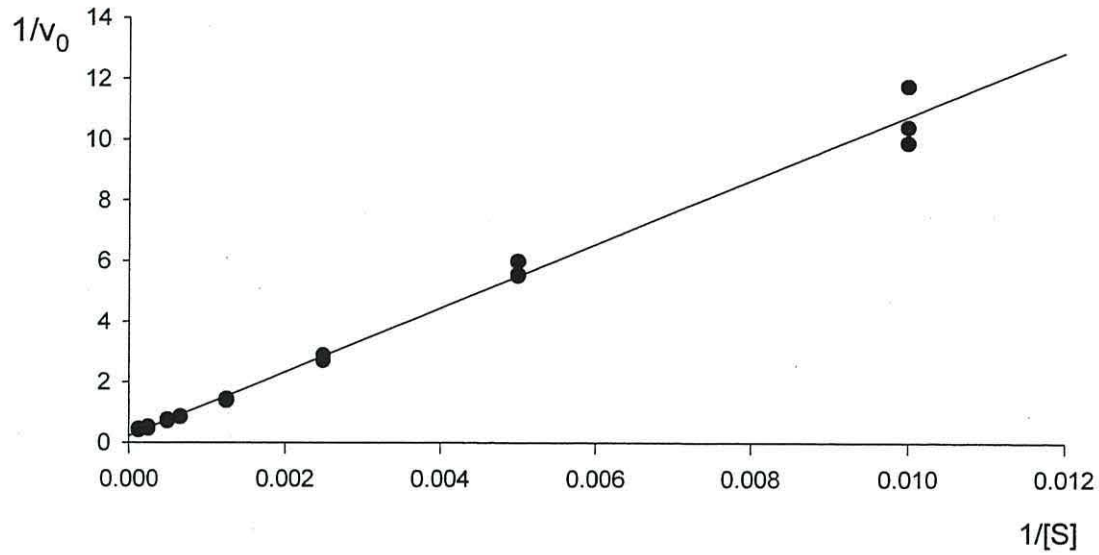

Figure 2.4: Lineweaver-Burk plot for porcine pancreatic lipase. Substrate was fluorescein diacetate. [S] was nM, v_0 was nM s^{-1}.

Table 2.2: Results of lipase inhibition assay for controlled decomposition light products. The initial velocity of the de-esterification of fluorescein diacetate by lipase in the presence of the sample has been divided by the initial velocity in the presence of buffer as sample to obtain the following ratios.

	Control	Cellulose	Chitin
5.9.1997	0.988	1.133	0.804[a]
11.9.1997	1.158	1.232[a]	1.080

[a] significantly different at the 5 % level when compared to the blank

	Lignin	Lignin & Cellulose	Lignin & Chitin
5.9.1997	0.702[a]	0.681	0.442[a]
11.9.1997	0.862[a]	0.960	0.678[a]

[a] significantly different at the 5 % level when compared to the blank

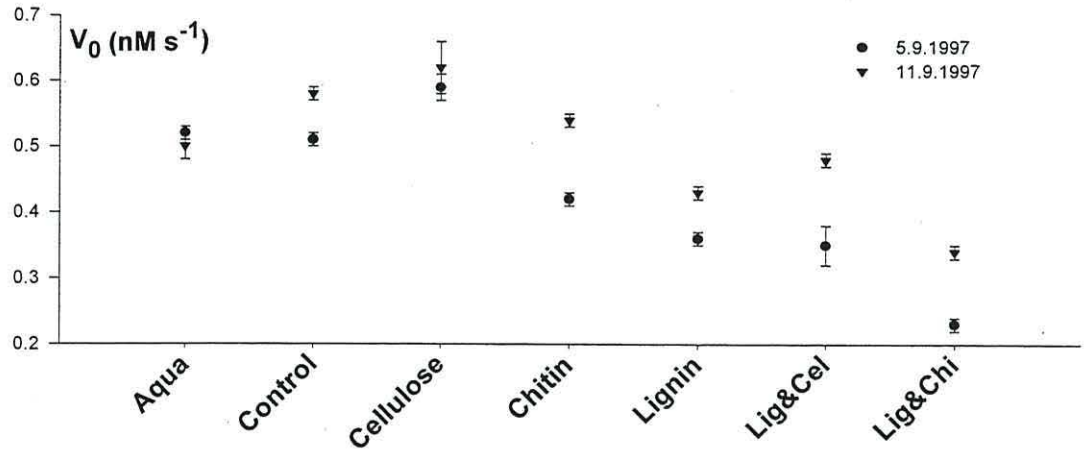

Figure 2.5: Results for lipase assay on controlled decomposition 'light' products obtained on 2 different days to check repeatability. Shown are initial velocities with ± 1 standard error.

The lipase concentration employed was 1 g l^{-1}. The aim of the assay was to investigate the effect of DOM, at naturally occurring concentrations of 0 to 40 mg l^{-1}, on lipase activity. The small effects determined during the kinetic study were likely to originate from the high concentration of lipase when compared with DOM concentrations. Thus for a fixed-time assay the lipase concentration was reduced to 0.1 g l^{-1} after filtration. The increase in fluorescence originating from fluorescein for the reduced lipase concentration was monitored over 60 min and found to be linear. Additionally, measuring the de-esterification of fluorescein diacetate when no lipase was added (Lipase absent) was included; this was deduced from the processed substrate after 60 min. DOM was known to quench fluorescence, thus a calibration for fluorescein was taken in the presence of each sample in order to account for any quenching.

2.4.4 Conclusions

An enzyme assay was developed that measured the effect of DOM on porcine pancreatic lipase. It was a fixed time assay (60 min) with a substrate concentration that saturated the enzyme. The overall design consisted of a calibration in the presence of the sample, buffer blank and 3 concentrations of fluorescein in duplicate, addition of substrate without added lipase and addition of substrate and lipase to the sample in triplicate. By comparing concentration of fluorescein released from fluorescein diacetate after 60 min in the presence of DOM with the control, a stimulating or inhibiting influence of DOM onto lipase activity was determined.

2.5 Protein Precipitation Assay

2.5.1 Introduction

DOM in river water is thought to be primarily of allochthonous origin, meaning derived from vegetation on the watershed (Wetzel 1991). As part of plant matter, polyphenolics are known to interact with proteins inducing precipitation. Methods to quantify these polyphenolics present utilise interactions between extractable tannins and Bovine Serum Albumin (BSA), to assay for protein precipitation (Hagerman and Butler 1978, Stadler Martin and Martin 1982, Mole and Waterman 1987c, Makkar 1989).

For this study the protein precipitation assay was adapted to be used directly on natural water samples. During humus synthesis, polyphenolics are altered via oxidation, complexation and polymerisation (Stevenson 1994). The aim was thus to investigate whether the protein precipitating ability of polyphenolics persisted after they were leached from plants (Serrano 1992) to form part of DOM. Secondly, as illustrated in Section 2.3, the iron complexation assay required higher concentrations to be applicable to the sites studied. In the current assay the polyphenolics protein complex was centrifuged and separated from the supernatant. Thus it represented a means to pre-concentrate polyphenolics for the iron-complexation assay.

According to existing theories, the complexation between proteins and tannins was based primarily on hydrogen bonding at naturally occurring pH (Mole and Waterman 1994, Haslam 1998). No fixed stoichiometry for the interaction was known and it was suspected that there were two stages of interaction. Initially tannins bonded to proteins via hydrogen bonds of the OH-groups and hydrophobic bonding occurred between the aromatic nuclei of the tannins and hydrophobic areas in the protein. This type of complex was soluble and its association could be separated via, e.g., increases in pH or addition of a surfactant, indicating that weak hydrogen bonding, but no polymerisation, occurred. If covalent bonds formed (polymerisation) between

the protein and the tannins, which happened at high tannin to protein ratios, an insoluble cross linked complex occurred and precipitated (Mole and Waterman 1994).

For the present work, the existing protein precipitating assay utilising BSA was adapted for filtered natural water samples. To achieve high complex formation, the assay was performed at the isoelectric point of BSA (pH 5) in acetate buffer (Hagerman and Butler 1978). Complex formation also depends on conductivity. Sodium chloride was therefore used to keep ionic strength constant during the assay.

Due to the lower levels of polyphenolics in natural water samples - as determined with the Folin-Ciocalteau assay the highest concentration was 8 mg l^{-1} in the sites studied (Chapter 3) compared to extracts of plant matter averaging 100 mg l^{-1} (Mole and Waterman 1987b, Stadler Martin and Martin 1982) - the concentrations of the reagents employed were lowered. Hagerman and Butler (1978) worked with a ratio of 1 tannic acid to 2 protein by weight. In the natural water samples studied, the amount of polyphenolics determined with the Prussian blue assay was up to 1.7 mg l^{-1}. Thus a concentration of 4 mg l^{-1} BSA was chosen to assure an excess was present. Additionally a standard addition of 2 and 4 mg l^{-1} TA (Tannic Acid) was included to raise the level of polyphenolics in the sample.

The protein precipitation assay was a means to investigate the biochemical activity of DOM to complement chemical parameters as described in Sections 2.2 and 2.3.

2.5.2 Materials and Methods

Filtered freshwater sample (25 ml), for which a table of site characteristics is given in the appendix, or spiked sample (spiking levels were 2 and 4 mg l^{-1} of tannic acid) were mixed with 5 ml BSA solution (10 mg bovine serum albumin, Fraction V from Sigma, and 0.17 M NaCl (4.967 g NaCl (Analar from BDH) in 500 ml 0.02 M acetate buffer pH 5.0 (prepared from 700 ml

2.722 g/l CH_3COONa * 3 H_2O, Analar from BDH, and 300 ml 0.02 M CH_3COOH, diluted from glacial acetic acid from Fischer)) left standing for 30 min and put in the fridge overnight. The tubes were centrifuged at 10,000 rpm for 10 min (Beckman Model J2-21 Centrifuge). The supernatant was decanted and saved. The tubes were placed upside down on adsorbent paper to remove as much supernatant as possible. The precipitate was dissolved in 1 ml SDS / triethanolamine (1 % (w/v) Sodium Dodecyl Sulfate, from Fluka, and 5 % (v/v) triethanolamine from Fischer) and 0.25 ml $FeCl_3$ solution (0.01 M $FeCl_3$ in 0.01 N HCl) was added and mixed. After 15 to 30 min, 0.35 ml was transferred into one well without using the second Eppendorf pipette point. Two wells were filled per centrifuge tube while three replicates were run per sample. The absorbance was measured after 15 to 30 min at 540 nm. Tannic acid (0 to 5 mg l^{-1}) was used for calibration.

The Folin-Ciocalteau assay was performed on the saved supernatant. Tannic acid (0 to 10 mg l^{-1}) was used for calibration.

Table 2.3: Result for repeated calibration of TA (Tannic Acid) in the adapted protein precipitation assay (standard error)

Actual [TA}	found [TA] (se)	found [TA] (se)
0 mg/l	0.00 (0.11) mg l^{-1}	0.37 (0.03) mg l^{-1}
1 mg/l	0.66 (0.05) mg l^{-1}	0.88 (0.12) mg l^{-1}
2 mg/l	2.06 (0.05) mg l^{-1}	1.41 (0.07) mg l^{-1}
3 mg/l	3.67 (0.12) mg/l	3.03 (0.16) mg/l
4 mg/l	3.90 (0.03) mg/l	4.36 (0.06) mg/l
5 mg/l	4.72 (0.18) mg/l	4.95 (0.20) mg/l

2.5.3 Results and Discussion

Initial evaluation of the assay used standard solutions of a hydrolysable tannic acid (TA) to investigate whether protein precipitation took place and whether it was repeatable in the concentration range of interest. Table 2.3 shows that the adapted protein precipitation assay was neither repeatable nor particularly accurate; the regression coefficient for both calibrations was 0.96. The reason for these inaccuracies could be twofold: either the protein precipitation itself or the detection of the precipitated tannic acid as $Fe(OR)_6^{3-}$ (Hagerman and Butler 1978) could be irreproducible. On performing only the iron complexation part of the assay with tannic acid standards ranging from 0.5 mg l^{-1} to 50 mg l^{-1}, a linear calibration curve (r^2=0.996) was produced. Thus the absence of a fixed stoichiometry for polyphenolics protein interactions was substantiated (Mole and Waterman 1994). The sensitivity of the iron complexation was low and a concentration of tannic acid of 3 mg l^{-1} was required to detect a significantly different absorption from the blank.

As an alternative to performing the iron complexation assay on the precipitated polyphenolics the analysis of the total phenolics content in the supernatant was performed, to establish whether the added spike was precipitating or stayed in solution. Initially it was suspected that the levels of protein precipitating polyphenolics in natural water samples were too small for the accurate performance of the protein precipitation assay. Performing the Folin-Ciocalteau assay on the supernatant of the standards substantiated incomplete precipitation, a concentration of 1.0 mg TAE l^{-1} for the 2 mg l^{-1} tannic acid spike and 2.4 mg TAE l^{-1} for the 4 mg l^{-1} tannic acid spike was found. This confirmed the poor calibration, with a regression coefficient below 0.99 for the iron complexation assay on the pellet of the precipitated polyphenolics.

For ecological research the fate of the added tannic acid could illustrate the complexation behaviour of DOM, but required a more precise analytical methodology. Figure 2.6 shows the difference in concentration of total

phenolics after the protein precipitation as a Δ value. The concentration of total phenolics measured in the sample before treatment, plus the concentration of added tannic acid was taken as zero. Negative Δ values thus indicated precipitation, while positive values showed increase in total phenolics as measured with the Folin-Ciocalteau assay. The blank and the 2 and 4 mg tannic acid l^{-1} standards are also included, verifying a positive response of the Folin-Ciocalteau assay towards protein (Serrano 1992).

2.5.4 Conclusions

A protein precipitation assay was developed that analysed the interaction between polyphenolics and the protein BSA. The filtered water samples were spiked with tannic acid at two levels prior to performing the assay. The interaction between DOM and protein and DOM and added tannic acid was assessed in the assay. Some of the DOM investigated showed such a strong interaction with either the added tannic acid or the added BSA, that it rendered the tannic acid non-protein precipitating.

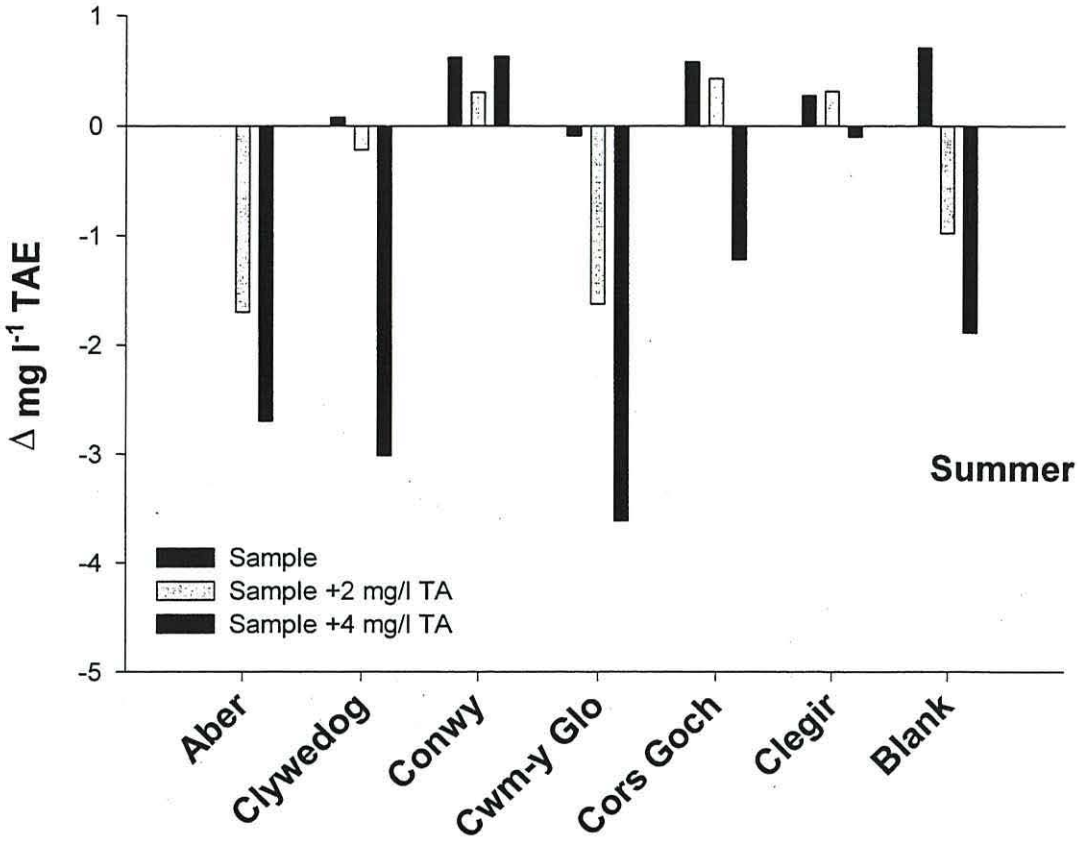

Figure 2.6: Result of Folin-Ciocalteau assay on supernatant of protein precipitation (sampled 27.7.1998). Shown are the differences between the Folin-Ciocalteau assay on the sample, sample + 2 mg TA l^{-1} and sample +4 mg TA l^{-1} on the untreated sample to the supernatant after the protein precipitation. Positive values indicate no polyphenolics precipitated, negative values indicate polyphenolics precipitated. Error bars are ± 1 standard error.

2.6 A Fingerprint

2.6.1 Introduction

The chemical and biochemical analysis of DOM was investigated as described in the preceding sections. The primary focus during the development of the analysis was the underlying reaction mechanisms of oxidation, complexation and polymerisation in addition to the analysis being quick and simple and working on natural matrices.

The application of the methodology led to large data sets. For the 'fingerprint' the chemical characteristics of DOM, the assays described in Sections 2.1, 2.2 and 2.3, were combined, because earlier work by Stadler Martin and Martin (1982) showed that there was no correlation between chemical and biochemical characteristics for polyphenolics. There were thus 12 assays in total. A data set describes the combined results for each assay for each sample. The statistical evaluation of those data sets was challenging, as was their visual display. Multivariate statistics were applied to the chemical characteristics. For the statistics, independence of all variables was assumed. This independence of the assay was a prerequisite to use principal component analysis (PCA) and cluster analysis (Vega et al. 1998). This assumption was thought justifiable, because a data set included sites with different vegetation and matrices. Even though some assay were found to correlate well, for example Prussian blue for polyphenolics and Folin-Ciocalteau for total phenolics had a correlation coefficient of 0.989, the ratio of poly- to total phenolics ranged from 0.15 to 0.60 illustrating differences for each sample. Thus independence of the assays was thought appropriate for this study.

The results gained from the (physico-) chemical analysis were then used to investigate the biochemical behaviour of DOM.

2.6.2 Materials and Methods

Assays for Fingerprint

Environmental parameters: pH, Conductivity, Temperature; *Bulk carbon characteristics*: Complexed phenol, fluorescence, TOC; *UV absorbance*: 200 nm, 240 nm, colour in Hazen units; *Phenolics*: Folin-Ciocalteau, Prussian blue, Gibbs

Data treatment and visualisation

In a 'fingerprint' the results of the 12 assays that chemically characterise DOM are condensed into one diagram. The type of diagram chosen was a radial plot. There were differences in the concentrations measured and units used between the assays. To allow the usage of a radial plot the data was normalised in order to obtain a value between 0 and 1 for each data point.

All data relating to one experiment made up a data set. A range of data points was obtained for each assay, one data point for each sample. These data points were normalised by dividing the difference between the data point and the minimum value for the assay by the range. For example, temperature in the seasonal variation ranged from 1 to 17 °C for all samples throughout the year, thus each data point minus 1 was divided by 16.

The normalised data was imported into Sigma plot 4.01. A radial plot was chosen with the normalised values as radial component and predefined theta values for the angular component (Table 2.4).

Statistics

Multivariate statistical methods cluster analysis and principle component analysis was performed on the data set with Minitab 11 for Windows.

Table 2.4: Angular and radial data input for 'fingerprint'

Radial component	Angular component
Temperature	0 °
pH	30 °
Conductivity	60 °
Monophenolics	90 °
Polyphenolics	120 °
Total Phenolics	150 °
UV absorbance 200 nm	180 °
UV absorbance 240 nm	210 °
Colour	240 °
TOC	270 °
Fluorescence	300 °
Complexation	330 °

2.6.3 Results and Discussion

The heterogeneous character of DOM leads to the need for caution in the interpretation of single aspects of water and DOM chemistry. The environmental parameters for DOM genesis, water temperature, pH and conductivity, in combination with the (physico-) chemical aspects of DOM, complexing strength towards phenol, fluorescence, TOC, colour in Grade Hazen, UV absorption at 240 and 200 nm and the phenolics pool of total, poly- and monophenolics were condensed into a graphical 'fingerprint' (Figure 2.7). In the 'fingerprint' a circle is displayed. The top right hand quadrant contains the environmental parameters: Conductivity, pH and water temperature (starting at one o'clock and proceeding clockwise). Clockwise the next quadrant contains complexing strength towards phenol, fluorescence and TOC, the bulk carbon characteristics. UV absorption parameters are displayed in the third quadrant, colour, UV absorption at 240 nm and 200 nm. The fourth quadrant contains the phenolic pool of total, poly- and monophenolics. The data had been normalised over the range of data points for each set of 'fingerprints'.

Initially the data was submitted to Principle Component Analysis (PCA). In PCA a correlation matrix of the 12 variables was calculated and converted into the 12 corresponding eigenvalues. Each eigenvalue thus has a contribution from each parameter. The results given for those eigenvalues describe the contribution of each parameter to the eigenvalue and the percentage of variance that can be explained with the eigenvalue. PCA is a useful means for preliminary data investigation because it shows the contribution of the assays to the overall result (Vega et al. 1998).

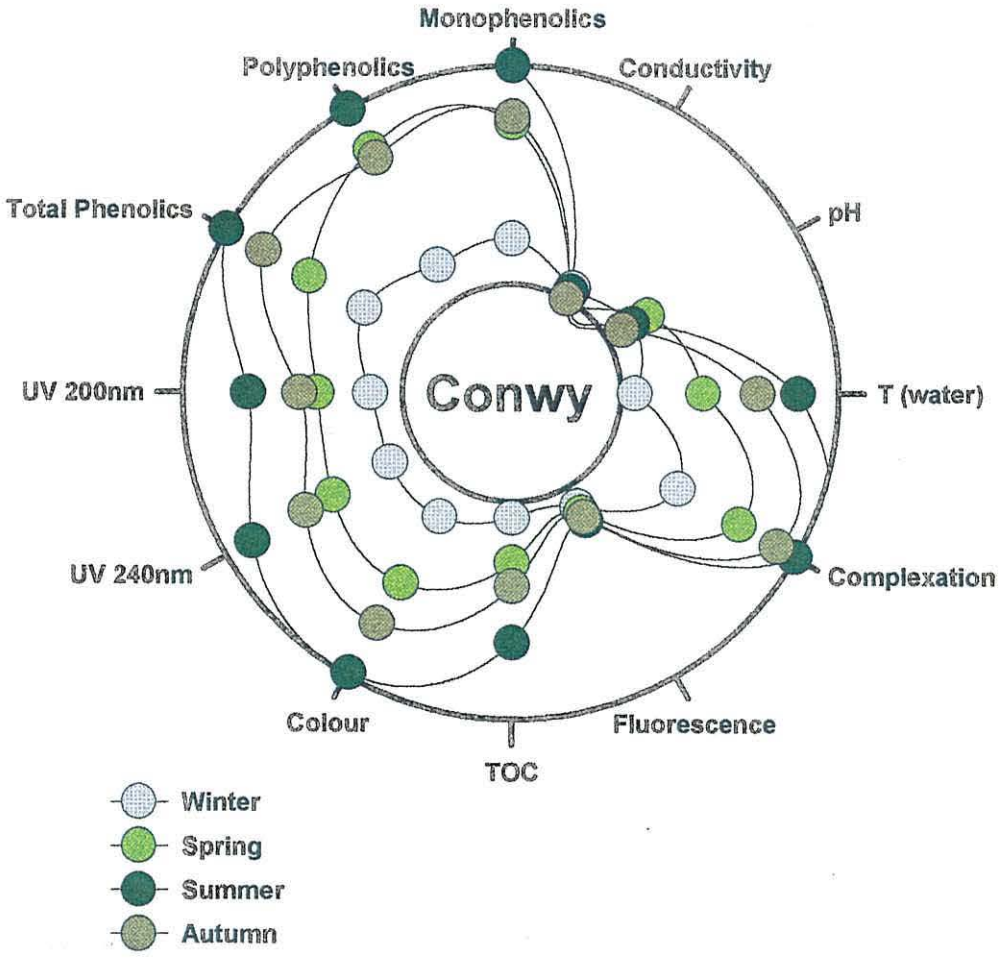

Figure 2.7: Fingerprint for Conwy (seasonal variation data)

Additionally the results were analysed using cluster analysis, which assumed each assay to be independent of each other assays. The twelve variables made a 'twelve dimensional room', thus there were twelve clusters. These clusters were linked by complete linkage, meaning the furthest apart points in each cluster are joined. Complete linkage is a method based upon the discrepancies of the samples as opposed to the similarities. Overall cluster analysis was used to establish which samples were most similar to each other. Additionally the variables themselves could be submitted to a cluster analysis, comparable to correlation.

Combining the 'fingerprints' with cluster analysis gave a visual impression of the chemical characteristics of each sample, while assuming independence of variables based upon one set of data.

Although no data was available from this section, the fingerprint was included as part of the analysis. Going through the process of an analysis entailed definition of the matrix and the analyte, defining the methodology of the analysis and applying the necessary statistics in order to be able to present the result in an understandable form. Visual means of data representation in ecological work with large multi-variable data sets tend to lose the context over too many graphs. In the application of the assays to the seasonal variation (Chapter 3) and the controlled decomposition (Chapter 4) a full set of 'fingerprints' will be displayed and interpreted.

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Chapter 3: Seasonal Variation

3.1 Introduction

DOM was described as being heterogeneous (Thurman 1985) and the largest part of DOM consisted of high molecular weight humic substances. In order to better define the ecological functioning of DOM, research into its bulk characteristics was undertaken (Perdue and Gjessing 1990) as it was suspected that the extensive processing of water samples could lead to artefacts (De Haan 1992). The 'fingerprinting' methodology (Chapter 2) matched onto the reaction mechanisms of humification (Stevenson 1994) was applied to filtered natural freshwater samples, thus working with natural matrices. Chemical and biochemical characteristics of DOM were determined throughout the year to investigate seasonal fluctuations.

According to Stevenson (1994), the starting material for humification is plant debris. The main reaction pathways were oxidation of polyphenolics to quinones and polymerisation with N-containing compounds. Two assays based on a redox mechanism, Folin-Ciocalteau (Box 1983) and Prussian blue (Price and Butler 1977), were included to account for oxidation. Regarding complexation reactions the lack of an appropriate methodology to measure the interaction between DOM and a complexor was identified and an assay measuring the quenching of phenol added at naturally occurring concentration of mononphenolics was developed (Chapter 2). Fluorescence quenching originating from humic metal complexes served as starting point (Ryan and Weber 1982, Ryan et al. 1983) but, instead of measuring the fluorescence quenching of humic acid, the quenching of phenol fluorescence was taken (Daharu and Sporns 1984, Eftink 1991). The measurement of TOC was included in the fingerprint, due to its common use in ecological studies to assess the size of the carbon pool (Thurman 1985, Freeman et al 1990, Allan 1995). The involvement of lignin in the genesis of humic substances was established prior to this work (Ertel et al. 1984). Keeping with the aim of non-intrusive, quick and simple analysis, two assays

measuring lignin-derived monophenolics were included. Fluorescence at excitation 370 nm and emission 445 nm was thought to measure caffeic acid derivatives (Larson and Weber 1994), while the Gibbs assay (Gibbs 1927) was responsive towards monomeric phenolic acids of lignin origin (Chapter 2 this work). UV absorbance of DOM at 200 and 240 nm was included to use the only available spectroscopic technique to be applied directly onto DOM in the low mg l^{-1} concentration range. Colour in Hazen units was included, as commonly performed for drinking water analysis (APHA). The environmental parameters of the sites studied, pH, conductivity and water temperature, were also included in the 'fingerprint' to assess water chemistry (Jenkins and Suberkropp 1995).

This suite of assays to measure the chemical composition of DOM used 12 different parameters. These created large data sets. Assuming independence of all variables, multivariate statistical techniques, cluster analysis and principle component analysis (Vega et al. 1998), were chosen as most appropriate for this study.

Degradation processes in freshwater bodies are microbially driven. The main mode of action for decomposition is considered to be enzymatic attack of plant debris. Thus one assay to elucidate DOM-BSA (Bovine Serum Albumin) interaction, adapted from tannin analysis (Hagerman and Butler 1978, Mole and Waterman 1987, 1994), was performed on the samples to assay the protein precipitating ability of DOM. To examine enzyme activity a model enzyme, porcine pancreatic lipase, was chosen and its activity in the presence of DOM assayed. The fact that maximum activity for this enzyme occurred at pH 7 (Guilbault and Kramer 1964) was an advantage, as it fell into the pH range of natural waters. The two assays investigating the biochemical behaviour of DOM were separated from the chemical fingerprint, because studies of plant polyphenols showed a lack of correlation between bulk chemical characteristics and biochemical activity (Stadler Martin and Martin 1982, Mole and Waterman 1987).

The amorphous analyte DOM varied spatially and temporally (Randke and Larson 1984). The employment of a single assay to quantify humic substances based upon the reactivity with phosphomolybdic and phosphotungstic acid (Box 1983) was criticised by Randke and Larson (1984) primarily due to the heterogeneous nature of humic substances. Thus employing multiple assays based upon different reaction mechanisms, as occurring during humification (Stevenson 1994), while working with natural matrices was thought to be more appropriate. A study of the seasonal variation of DOM in three rivers and three wetland sites was taken to investigate the performance of all assays. Data on the seasonal changes of enzyme activity or phenolic content of the study sites was available for comparison of the 'fingerprint' results, relating to the three rivers (Freeman et al 1990, Freeman and Lock 1992). In addition 4 humic substances samples, obtained from the lake 'Skjervatjern' experiment (Hayes et al. 1997), were included to compare their responses to the methodology developed in this study.

3.2 Materials and Methods

Sites

Aber, an oligotrophic mountain stream at longitude 4° 02' W and latitude 53° 13' N. Clywedog, a eutrophic lowland river at longitude 3° 21' W and latitude 53° 08' N. Conwy, a dystrophic brown-water river at longitude 3° 49' W and latitude 52° 59' N (more details for the three rivers in Freeman et al. 1990). Cwm-y Glo, a riparian wetland at longitude 4° 09' W and latitude 53° 08' N. Cors Goch, an alkaline fen at longitude 4° 16' W and latitude 53° 19' N. Clegir, an acid fen at longitude 3° 49' W and latitude 52° 59' N.

'Fingerprint'

For the 'fingerprint' a protocol was developed based upon the results presented in Chapter 2. Monophenolics were found to degrade rapidly, thus the Gibbs assay was performed the day after sampling. Poly- and total

phenolics were thought more stable and their respective concentration was measured two days after sampling. Colour, UV absorbance and TOC were analysed last. Samples were taken during each season: winter 27.1.1998, spring 27.4.1998, summer 27.7.1998 and autumn 13.10.1998. A total volume of 500 ml was estimated sufficient to carry out the analysis.

Protocol for 'Fingerprint'

On day 1, samples were taken at all sites. On-site the water temperature, pH and conductivity were measured (Hanna Water tester). Samples were taken in acid washed, brown Winchester bottles (2.5 l volume) and brought back to the laboratory.

On day 2, samples were filtered through Whatman GF/F. Each sample was spiked with 0.1 and 0.2 mg phenol l^{-1} (AnalaR from BDH) and standards prepared (0 to 1 mg l^{-1}). The Gibbs assay (Daharu and Sporns 1984) for monophenolics was performed and the absorbance measured at 620 nm. For the complexed phenol assay the fluorescence of the samples and standards at excitation 270 nm, slit width 15 nm, and emission at 300 nm, slit width 15 nm, taken. A linear regression was performed for each site and the standards. Results were calculated according to the following equation.

Complexing strength towards phenol =

$$[1-(\text{slope regression site} / \text{slope regression standards})] \times 100$$

Fluorescence of the samples at excitation 370 nm, slit width 5 nm, and emission at 445 nm, slit width 15 nm, was taken (Ryan and Weber 1982).

On day 3 samples were spiked with 1 and 2 mg l^{-1} TA (Tannic Acid BP 73 from Roy Wilson Dickson, Chester, England) and TA standards (0 to 5 mg l^{-1}) were prepared. The Folin-Ciocalteau (Box 1983) for total phenolics and the Prussian blue assay (Price and Butler 1977) for polyphenolics were performed. The reagent concentrations for the Prussian blue assay were adjusted to 0.0025 M $K_3[Fe(CN)_6]$ and 0.1 M $FeCl_3$ in 0.1 M HCl. The

absorbance at 750 nm was taken for both assays.

The concentration of mono-, poly- and total phenolics was calculated from the standard addition (Caulcutt and Boddy 1983).

On day 4 colour at 350 nm (APHA) and UV absorption at 200 and 240 nm were taken. For the TOC analysis the samples and standards, 0 to 25 mg l^{-1} of potassium hydrogenphthalate, were acidified with 1/1000 of their volume conc. HNO_3 and purged with N_2 for 15 min. The total carbon content of the solutions was determined with a Shimadzu Total Organic Carbon Analyser.

All statistics were performed with Minitab 11.

Protein Precipitation

The protein precipitation assay was adapted from Hagerman and Butler (1978). The concentration of samples and reagents was altered to account for the lower levels of analyte. Sample, spiked sample (2 and 4 mg TA l^{-1}), and standards (0, 2 and 4 mg TA l^{-1}) of 25 ml volume were mixed with 5 ml standard protein solution (4 mg BSA l^{-1}). Mixtures were left in the fridge overnight before being centrifuged. The Folin-Ciocalteau assay was performed on the supernatant, the iron complexation assay was performed on the pellet.

Lipase Assay

Solutions used were **phosphate buffer** pH 7 (0.01 M), made up from 50 ml 0.1 M KH_2PO_4 and 29.1 ml 0.1 M NaOH in1000 ml. **Lipase solution** (0.1 gl^{-1}) made up from porcine pancreatic lipase (crude Type II from Sigma) in buffer. **Substrate solution** Fluorescein diacetate (from Sigma) was dissolved in warm acetone and diluted with buffer to final concentration of 10 µM. **Product solution** Fluorescein (from Sigma) was dissolved in acetone and diluted with buffer to concentrations of 40, 80 and 120 nM.

Procedure: For the calibration 1.5 ml of each sample, 0.75 ml product

solution for each concentration and 0.75 ml lipase solution were used. For *lipase absent* 1.5 ml sample, 0.75 ml buffer and 0.75 ml substrate solution were used. For *lipase present* 1.5 ml sample, 0.75 ml substrate solution and 0.75 ml lipase solution were used. Buffer solution was used as a blank. The cuvettes were inverted and left for 60 minutes. The fluorescence was measured at excitation 490 nm, slit width 5 nm, and emission 520 nm, slit width 15 nm.

The fluorescence measured after 60 minutes was converted into concentration according to the calibration for each sample. The amount of fluorescein produced, *lipase present* less *lipase absent*, for the sample was divided by the value for the buffer blank to obtain a ratio. Triplicate samples were run.

3.3 Results

3.3.1 'Fingerprint'

The seasonal variation of DOM in three rivers and three wetland sites in North Wales was studied and graphically condensed into a 'fingerprint' by site (Figure 3.1) and by season (Figure 3.2). Table 3.1 contains the data. The assays performed for the 'fingerprint' had simplicity and speed as their primary aim. Bulk chemical characteristics of DOM or the conditions in which DOM was synthesised were determined.

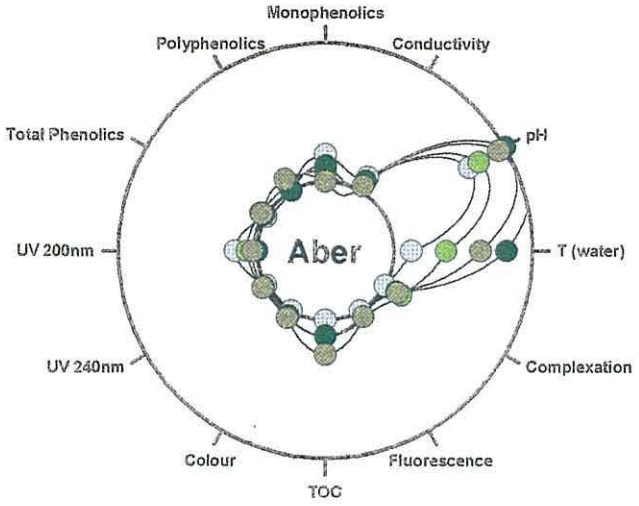
Each 'fingerprint' could be split into four quadrants. The top right hand corner contained the environmental parameters. In analogy to a clock, at 1 o'clock conductivity could be found, at 2 o'clock pH and at 3 o'clock water temperature. Contrasting rivers and wetlands were used in this study. The Aber was an oligotrophic mountain stream with circumneutral pH and low conductivity. The mildly eutrophic lowland river Clywedog had slightly alkaline pH and showed higher conductivity at all seasons. The upper Conwy, a brown water dystrophic river, had a low pH and low conductivity. The water temperature showed a broad spread for all three rivers throughout

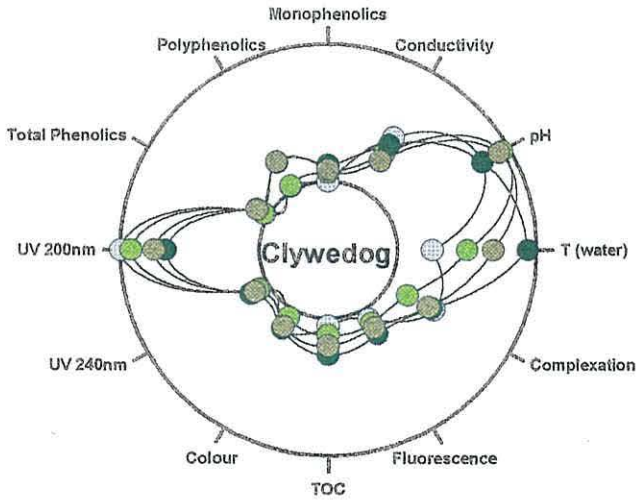
the seasons. The other three sites were wetlands. Cwm-y Glo was a riparian wetland. Low conductivity and slightly acid pH were found throughout all seasons. Cors Goch was classified as an alkaline fen. High pH and high conductivity were found. An acid fen, Clegir, showed low pH and low conductivity. The water temperature, apart from during winter, was at the higher end of the range for all wetland sites. The difference to the well mixed rivers was usage of sampling holes (0.5 m x 0.5 m x 0.5 m) for Cwm-y Glo and Clegir and sampling from a man-made pond (surface area ca. 15 m^2 and max 1.5 m deep) at Cors Goch. Regarding the overall climate, 1998 was a particularly cold (water temperature did not increase above 17 °C (Table 3.1)) and wet year.

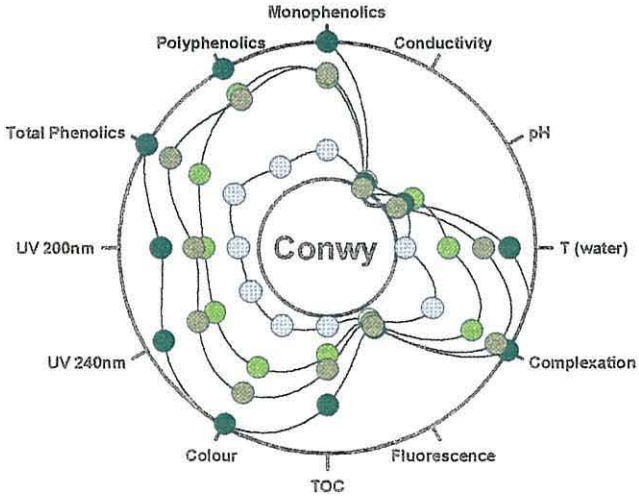
Proceeding clockwise to the next quadrant, bulk carbon characteristics are found: complexed phenol, fluorescence and TOC. The complexed phenol assay measured the interaction between naturally occurring phenol concentrations (0.1 and 0.2 mg l^{-1}) and DOM via fluorescence quenching. The Aber had the lowest complexing ability, followed by Clywedog. The highest complexing ability was shown by Cors Goch, which equally had the highest levels of fluorescence and TOC. The complexing strength of Conwy, Cwm-y Glo and Clegir showed more dependence upon the season and were overall towards the higher end of the range. Regarding the fluorescence of the six sites, only Cors Goch displayed strong fluorescence independent of the season. TOC levels were low for Aber and Clywedog, increasing for Cwm-y Glo, Clegir and Conwy and highest for Cors Goch.

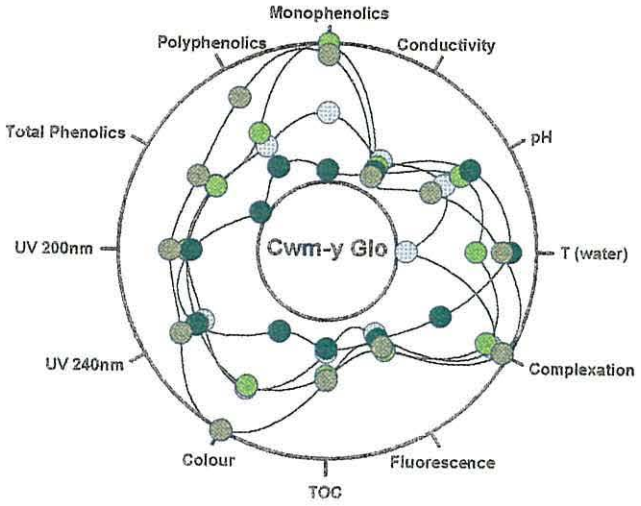
The third quadrant displayed UV - VIS absorbance characteristics of DOM: Colour, absorbance at 240 and 200 nm. Aber showed the lowest values of the range and Cors Goch showed the highest. Regarding the values as a spectrum going clockwise, Conwy, Cwm-y Glo and Clegir showed a decreasing trend. Clywedog, on the other hand, showed high absorbance at 200 nm. A similar increase was found for the Aber, but the absorbance at 200 nm was low.

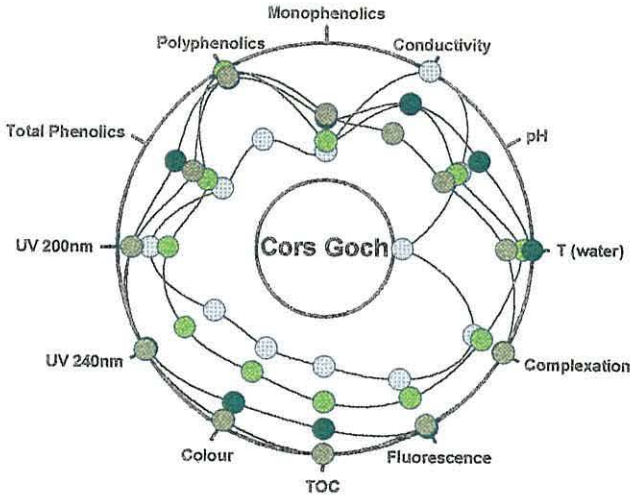
Seasonal Variation











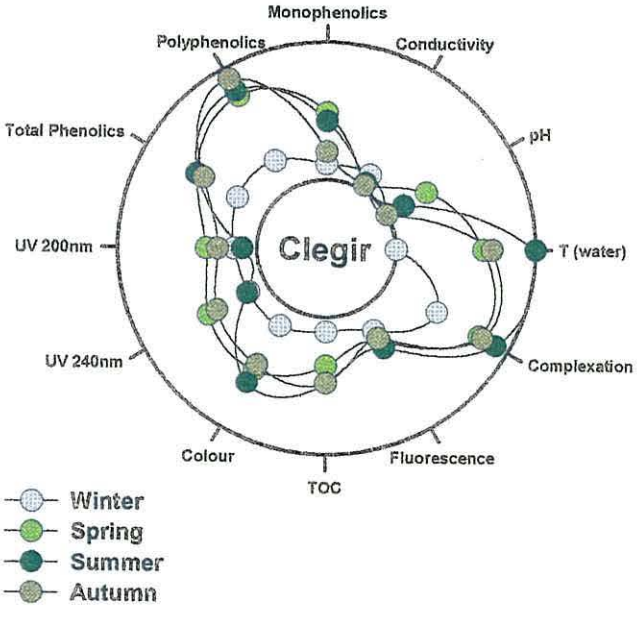


Figure 3.1: 'Fingerprint' for the seasonal variation of bulk characteristics of DOM. Displayed are the results of 12 assay measured on samples taken in winter (27.1.1998), spring (27.4.1998), summer (27.7.1998) and autumn (13.10.1998) on the six different sites.

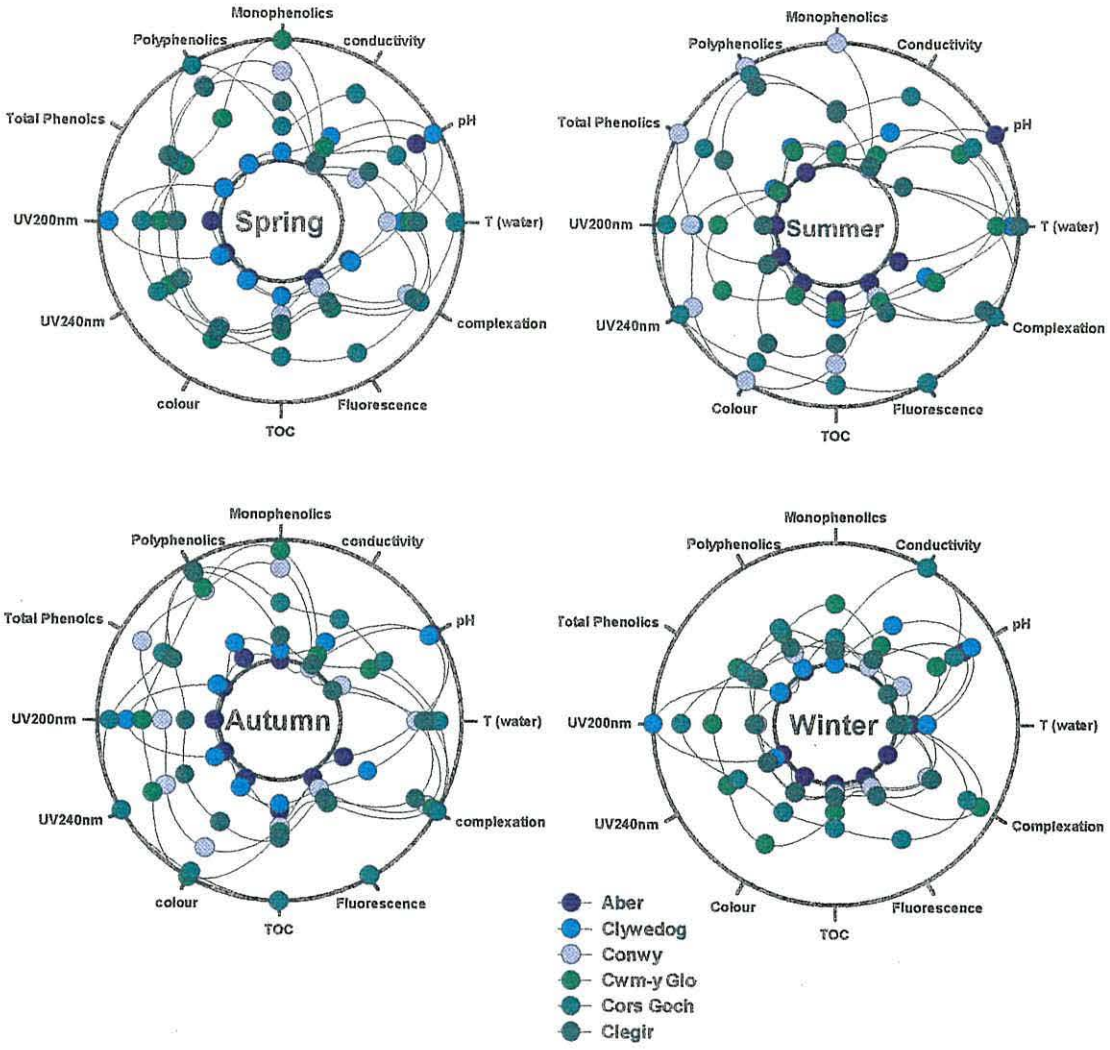


Figure 3.2: 'Fingerprint' for the seasonal variation of bulk characteristics of DOM. Displayed are the results of 12 assay measured on the six different wetland sites grouped according to the four seasons.

Table 3.1: Results of assays for 'fingerprint' for each site and season. Units, where applicable, were given at the top column (PE = Phenol Equivalents, TAE = Tannic Acid Equivalents). Standard error given in brackets. Monophenolics were determined one day after sampling with the Gibbs assay, two days after sampling polyphenolics were determined with the Prussian blue assay and total phenolics with the Folin-Ciocalteau assay. Dates for sampling: winter 27.1.1998, spring 27.4.1998, summer 27.7.1998, autumn 13.10.1998.

	T (°C)	pH	Conductivity (µS)	Monophenolics (± se, mg l^{-1} PE)	Polyphenolics (± se, mg l^{-1} TAE)	Total Phenolics (± se, mg l^{-1} TAE)
Aber Winter	3	6.8	67.8	0.041 (0.008)	0.195 (0.022)	0.367 (0.007)
Aber Spring	7	7.1	47.0	0.015 (0.012)	0.251 (0.042)	0.708 (0.065)
Aber Summer	14	7.9	53.4	0.024 (0.003)	0.182 (0.035)	0.517 (0.031)
Aber Autumn	11	7.7	32.0	<LoD	0.328 (0.022)	0.639 (0.030)
Clywedog Winter	5	7.1	236.0	<LoD	0.238 (0.023)	0.570 (0.033)
Clywedog Spring	9	7.7	165.0	0.014 (0.005)	0.243 (0.030)	0.653 (0.042)
Clywedog Summer	16	7.1	204.0	0.025 (0.002)	0.548 (0.063)	1.030 (0.024)
Clywedog Autumn	12	7.6	137.0	0.018 (0.004)	0.555 (0.036)	1.006 (0.027)
Conwy Winter	2	4.7	39.5	0.044 (0.007)	0.456 (0.049)	2.333 (0.071)
Conwy Spring	7	5.0	28.0	0.156 (0.034)	1.399 (0.146)	4.434 (0.089)
Conwy Summer	14	4.7	31.2	0.211 (0.007)	1.728 (0.194)	7.533 (0.176)
Conwy Autumn	11	4.5	12.0	0.162 (0.007)	1.330 (0.085)	6.141 (0.312)
Cwm-y Glo Winter	2	5.9	140.0	0.105 (0.011)	0.750 (0.043)	3.425 (0.126)
Cwm-y Glo Spring	10	6.4	114.0	0.210 (0.026)	0.918 (0.064)	3.370 (0.243)
Cwm-y Glo Summer[a]	14[a]	6.7[a]	95.0[a]	0.018 (0.004)[a]	0.489 (0.104)[a]	0.744 (0.010)[a]
Cwm-y Glo Autumn	13	5.5	72.0	0.193 (0.013)	1.374 (0.119)	4.455 (0.164)

[a] **Different location from winter, spring and autumn sampling, due to dry sampling hole.**

Table 3.1 (cont.): Results of assays for 'fingerprint' for each site and season. Units, where applicable, were given at the top column (PE = Phenol Equivalents, TAE = Tannic Acid Equivalents). Standard error given in brackets. Monophenolics were determined one day after sampling with the Gibbs assay, two days after sampling polyphenolics were determined with the Prussian blue assay and total phenolics with the Folin-Ciocalteau assay. Dates for sampling: Winter 27.1.1998, spring 27.4.1998, summer 27.7.1998, autumn 13.10.1998.. Humic (HA) and fulvic acid (FA) samples came from the lake Skjervatjern experiment.

	T (°C)	pH	Conductivity (µS)	Monophenolics (± se, mg l^{-1} PE)	Polyphenolics (± se, mg l^{-1} TAE)	Total Phenolics (± se, mg l^{-1} TAE)
Cors Goch Winter	2	6.5	511.0	0.045 (0.022)	0.823 (0.085)	2.970 (0.085)
Cors Goch Spring	16	6.4	370.0	0.060 (0.027)	1.701 (0.176)	3.885 (0.279)
Cors Goch Summer	17	7.1	374.0	0.095 (0.003)	1.612 (0.237)	5.800 (0.263)
Cors Goch Autumn	14	6.0	248.0	0.101 (0.015)	1.631 (0.140)	4.743 (0.451)
Clegir Winter	1	4.2	73.0	0.024 (0.014)	0.543 (0.037)	2.045 (0.037)
Clegir Spring	11	5.4	38.0	0.103 (0.030)	1.376 (0.152)	4.496 (0.202)
Clegir Summer	17	4.7	40.2	0.090 (0.004)	1.447 (0.085)	4.451 (0.036)
Clegir Autumn	12	4.2	29.0	0.043 (0.004)	1.595 (0.107)	4.086 (0.173)
FA background	n/a	n/a	n/a	0.099 (0.003)	2.090 (0.188)	4.003 (0.356)
FA acid soil	n/a	n/a	n/a	0.127 (0.004)	2.110 (0.536)	4.206 (0.470)
HA background	n/a	n/a	n/a	0.126 (0.007)	1.865 (0.208)	4.635 (0.261)
HA acid soil	n/a	n/a	n/a	0.099 (0.004)	1.419 (0.146)	4.473 (0.208)

Table 3.1 (cont.): Results of assays for 'fingerprint' for each site and season. Units, where applicable, were given at the top. Standard error given in brackets. Dates for sampling: Winter 27.1.1998, spring 27.4.1998, summer 27.7.1998, autumn 13.10.1998.

	UV Absorbance 200 nm	UV Absorbance 240 nm	Colour (Grade Hazen)	Total Organic Carbon (mg C l^{-1})	Fluorescence	Complexed Phenol (%)
Aber Winter	0.745	0.116	4	0.452	10.62	0.133
Aber Spring	0.563	0.159	10	3.714	24.64	0.243
Aber Summer	0.403	0.145	11	3.617	24.91	0.209
Aber Autumn	0.477	0.153	12	7.288	24.91	0.217
Clywedog Winter	2.565	0.205	25	1.929	34.41	0.433
Clywedog Spring	2.393	0.222	13	3.778	45.67	0.262
Clywedog Summer	1.860	0.306	26	7.775	72.95	0.416
Clywedog Autumn	2.044	0.262	23	5.876	56.95	0.398
Conwy Winter	0.712	0.299	25	2.511	40.62	0.433
Conwy Spring	1.244	0.696	72	8.147	50.95	0.694
Conwy Summer	1.916	1.251	136	18.289	62.60	0.931
Conwy Autumn	1.411	0.883	101	11.153	57.85	0.854
Cwm-y Glo Winter	1.501	0.798	91	6.780	66.65	0.825
Cwm-y Glo Spring	1.479	0.862	88	11.490	108.60	0.789
Cwm-y Glo Summer[a]	1.424	0.879	27	6.054	79.39	0.482
Cwm-y Glo Autumn	1.760	1.040	138	12.363	98.08	0.896

[a] **Different location from winter, spring and autumn sampling, due to dry sampling hole.**

Table 3.1 (cont.): Results of assays for 'fingerprint' for each site and season. Units, where applicable, were given at the top column. Standard error given in brackets. Dates for sampling: Winter 27.1.1998, spring 27.4.1998, summer 27.7.1998, autumn 13.10.1998. Humic (HA) and fulvic acid (FA) samples came from the lake Skjervatjern experiment.

	UV Absorbance 200 nm	UV Absorbance 240 nm	Colour (Grade Hazen)	Total Organic Carbon (mg C l^{-1})	Fluorescence	Complexed Phenol (%)
Cors Goch Winter	2.066	0.671	50	10.413	190.40	0.732
Cors Goch Spring	1.789	0.998	77	17.559	236.80	0.783
Cors Goch Summer	2.350	1.410	112	22.962	310.70	0.926
Cors Goch Autumn	2.347	1.432	132	28.000	304.70	0.932
Clegir Winter	0.751	0.298	24	3.373	66.31	0.464
Clegir Spring	1.184	0.734	74	10.172	91.11	0.742
Clegir Summer	0.623	0.317	88	13.595	109.10	0.853
Clegir Autumn	1.004	0.646	68	13.675	85.35	0.766
FA background	0.897	0.549	58	7.062	98.60	0.700
FA acid soil	0.884	0.535	58	8.567	85.01	0.740
HA background	0.978	0.622	76	10.117	45.45	0.770
HA acid soil	0.850	0.860	66	9.323	33.91	0.720

The last quadrant contained the phenolics pool of DOM. Going clockwise, total, poly- and monophenolics were displayed. Again Aber and Clywedog showed the lowest values throughout all seasons. Conwy included the highest values for each assay. For Cwm-y Glo an increasing trend was found going clockwise. Clegir and Cors Goch showed a peak for polyphenolics. Working with the actual data, a ratio of polyphenolics to total phenolics gave high values for the Aber and Clywedog, 0.4 to 0.6. Cors Goch and Clegir had ratios of 0.3 to 0.4 and Conwy and Cwm-y Glo, apart from the summer sample that was taken at a different location, had the lowest ratio from 0.2 to 0.3.

As illustrated in Figure 3.1 a fingerprint for DOM was obtained. A different pattern for each site in four different seasons was shown using bulk characteristics. Figure 3.2 used the same illustrative technique, but sorted the data into seasons. The winter diagram showed values towards the lower end of the range, while the 'fingerprints' for spring, summer and autumn encompass the whole range.

In Figure 3.1 a colour coding for the seasons was applied. The seasonal variation for each site according to each parameter will be described in the following part. The seasonal trends depend on the changing of parameters such as temperature, sun light and leaf fall.

The pH in the Aber increased from winter to summer and decreased for autumn. The pH in the Clywedog was constant with a slight drop in summer. For the Conwy pH was lowest in autumn. The wetland sites showed a broader spread throughout the year. For all sites pH increased from winter to summer and decreased in autumn.

Conductivity was constant for the rivers and for Clegir for all seasons. Cwm-y Glo showed a small decrease from winter to autumn. Cors Goch showed the same trend but over a broader range.

The next quadrant, 12 o'clock anticlockwise to 10 o'clock, contains the

phenolics pool of mono-, poly- and total phenolics. For the Aber the range was small, monophenolics decreased from winter to autumn. Polyphenolics peaked in autumn. Total phenolics stayed constant. In the Clywedog mono- and total phenolics stayed constant, while polyphenolics equally peaked in autumn. The concentrations in the Conwy encompassed the whole range, increasing from winter to summer and decreasing in autumn. In the wetland site Cwm-y Glo the summer sample was not considered because the sample hole had dried up. Samples were taken from inside a tree root. Thus the phenolics measured were considered non-comparable with the other seasons. All concentrations of phenolics increased from winter to autumn. The range of monophenolics was highest, followed by poly- and total phenolics. Cors Goch and Clegir showed a similar pattern. Polyphenolics levels were highest of the three, increasing slightly towards autumn for Clegir. Total phenolics were lowest in winter and increased towards summer to drop again in autumn. Monophenolics increased from winter to autumn for Cors Goch. In Clegir they increased from winter to spring and then decreased towards autumn.

The quadrant in the 'fingerprint' anticlockwise from 9 to 7 o'clock, encompasses UV absorbance at 200 and 240 nm and colour in grade hazen. For the Aber 200 nm was highest in winter and colour was highest in autumn, although small values were determined throughout the year. The Clywedog was similar regarding 240 nm and colour. UV absorbance was very strong at 200 nm with the highest level found in winter, declining towards summer to increase again in autumn. Conwy showed an increase from winter to summer and a decrease in autumn. Cwm-y Glo increased from winter to autumn, summer was ignored as explained earlier. For Cors Goch colour and 240 nm increase gradually throughout the year. For 200 nm the lowest absorbance was in spring increasing towards equal readings for summer and autumn and a decrease again in winter. Clegir showed at 200 and 240 nm an increase from winter to spring, a drop for summer and again an increase for autumn. Colour displayed a different pattern increasing from

winter to summer to decrease in autumn.

The fourth quadrant, anticlockwise from 6 to 4 o'clock, contained bulk carbon characteristics: TOC, fluorescence and complexation of phenol. For the Aber TOC increased towards autumn. Fluorescence and complexation stayed constant with slightly higher levels in autumn. The Clywedog showed an increase from winter to summer for TOC and fluorescence declining in autumn. Complexation was lowest in spring in comparison to the other seasons, which had the same values. The Conwy showed a broad range for TOC and complexation, with increasing values from winter to summer, decreasing in autumn. Fluorescence showed little variation throughout the year. For Cwm-y Glo the summer sample was ignored again, due to the different sample location. TOC and complexation increased from winter to autumn. Fluorescence increased from winter to spring and dropped slightly in autumn. Cors Goch showed an increasing trend from winter to autumn for all three assays encompassing a substantial range. At Clegir TOC values increased from winter to summer and stayed level in autumn. Fluorescence and complexation increased from winter to summer and decreased in autumn.

The statistics applied to the seasonal data were principle component analysis (PCA) and cluster analysis. Both types of statistic were multivariate in nature. During the seasonal variation a large data set was accumulated. Multivariate statistical techniques assume independence of data, desirable to evaluate the new methodology employed in this study. Additionally Gaussian distribution of the data was not required.

PCA was a means to analyse the data regarding the contributions of each assay towards the correlation matrix. As opposed to using the 12 original parameters, PCA converted the 12 assays into 12 new eigenvalues and calculated the correlation of each assay for each eigenvalue. The scree plot (Figure 3.3) illustrates the contribution of each eigenvalue towards the

overall variation. A change in the slope of the scree plot was seen after eigenvalue four, thus the first four eigenvalues were chosen that accounted for 93 % of the variation within the data (Table 3.2).

Eigenvalue 1 showed a positive correlation for pH and negative correlations for the other assays, meaning that pH was the leading parameter. Six assays showed correlations above -0.3: polyphenolics, total phenolics, UV absorbance at 240 nm, colour, TOC and complexed phenol. These assays are strongly linked to the theory of humification (Stevenson 1994). Eigenvalue 1 explained 59 % of the overall variation within the data.

Eigenvalue 2 (Table 3.2) was most strongly correlated with pH, conductivity, UV absorbance at 200 nm and fluorescence, all above -0.3. The pH and conductivity mirror the environmental conditions on site. The UV absorbance at 200 nm was thought to originate from microbial activity. Fluorescence was thought to originate from a chemical transformation of caffeic acid. Eigenvalue 2 thus included the environmental parameters and their effect on microbial and chemical activity, separated from the chemical characteristics of DOM in eigenvalue 1. Eigenvalue 2 explained 20 % of the overall variation within the data.

Eigenvalue 3 had temperature as the only contributing factor, correlation 0.811, and explained 9 % of the overall variation within the data.

Eigenvalue 4 had pH, monophenolics, UV 200 nm and fluorescence as contributing assays above ± 0.3. Again these assays were considered to reflect water chemistry and microbial activity. Eigenvalue 4 explained 6 % of the overall variation within the data.

PCA was a means of investigating the underlying structure of the data. The initial approach to the suite of assays based upon the reaction mechanisms of humification was substantiated with eigenvalue 1. Eigenvalues 2,3, and 4 related to the water chemistry.

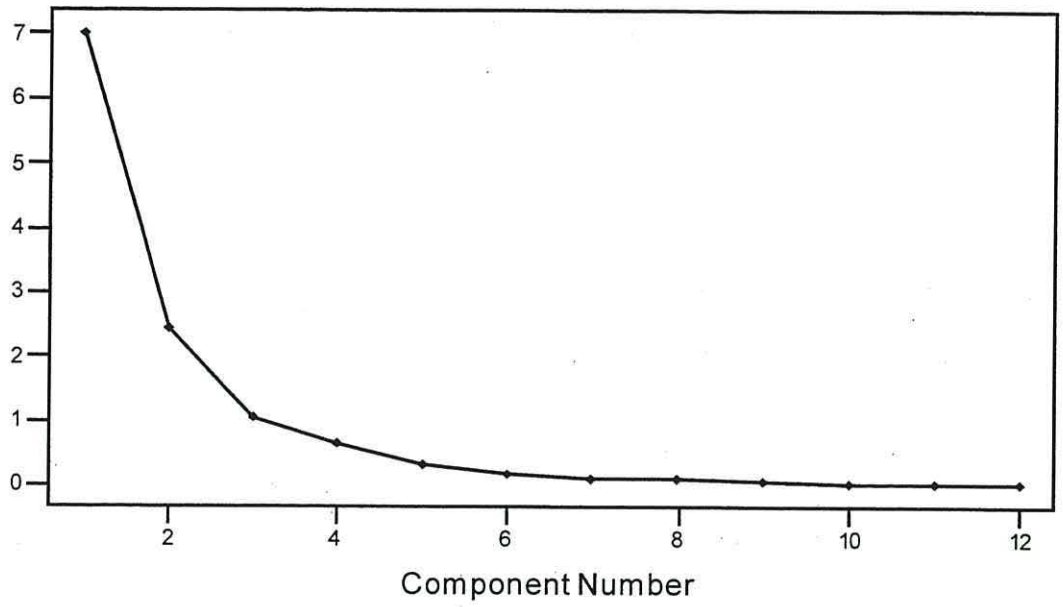


Figure 3.3: Scree plot for principle component analysis showing the contribution of each eigenvalue towards the overall correlation (12, the sum of all parameters).

Table 3.2: Result of principle component analysis for the 12 assays comprising the fingerprint as calculated by Minitab version 11. Shown are the 4 eigenvalues contributing 93 % of the overall variation amongst the data.

Variable	PC1	PC2	PC3	PC4
T (water)	-0.177	-0.133	0.811	-0.077
pH	0.173	-0.464	0.283	-0.377
Conductivity	-0.098	-0.549	-0.329	0.195
Monophenolics	-0.279	0.278	-0.071	-0.499
Polyphenolics	-0.355	0.094	0.094	0.196
Total Phenolics	-0.347	0.193	-0.061	0.013
UV 200nm	-0.166	-0.414	-0.271	-0.531
UV 240nm	-0.350	-0.075	-0.041	-0.107
Colour	-0.361	0.099	-0.022	-0.181
TOC	-0.344	-0.142	0.202	0.191
Fluorescence	-0.275	-0.359	-0.020	0.403
Complexed Ph.	-0.361	0.071	-0.141	0.023
Eigenvalue	7.0362	2.4288	1.0465	0.6668
Proportion	0.586	0.202	0.087	0.056
Cumulative	0.586	0.789	0.876	0.932

After PCA, cluster analysis was performed to evaluate the similarities between the samples. The results of the cluster analysis (Figure 3.4 and Table 3.3) show a dendrogram of samples linked via similarity levels. Two sites, Aber and Cors Goch formed distinct clusters. The samples for the Clywedog were clustered with the Cwm-y Glo summer sample, a sample taken at a different location at Cwm-y Glo than winter, spring and autumn. As a rough grouping two clusters could be seen, the left hand one containing the low carbon content samples and the right hand one the high carbon content samples. The sites at Conwy and Clegir has similar vegetation present, comprising primarily Sphagnum moss, heather and soft rush *Juncus Effusus*. However, while Conwy was a river Clegir was an acid fen and samples were taken from a man-made sampling hole. For their respective winter samples they fell into the low carbon cluster, showing high similarity. The spring samples showed close similarity joined subsequently by the Conwy autumn sample. While for Clegir the summer and autumn sample were similar, the Conwy summer sample was segregated, a reminder of the cold and wet summer. For Cwm-y Glo similarity was found for all seasons sampled at the same location. The spring and autumn sample were most similar to each other with the winter sample joining after Conwy and Clegir spring and Conwy autumn.

The results of the cluster analysis substantiate the allochthonous origin of DOM on site. The seasonal influence of DOM, as analysed in this study, was less important than the surrounding vegetation of the sites. For Conwy and Clegir with similar vegetation the seasons influenced similarity.

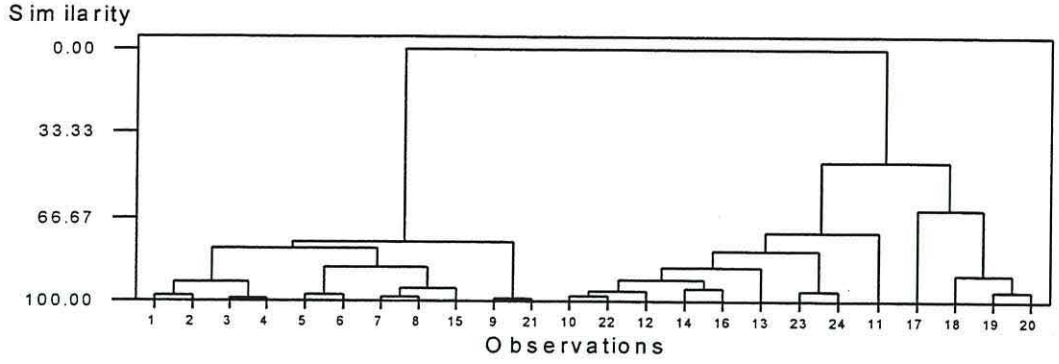


Figure 3.4: Result of analysis based on the suite of assays. The dendrogram shows similarities between the samples and thus groups the samples into clusters. The linkage method was complete linkage for the normalised data (squared Pearson algorithm). The numbers used for the observations are listed in Table 3.3 shown below.

Table 3.3: Number system for observations used in the dendrogram (Figure 3.5).

	Winter	Spring	Summer	Autumn
Aber	1	2	3	4
Clywedog	5	6	7	8
Conwy	9	10	11	12
Cwm-y Glo	13	14	15	16
Cors Goch	17	18	19	20
Clegir	21	22	23	24

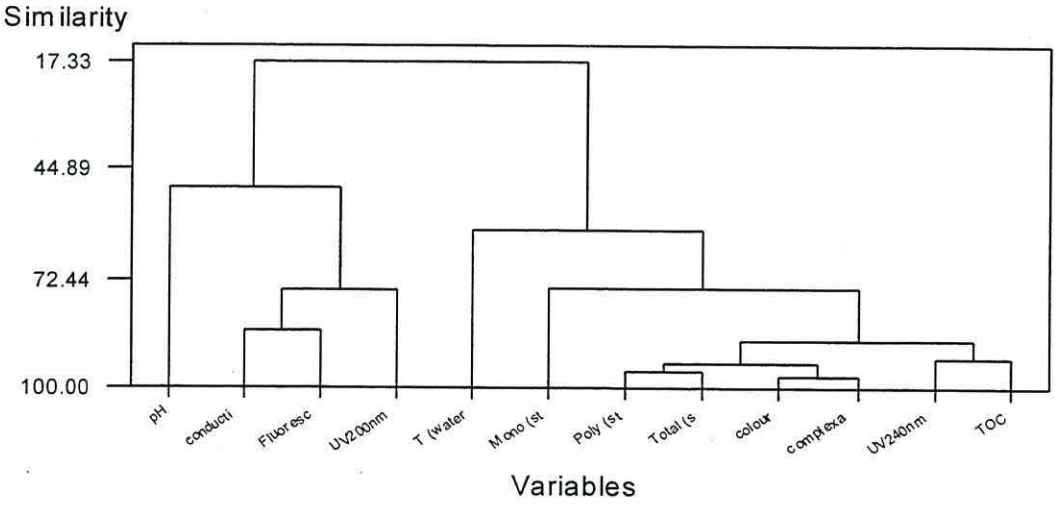


Figure 3.5: Results of cluster variables analysis

In the same way as the data for the sites and season was clustered, the assays themselves were submitted to cluster variable analysis. Figure 3.5 confirms the results of the PCA analysis. The left-hand cluster contained the eigenvalue 2 variables. The right hand cluster shows the strong similarity between poly- and total phenolics, colour and complexation, and UV absorbance at 200 nm and TOC, as were bulked for eigenvalue 1. Monophenolics join the right hand cluster next. They are only contributing to eigenvalue 4. Temperature shows the least similarity and was the last assay to join the right hand cluster. In PCA it forms its own eigenvalue 3. It is interesting to note that two multivariate statistical techniques, principle component analysis and cluster analysis, give very similar results regarding the variables measured in the suite. However, both methods were based upon the correlation matrix and are thus two similar means to investigate multivariate data.

3.3.2 Protein Precipitation

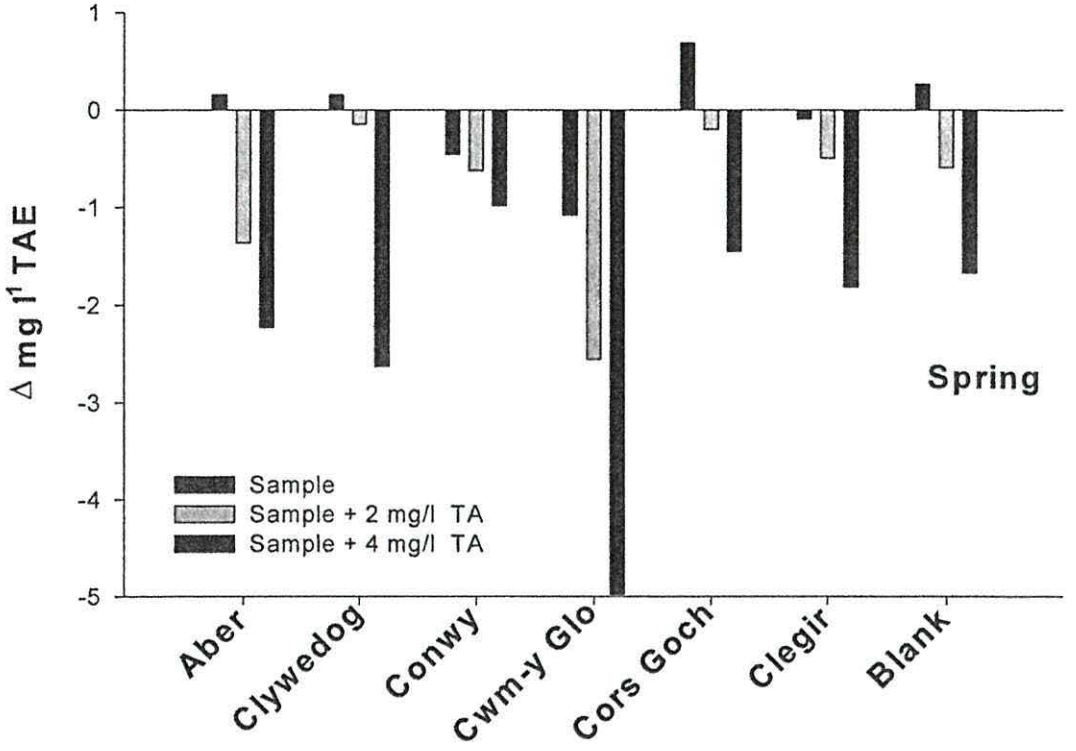
The protein precipitation assay was adapted from tannin analysis. The thoughts behind the adaptation were twofold. Firstly tannins, being recalcitrant by virtue of their polyphenolic nature, leach from plant and plant debris into natural waters and thus form part of DOM (Serrano 1992). Secondly, the key process of humification, complexation of oxidised phenolics with amino containing compounds, is mirrored in the protein precipitation.

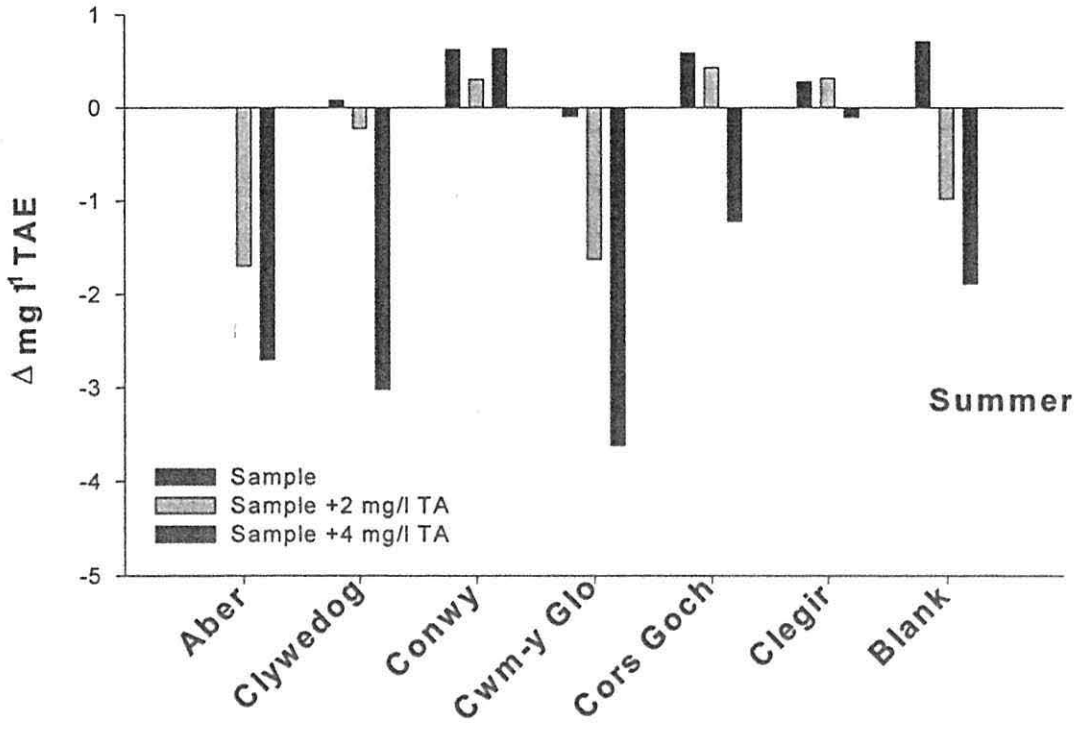
In the assay the sample was mixed with a standard protein solution in order to induce [DOM*BSA] complexation. Due to the low levels of DOM and BSA the samples were additionally spiked with TA to achieve the appropriate concentration levels for precipitation. The mixtures were placed in the fridge to aid complexation. After a minimum of 12 hours, centrifugation was performed and the supernatant decanted from the pellet. Following the

procedure of Hagerman and Butler (1978), the iron complexation assay was performed on the pellet. Poor repeatability was found. As an alternative, the total phenolics assay was performed on the supernatant, this having the advantage of being directly comparable to the total phenolics assay performed on the untreated sample which formed part of the 'fingerprint'.

The raw data for all seasons, analysis of supernatant and pellet, are listed in Table 3.4. For spring, summer and autumn the results of the protein precipitation are displayed in Figure 3.6. For each site, three bars are displayed showing sample and spiked samples, 2 and 4 mg TA l^{-1}. The y-axis represents a difference measure between the concentration of total phenolics of the samples and spiked samples before and after protein precipitation. Positive values indicated an increase in total phenolics thought to originate from the added protein. Negative values indicate total phenolics precipitated. It was expected that the spiked TA precipitated fully, in which case a difference in concentration of -2 and -4 mg TA l^{-1} should have been found for the spiked samples. The results for the blank (Figure 3.6) show an increase in total phenolics while the spiked samples show a lower reduction than expected, confirming the positive response of the total phenolics assay towards protein and the incomplete precipitation. Additionally, a comparison of the blanks, measured on three different occasions, confirmed the poor repeatability of TA protein precipitation.

Seasonal Variation





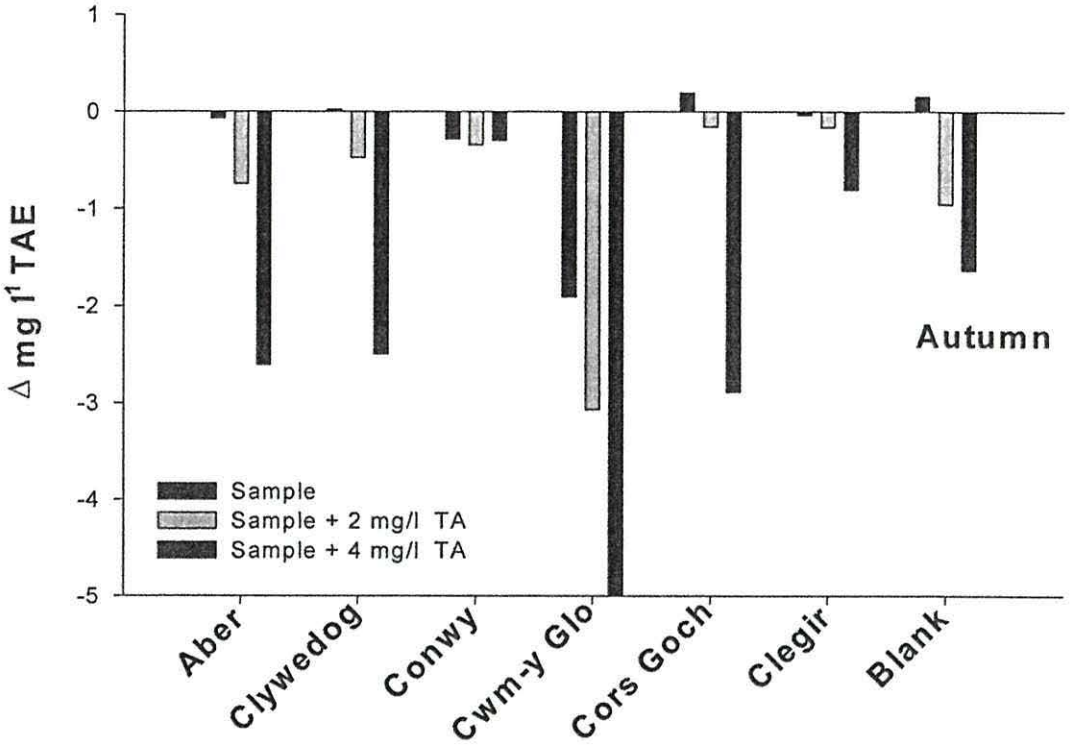


Figure 3.6: Result of the Folin-Ciocalteau assay on the supernatant after performing the protein precipitation assay. Shown are the differences between the Folin-Ciocalteau assay of the sample, sample + 2 mg TA l^{-1} and sample +4 mg TA l^{-1} on the untreated sample to the supernatant after the protein precipitation. Negative values indicate polyphenolics removed from sample. Positive values indicate response of Folin-Ciocalteau assay to non-phenolic compounds.

Table 3.4: Results of protein precipitation assay using BSA for each season per site including TA standards. Given are the concentration (mg l^{-1} with se) in TAE obtained in the iron-complexion assay for the dissolved pellet and the Folin-Ciocalteau assay for the supernatant.

		Pellet			Supernatant		
Seaon	Site	Sample	Spike 2 mg/l TA	Spike 4 mg/l TA	Sample	Spike 2 mg/l TA	Spike 4 mg/l TA
Winter	Standard	0.00 (0.04)	2.04 (0.01)	3.98 (0.04)			
Winter	Aber	0.40 (0.04)	0.95 (0.07)	4.11 (0.09)			
Winter	Clywedog	0.36 (0.05)	0.74 (0.08)	4.48 (0.09)			
Winter	Conwy	0.41 (0.07)	4.97 (0.08)	6.18 (0.11)			
Winter	Cwm-y Glo	2.90 (0.05)	9.90 (0.08)	15.55 (0.16)			
Winter	Cors Goch	0.23 (0.05)	0.48 (0.04)	2.37 (0.07)			
Winter	Clegir	0.13 (0.01)	2.77 (0.06)	4.02 (0.06)			
Spring	Standard	0.58 (0.08)	0.85 (0.07)	4.43 (0.09)	0.27 (0.01)	1.41 (0.00)	2.33 (0.07)
Spring	Aber	0.63 (0.03)	3.92 (0.09)	6.58 (0.09)	0.77 (0.01)	1.25 (0.01)	2.39 (0.07)
Spring	Clywedog	0.70 (0.03)	1.66 (0.09)	7.61 (0.61)	0.85 (0.01)	2.54 (0.01)	2.06 (0.01)
Spring	Conwy	1.03 (0.12)	1.35 (0.18)	1.45 (0.25)	4.15 (0.03)	5.98 (0.05)	7.62 (0.06)
Spring	Cwm-y Glo	1.26 (0.05)	6.58 (0.17)	15.80 (1.70)	1.93 (0.02)	2.45 (0.04)	2.03 (0.01)
Spring	Cors Goch	1.00 (0.08)	1.45 (0.11)	3.06 (0.18)	4.53 (0.61)	5.64 (0.12)	6.39 (0.05)
Spring	Clegir	1.08 (0.15)	1.58 (0.24)	1.96 (0.13)	3.90 (0.03)	5.49 (0.02)	6.17 (0.05)

Table 3.4: Results of protein precipitation assay using BSA for each season per site including TA standards. Given are the concentration (mg l^{-1} (se)) in TAE obtained in the iron-complexion assay for the dissolved pellet and the Folin-Ciocalteau assay for the supernatant.

		Pellet			Supernatant		
Seaon	Site	Sample	Spike 2 mg/l TA	Spike 4 mg/l TA	Sample	Spike 2 mg/l TA	Spike 4 mg/l TA
Summer	Standard	0.22 (0.01)	1.56 (0.03)	4.22 (0.19)	0.71 (0.16)	1.02 (0.13)	2.11 (0.09)
Summer	Aber	0.23 (0.01)	2.92 (0.09)	5.43 (0.31)	0.41 (0.01)	0.79 (0.03)	1.72 (0.08)
Summer	Clywedog	0.29 (0.02)	1.32 (0.03)	5.83 (0.02)	1.06 (0.01)	2.76 (0.03)	1.97 (0.03)
Summer	Conwy	0.40 (0.02)	0.52 (0.01)	0.56 (0.23)	7.08 (0.02)	8.76 (0.35)	11.09 (0.03)
Summer	Cwm-y Glo	0.40 (0.02)	4.20 (0.05)	8.23 (0.02)	0.63 (0.01)	1.09 (0.03)	1.10 (0.02)
Summer	Cors Goch	0.37 (0.02)	0.62 (0.01)	3.64 (0.10)	4.94 (0.07)	6.78 (0.08)	7.13 (0.08)
Summer	Clegir	0.32 (0.02)	0.78 (0.01)	1.91 (0.10)	4.38 (0.06)	6.42 (0.05)	8.00 (0.40)
Autumn	Standard	0.17 (0.09)	1.67 (0.01)	4.17 (0.09)	0.16 (0.06)	1.05 (0.02)	2.37 (0.08)
Autumn	Aber	0.11 (0.02)	0.98 (0.13)	0.88 (0.29)	0.56 (0.01)	1.88 (0.05)	2.02 (0.02)
Autumn	Clywedog	0.15 (0.06)	0.54 (0.06)	5.06 (0.05)	1.10 (0.08)	2.60 (0.02)	2.58 (0.08)
Autumn	Conwy	0.28 (0.08)	0.32 (0.06)	0.47 (0.80)	5.00 (0.02)	6.93 (0.08)	8.97 (0.03)
Autumn	Cwm-y Glo	1.17 (0.14)	4.02 (0.15)	8.47 (0.14)	1.88 (0.02)	2.72 (0.07)	2.76 (0.01)
Autumn	Cors Goch	0.18 (0.01)	0.79 (0.26)	5.08 (0.04)	4.89 (0.02)	6.54 (0.08)	5.81 (0.01)
Autumn	Clegir	0.34 (0.03)	0.64 (0.06)	1.09 (0.13)	3.31 (0.01)	5.18 (0.04)	6.54 (0.11)
n/a	FA background	0.90 (0.16)	0.48 (0.06)	0.40 (0.06)	4.05 (0.04)	5.53 (0.05)	6.76 (0.01)
n/a	FA acid soil	0.27 (0.03)	0.54 (0.03)	0.78 (0.07)	4.66 (0.03)	6.01 (0.05)	7.14 (0.11)
n/a	HA background	0.45 (0.11)	0.52 (0.18)	0.40 (0.02)	4.79 (0.05)	6.58 (0.11)	8.44 (0.05)
n/a	HA acid soil	0.20 (0.02)	0.34 (0.07)	0.51 (0.01)	4.53 (0.02)	6.46 (0.02)	8.17 (0.06)

Regarding the results for the sites, the first impression shows a distinct pattern independent of the season. Only at the Cwm-y Glo site in spring and autumn were more total phenolics precipitated than the added spike. In summer a different sampling location was used at Cwm-y Glo, not allowing direct comparison with the other three seasons. The pattern obtained for the Aber was similar to the blank. For the Clywedog the 2 mg TA l^{-1} spike was fully retained in the sample. Precipitation occurred at 4 mg TA l^{-1} added. The Conwy was the site where the addition of TA induced the least precipitation; the concentration difference of total phenolics remained constant. During spring and autumn an increase in total phenolics in the supernatant was found, while during summer a slight precipitation was measured. The Cors Goch site showed an increase in total phenolics for the sample for all three seasons. At the 2 mg TA l^{-1} spiking level, a slight precipitation was noted in spring and autumn with an increase in total phenolics concentration in summer. At the higher spiking level no difference was found between the seasons. Clegir showed a similar pattern for spring and autumn with zero levels for the sample, slight precipitation at 2 mg TA l^{-1} spike and increased precipitation at 4 mg TA l^{-1} spike. During summer zero levels were obtained for sample and spiked sample, indicating that no precipitation occurred. Overall the protein precipitation illustrated the interaction between DOM, protein and added polyphenolics. A seasonal dependence was found for Conwy, Cors Goch and Clegir, while no change was recorded at Aber, Clywedog and Cwm-y Glo for the three seasons studied.

3.3.3 Lipase Assay

In the lipase assay, a solution of lipase was added to each sample and the amount of substrate, fluorescein diacetate, converted into fluorescein after 60 minutes was measured. DOM was known to quench fluorescence, thus a calibration for fluorescein in the presence of each sample was taken. The concentration of fluorescein measured after 60 minutes for each sample was divided by fluorescein diacetate converted to fluorescein in the presence of

carbon free water, to determine inhibition or stimulation.

Figure 3.7 shows the ratios of lipase activity for each site by season compared to the blank. Table 3.5 contains the relevant raw data, while Table 3.6 contains the ratios. The six sites showed two different types of behaviour. While Aber and Clywedog showed slight inhibition in winter, increasing towards slight stimulation in summer and decreasing again in autumn, Conwy, Cwm-y Glo, Cors Goch and Clegir showed inhibition in winter and summer and neutral or slight stimulation in spring and autumn. Cwm-y Glo differed from the three other sites because the ratio obtained for autumn was less than the ratio for spring, while Conwy, Cors Goch and Clegir showed the highest ratio for autumn. Overall the highest inhibition was measured for Cors Goch in summer with a ratio of 0.71. Cors Goch showed equally the highest stimulation with a ratio of 1.10 in autumn. Thus Cors Goch encompassed the broadest range of all sites with a difference in the ratio of 0.39. Conwy, Cwm-y Glo and Clegir showed a difference in ratio of 0.21, 0.27 and 0.21, respectively. Aber and Clywedog had the least variation for the seasons with a difference of 0.13 and 0.10, respectively.

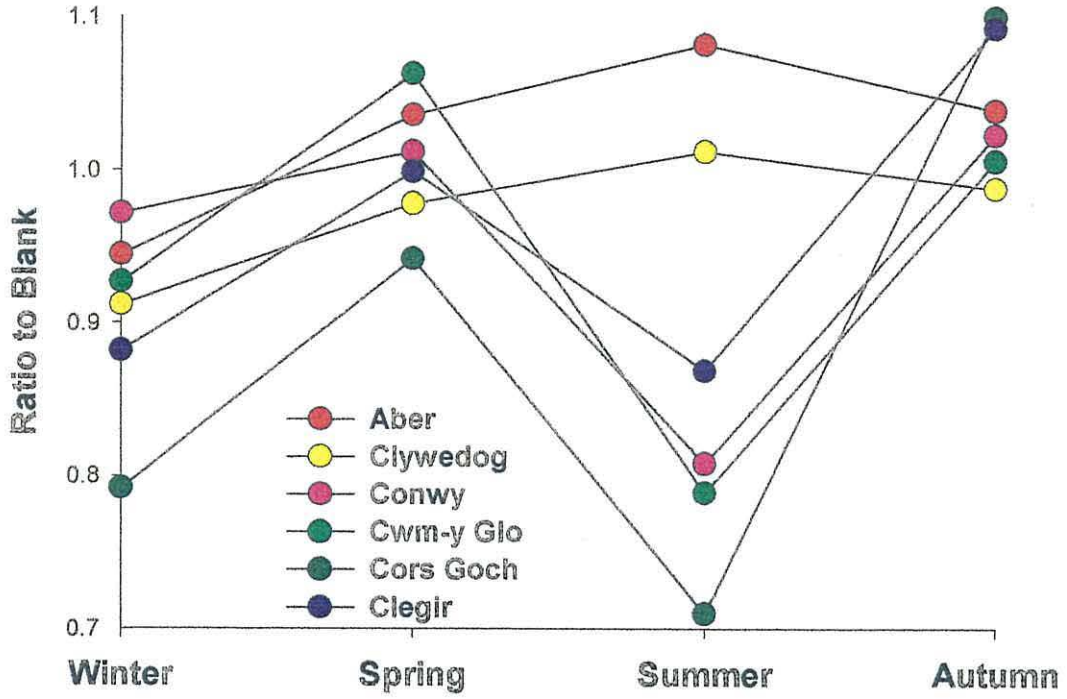


Figure 3.7: Result of lipase inhibition assay for each site by season. Shown was the ratio of the concentration of substrate, fluorescein diacetate, converted after 60 minutes divided by the concentration of substrate converted by lipase in a buffer blank. Calibrations for fluorescein were obtained for each sample to account for any quenching.

Table 3.6: Results of lipase inhibition assay. The concentration of substrate converted by lipase in the presence of the sample after 60 min has been divided through the concentration of substrate processed in the presence of buffer after 60 min to obtain the following ratios.

Season	Aber	Clywedog	Conwy	Cwm-y Glo	Cors Goch	Clegir
Winter	0.945	0.912	0.972	0.927	0.792	0.882
Spring	1.036	0.978	1.012	1.063	0.942	0.999
Summer	1.082	1.012	0.808	0.789	0.710	0.869
Autumn	1.039	0.988	1.023	1.006	1.100	1.093

Table 3.5: Results of lipase inhibition assay. The calibration of fluorescein was determined in the presence of buffer as blank and the sample, intercept, slope and r^2 values are given. The concentration of fluorescein (nM) converted from fluorescein diacetate after 60 min in the absence and presence of lipase is given with standard error in parentheses.

Season	Site	Calibration Intercept	Slope	r^2	Lipase absent [Fluor.] ± se	Lipase present [Fluor.] ± (se)
Winter[a]	Blank	5.40	6.52	1.000	<LoD	17.8 ± 0.1 nM
Winter[a]	Aber	5.62	6.64	1.000	<LoD	16.8 ± 0.0 nM
Winter[a]	Clywedog	7.05	6.67	1.000	<LoD	16.3 ± 0.1 nM
Winter[a]	Conwy	7.52	6.50	1.000	<LoD	17.9 ± 0.2 nM
Winter[a]	Cwm-y Glo	5.44	6.21	1.000	<LoD	17.7 ± 0.2 nM
Winter[a]	Cors Goch	5.40	6.52	1.000	<LoD	14.1 ± 0.1 nM
Winter[a]	Clegir	8.86	6.39	1.000	<LoD	15.7 ± 0.2 nM
Spring	Blank	9.71	12.73	0.984	<LoD	54.6 ± 0.2 nM
Spring	Aber	22.13	12.02	0.980	<LoD	56.6 ± 0.7 nM
Spring	Clywedog	7.78	12.56	0.997	2.3 ± 0.2 nM	55.4 ± 0.2 nM
Spring	Conwy	8.87	11.67	0.999	3.8 ± 0.1 nM	58.7 ± 0.6 nM
Spring	Cwm-y Glo	10.34	11.67	0.999	3.7 ± 0.2 nM	60.4 ± 0.4 nM
Spring	Cors Goch	21.85	12.62	0.981	3.9 ± 0.1 nM	55.0 ± 0.3 nM
Spring	Clegir	13.54	11.27	0.998	<LoD	55.0 ± 0.4 nM

[a] **slit width excitation 5 nm emission 10 nm, calibration 0 to 150 nM, [fluorescein diacetate] 1 µM**

Table 3.5: Results of lipase inhibition assay. The calibration of fluorescein was determined in the presence of buffer as blank and the sample, intercept, slope and r^2 values are given. The concentration of fluorescein (nM) converted from fluorescein diacetate after 60 min in the absence and presence of lipase is given with standard error in parentheses.

Season	Site	Calibration Intercept	Slope	r^2	Lipase absent [Fluor.] ± se	Lipase present [Fluor.] ± (se)
Summer	Blank	15.05	12.45	0.989	3.3 ± 1.2 nM	69.0 ± 1.5 nM
Summer	Aber	44.41	10.91	0.991	<LoD	71.3± 0.9 nM
Summer	Clywedog	36.72	10.91	0.987	8.7 ± 1.0 nM	75.1± 0.8 nM
Summer	Conwy	14.87	11.04	0.976	6.3 ± 1.5 nM	59.4 ± 0.5 nM
Summer	Cwm-y Glo	17.58	11.83	0.977	6.8.± 1.3 nM	58.6± 1.3 nM
Summer	Cors Goch	0.335	14.01	0.988	11.9 ± 2.8 nM	58.5 ± 1.1 nM
Summer	Clegir	17.55	11.14	0.993	3.4 ± 0.6 nM	60.4 ± 0.6 nM
Autumn	Blank	2.50	11.93	0.999	0.6 ± 0.0 nM	62.6 ± 0.4 nM
Autumn	Aber	0.72	12.13	1.000	1.8 ± 0.0 nM	66.2 ± 0.9 nM
Autumn	Clywedog	2.64	11.99	1.000	3.5 ± 0.0 nM	64.7 ± 0.3 nM
Autumn	Conwy	4.91	11.32	0.999	3.0 ± 0.0 nM	66.4 ± 0.4 nM
Autumn	Cwm-y Glo	3.68	11.43	1.000	12.2 ± 0.1 nM	74.6 ± 0.6 nM
Autumn	Cors Goch	10.45	11.85	0.998	3.9 ± 0.0 nM	72.1 ± 0.6 nM
Autumn	Clegir	4.59	11.53	0.998	1.5 ± 0.0 nM	69.2 ± 0.7 nM
n/a	FA background	4.77	11.21	1.000	0.9 ± 0.0 nM	6.9 ± 0.1 nM
n/a	FA acid soil	3.61	11.29	1.000	0.9 ± 0.0 nM	7.1 ± 0.1 nM
n/a	HA background	5.76	10.73	1.000	0.9 ± 0.0 nM	6.5 ± 0.0 nM
n/a	HA acid soil	2.54	10.80	1.000	1.1 ± 0.0 nM	6.5 ± 0.1 nM

3.3.4 Humic and fulvic acids

In addition to the filtered natural water samples used for the study, humic and fulvic acid samples obtained from the 'Lake Skjervatjern' experiment (Hayes et al. 1997) were submitted to the same assays during the autumn sampling. In this experiment a humic lake and its watershed in western Norway were split into two parts, one of which was submitted to artificial acid rain by addition of sulphuric acid and ammonium nitrate. Humic and fulvic acid processed from the acidified part and the background were used.

Comparing the humic acids with the fulvic acids (Figure 3.8 and Table 3.1) at concentrations of 20 mg l^{-1} showed differences for the polyphenolics and fluorescence, with the concentration measured being higher for the fulvic acids, while the UV absorbance at 240 nm and colour were higher for the humic acids. In comparison to the sites in the current study the 'fingerprint' was most similar to the Conwy and Clegir spring samples, clustering at a similarity level of 98.21.

Performing the protein precipitation assay on the samples showed a different pattern for the humic acids when compared to the fulvic acids (Figure 3.9 and Table 3.4). The humic acids kept the added TA spike in solution, while the fulvic acids led to the precipitation of ca. half the added spike. Compared to natural water samples, the humic acid showed a similar pattern to Clegir summer while the fulvic acids behaved like Clegir spring.

For the lipase assay (Table 3.5), an inhibition of 90 % was obtained for both humic and fulvic acids. None of the natural water samples showed inhibition higher than 30 %, even though the concentrations employed were similar. The results of the lipase assay confirm that the processing of natural water samples leads to artefacts regarding the biochemical activity of the high molecular weight fraction of DOM.

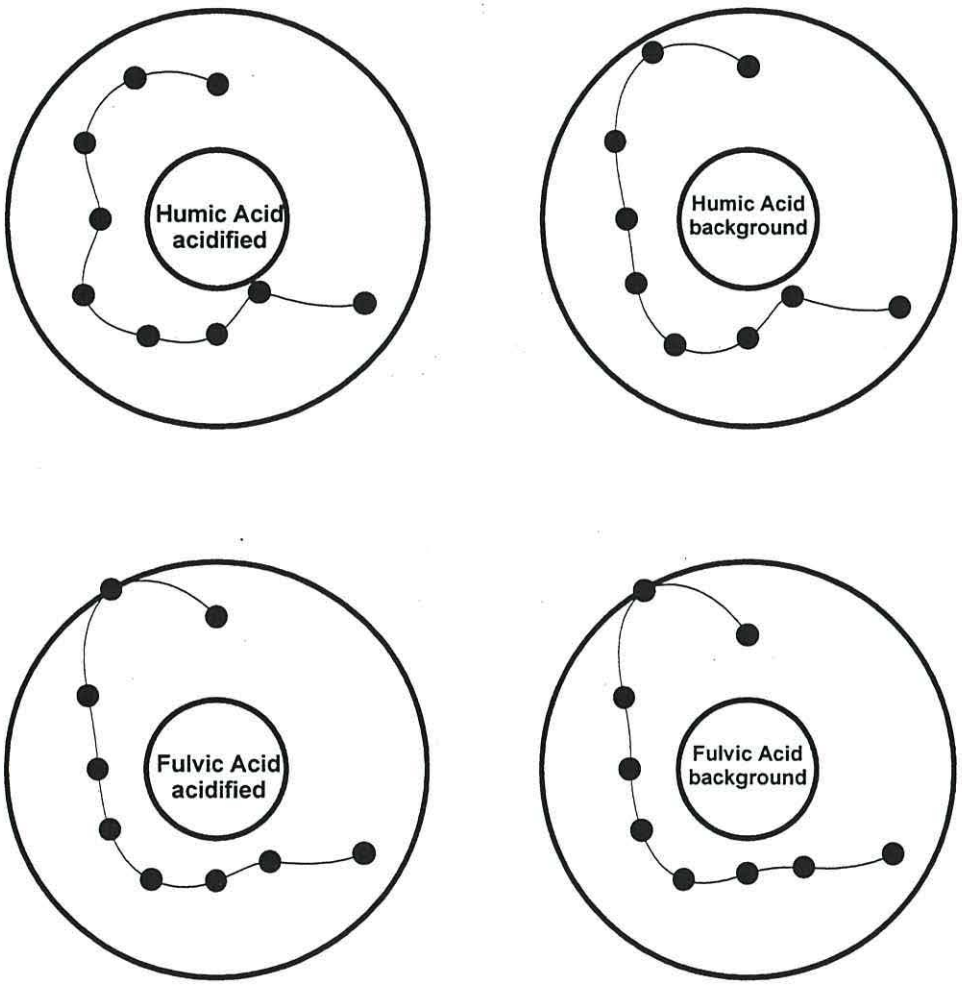


Figure 3.8: 'Fingerprint' for humic and fulvic acid samples from the lake 'Skjervatjern' experiment, in which the lake was split into two parts of which one was acidified. The data was normalised over the whole range including the river and wetland samples for each assay.

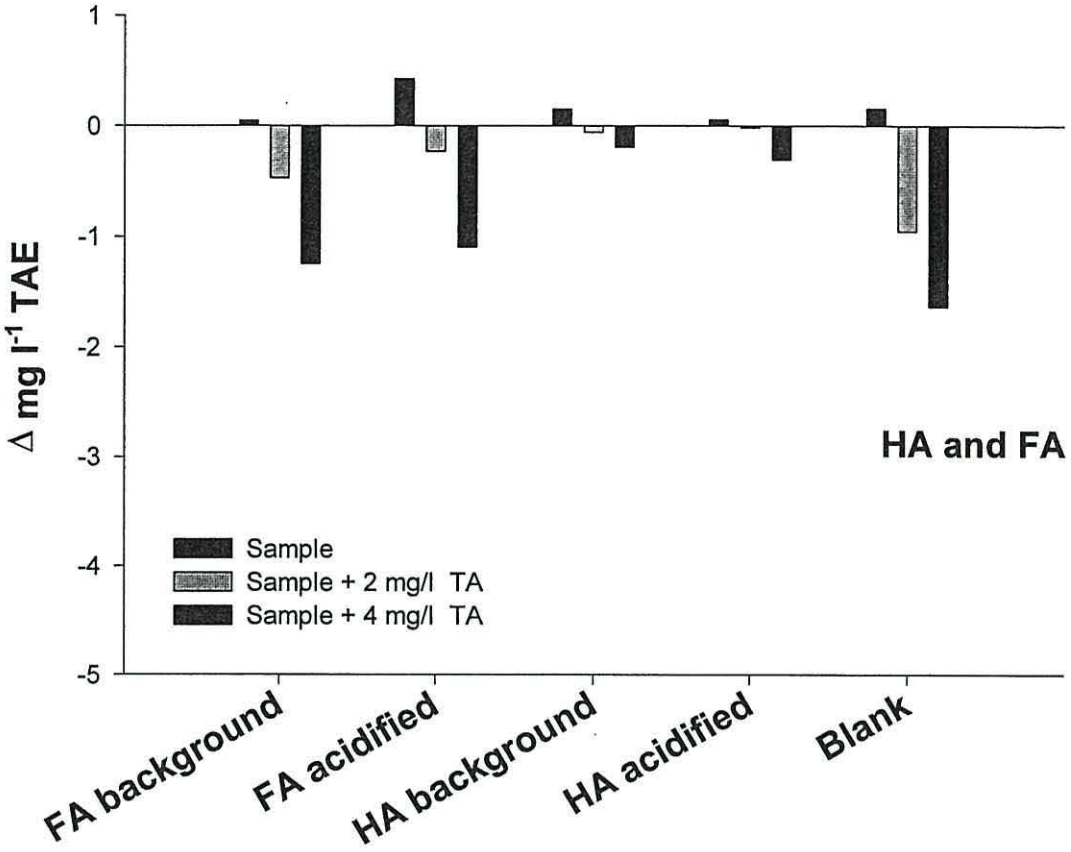


Figure 3.9: Result of Folin-Ciocalteau assay on supernatant of protein precipitation assay for humic and fulvic acid sample from the lake Skjervatjern experiment. Shown are the differences between the Folin-Ciocalteau assay on the sample, sample + 2 mg TA l^{-1} and sample +4 mg TA l^{-1} on the untreated sample to the supernatant after the protein precipitation. Negative values indicate polyphenolics removed from sample. Positive values indicate response of Folin-Ciocalteau assay to non-phenolic compounds.

3.4 Discussion

The methodology employed in this study used existing theories of humification (Stevenson 1994) to elucidate chemical and biochemical characteristics of DOM (Figure 1.2). As opposed to working with humic substances, the work was performed on natural matrices, allowing the investigation of the environmental parameters influencing microbial and chemical transformations of plant debris.

The application of PCA to the data enabled a distinction to be made between chemical characteristics and environmental parameters (Vega et al. 1998). Conductivity and pH were part of the environmental parameters as expected due to their influence on the water chemistry (Jenkins and Suberkropp 1995). Fluorescence also formed part of the environmental parameters. Working at excitation 350 nm and emission 445 nm was thought to determine the fluorescence of the coumarin derivative of caffeic acid, a lignin degradation product (Larson and Weber 1994). Only Cors Goch displayed high fluorescence, substantiating an allochthonous origin from the vegetation on site. The actual transformation of caffeic acid seemed to depend upon environmental parameters according to the results of PCA (Table 3.2). Equally surprising was the UV absorbance at 200 nm forming part of Eigenvalue 2, the environmental conditions. This absorbance was thought to derive from a high protein content of the sample. Freeman et al. (1990) described the Aber as an oligotrophic mountain stream and the Clywedog as a eutrophic lowland river. Investigating the effect of high molecular weight DOM upon microbial metabolism on a seasonal basis, little difference between the two rivers was found (Freeman and Lock 1992). The marked difference between the two rivers in this study was in the UV absorbance at 200 nm. Being eutrophic, the Clywedog received a higher carbon load in comparison to the Aber, while showing similar concentrations of TOC (Table 3.1). It was thus suspected that a higher turnover rate could be found in Clywedog for which a higher enzyme content was necessary. Supporting evidence for this hypothesis was found in the protein precipitation assay in

the Clywedog, where an added tannic acid spike of 2 mg l^{-1} was kept in solution, by the protein present in the sample in all seasons investigated (Figure 3.6).

Six of the assays in the fingerprint were related to the chemical characteristics of DOM: TOC, total phenolics, polyphenolics, UV absorbance at 240 nm, colour and complexed phenol (Table 3.2 and Figure 3.5). Phenolics were implicated in DOM enzyme interactions (Spencer et al. 1988, Freeman et al. 1990, Wetzel 1992, Wetzel 1993, Pillinger 1994). There were two assay that oxidised phenolics leading to a coloured species of the oxidant: the Folin-Ciocalteau assay working at alkaline pH and the Prussian blue assay at acid pH. Being redox based, the assays were non-specific for phenolics (Serrano 1992, Gallet and Lebreton 1995, Ohno and First 1998). In the protein precipitation assay the removal of total phenolics was investigated. A positive response of the Folin-Ciocalteau assay was found for the blank solution containing only BSA (Figure 3.6). Thus the results gained with either assay need careful interpretation. However, applying both assays and investigating their ratio in combination with the results from the protein precipitation allowed the following conclusions: the only sample rich in polyphenolics was Cwm-y Glo (Figure 3.6). Conwy and Clegir were low in phenolics. They showed a low ratio of polyphenolics to total phenolics and little precipitation as polyphenol protein complexes. Both sites had *Sphagnum* moss as the dominant vegetation on-site. *Sphagnum* moss does not produced phenolics as defense agents, instead it synthesises 5-keto-D-mannuronic acid (Painter 1995), a keto sugar acting via building polymer bridges between its carbonyl groups and amino groups, e. g., in protein.

The assays TOC and colour have been used extensively in ecological research. TOC represents the carbon pool and colour is important with regards to the amount of light penetrating the water. UV absorbance at 240 nm as well as colour could indicate the extent of polymerisation of DOM, but caution is necessary for any statement regarding an analyte as

heterogeneous as DOM (Appendix 1).

The complexed phenol assay was developed for this fingerprint to account for complexation. The lack of an assay to measure complexation in natural matrices was identified. During this work the amount of monophenolics was measured on the sites and thus the complexed phenol assays used a phenol concentration at naturally occurring levels. As the PCA (Table 3.2) and cluster analysis (Figure 3.6) shows, complexation is strongly correlated with the other parameters that chemically characterise DOM.

In order to discuss the biochemical activity of the DOM, the results will be compared with earlier work on three sites Aber, Clywedog and Conwy that investigated enzyme activity and microbial heat ouput of the high molecular weight fraction (> 1000 D) (Freeman et al. 1990, Freeman and Lock 1992). On a seasonal basis an increase in heat output was found for the Conwy throughout the year and for Aber and Clywedog in winter. The lipase assay gave the same response for the Aber and Clywedog, measuring slight inhibition in winter. The Conwy showed inhibition of lipase in winter and summer. The significant effect of the DOM upon microbial metabolism in river biofilms as determined for the Conwy by Fremann and Lock (1992) is better explained by the result of the protein precipitation, where throughout the year a strong complexing of protein and/or tannic acid was found. Regarding the other sites studied, it was noted that although a distinctly different pattern was obtained for Conwy, Cwm-y Glo, Cors Goch and Clegir in the protein precipitation, all four sites showed inhibition of lipase in summer. It is thus suspected that there are two possible causes for enzyme inhibition to occur. The complexing of enzymes by polyphenolics is already accepted (Wetzel 1991, 1992, 1993) and was displayed at Cwm-y Glo. Additionally there is another possible explanation based on the polymer bridges described earlier. Supporting evidence for that theory is found at the sites Conwy, Cors Goch and Clegir, where the DOM protein interaction led to soluble complexes. During summer a positive Δ value was obtained for Conwy and Clegir (Figure 3.6). Cors Goch showed a positive Δ value in

spring and summer. Lipase inhibition was measured for those sites when they showed soluble DOM protein complexes (Figure 3.7).

Humic and fulvic acid samples were also submitted to the same fingerprinting methodology and biochemical assays. In the fingerprint for the humic and fulvic acid samples, a similar pattern to Clegir was found (Figure 3.8), substantiating the estimated 50 % of DOM being of a recalcitrant nature (Thurman 1978). Differences between the humic and fulvic samples were found in the protein precipitation assay where a higher Δ value was obtained for fulvic acids. Humic acids showed the occurrence of soluble DOM protein complexes described earlier (Figure 3.9). The most surprising result was obtained in the lipase assay. While the freshwater samples had a maximum inhibition of 30 %, both humic and fulvic acid showed 90 % inhibition (Table 3.5). This inhibition is thought to originate from the conversion of phenolic groups to quinones at alkaline pH (Appel 1993) occurring during the processing of natural water samples into humic substances (Thurman and Malcolm 1981). Quinones themselves had been implicated with stronger biochemical activity than phenols (Pillinger et al. 1994). This hypothesis could not be proven with the experiments performed during this study, because it would require work to be carried out on filtered samples and humic and fulvic acids from the same site. Additionally the amount of humic and fulvic acid in the natural samples was not known. However, the fact that a similar 'fingerprint' had been obtained for the humic and fulvic acid samples and for the Clegir site, could be used as justification for comparing the results obtained in the lipase assay.

3.5 Conclusions

A 'fingerprinting' technique to measure DOM in natural matrices using simple assays was introduced and applied to freshwater samples. A distinct pattern was found for each site, varying over the year. The biochemical activity of DOM was studied using protein precipitation and the influence upon lipase activity. The results gained for three of the sites were compared with earlier

work regarding biochemical activity of DOM in biofilm matrices and comparable effects were measured. The biochemical assays allowed a differentiation between a toxicological cause, polyphenolics, and a physicochemical phenomenon, complexation. The 'fingerprinting' technique was equally applied to humic and fulvic acid samples and found to give similar responses, apart from lipase activity, which was inhibited to a larger extent.

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Chapter 4: Controlled Decomposition of Cellulose, Chitin and Lignin

4.1 Introduction

The decomposition of plant debris leads eventually to the production of DOM (Dissolved Organic Matter). Plant and crustacean debris consists largely of skeletal biopolymers, in particular the polysaccharide cellulose, the amino sugar chitin and the polyphenolic lignin. In order to investigate the contribution of the different pathways during humification (Stevenson 1994) and the importance of the nature of the starting material for the chemical characteristics of DOM, a decomposition study under controlled conditions was performed.

Chitin is a skeletal biopolymer mainly produced in marine environments. Boyer and Kator (1985) estimated the annual production of chitin by planktonic copepods to be about 10^9 tons $year^{-1}$. In addition chitin forms part of fungal cell walls, thus its total annual production could approach that of cellulose.

The skeletal biopolymers cellulose and lignin occur together in vascular plants, and are thus representatives of terrestrial environments. The annual production of cellulose is estimated to be about 10^{11} tons $year^{-1}$ (Leshine 1995). No figure for the annual production of lignin was found, but it is thought that on average 25 % of the weight of vascular plants derives from lignin (Hon and Hirashi 1991, Aber and Mellilo 1991, Harwood and Parales 1996). Lignin is thus the most abundantly produced aromatic biopolymer.

The current decomposition study aimed to investigate the effect of the different chemical properties of the starting materials. Cellulose is a polysaccharide consisting of joined glucose units. Chitin is a polysaccharide containing nitrogen. Cellulose and chitin are very similar in structure and in their ecological function, as providers of physical strength, and both substrates are insoluble in water. Lignin is a polyphenolic material and is

found in woody plants tightly bound to cellulose because it is thought to protect against external physical and chemical stresses (Mann 1996).

Degradation studies on cellulose, chitin and lignin had been performed prior to this study. Waksman (1946) performed lignin degradation studies and he suspected the recalcitrance of lignin to be a reason for its involvement during humification. The methodology employed used the determination of mass loss as an indicator of percentage degradation. The most recently used methods for determining mass loss utilise ^{14}C-labelled substrate and measure evolved carbon dioxide (Crawford and Crawford 1976, Benner et al. 1984, Boyer and Kator 1985, Colberg and Young 1985, Scheu 1993, Boyer 1994). Chitin was found to degrade rapidly; at a concentration of 100 mg l^{-1} all chitin was degraded in 14 days in river water (Boyer and Kator 1985, Boyer 1994). Cellulose was found to degrade more slowly with only 41 % of cellulose as part of plant litter having degraded in 150 days (Scheu 1993). In a long-term decomposition study of beech leaves and fir needles, 35 and 20%, respectively, of the lignin initially present remained after 7 years (Rutigliano et al. 1996). Benner et al. (1984) radio-labelled lignin of four phylogenetically different wetland plants and found 4% of [^{14}C-lignin]-lignocellulose mineralised after 10 days with mineralisation rate decreasing logarithmically. An alternative approach to study the degradation of lignin was used by Camarero et al. (1994), where wheat straw was submitted to solid state fermentation using two white rot fungi. After the separation of lignin from the fermented straw, it was first methylated and then either pyrolysed or oxidised using alkaline copper oxide. The resulting phenolic products were separated and identified using GC-MS. The aim of their study was to investigate the differences in lignin degradation of the two fungi employed.

Based upon the results of those earlier studies it was expected that chitin would degrade completely, cellulose would require more time for complete degradation while lignin, due to its recalcitrant nature, would only partially degrade in this experiment. The methodology is targeted at DOM and even

though the substrate might completely degrade, as it is expected for chitin, conversion of the carbon bound within the substrate into humic substances is expected. The aim was to study the genesis of DOM in order to investigate if its chemical characteristics depended upon the carbon quality of the substrate used.

The variables time period, 'light' and 'dark' treatment of the controlled decomposition study were related to natural conditions in temperate climates. The autumnal shedding of leaves provides a large input of dead matter in terrestrial environments. A variety of possibilities for the subsequent location of the fallen leaves exist, ranging from remaining on the surface were algae colonisation occurs under light provision, to burial in sediments of water bodies. The choice of length (15 month) for the time period employed was based upon the time lag between autumnal leaf shedding, while the 'light' and 'dark' treatments were thought to simulate the different possibilities that those leaves could encounter in the environment.

The choice of substrate for the decomposition study was twofold: apart from cellulose, chitin and lignin being the bulk carbon sources on the planet, their differences in chemical characteristics should allow for the occurrence of each pathway during humification (Figure 1.2). The pathways involving polyphenolics are accounted for by lignin as a substrate. The sugar-amine-condensation should be followed with cellulose or chitin as a substrate. The controlled decomposition study was designed to investigate the relative importance of the four pathways for humification.

According to Aber and Mellilo (1991), the addition of high-quality carbon substrates (sugars) increases lignin decomposition rates. Thus the effects of co-metabolism on the degradation of all substrates were investigated in the 'light' treatment, because on exposure over 12 weeks readily biodegradable algal biomass was synthesised in-situ. Additionally adding cellulose or chitin to lignin should equally result in faster degradation due to co-metabolism.

To conclude, an experiment was designed that investigated the importance

of the carbon-quality of the substrate for the chemical characteristics of the resulting DOM, in addition to its biochemical activity for protein precipitation and influence upon lipase activity. The substrates, the time period employed and the two treatments provided were chosen to simulate decomposition occurring in terrestrial and marine environments.

4.2 Materials and Methods

Experimental design

The experimental set-up is shown in Figure 4.1 and 4.2. Apart from unit 1, the control, the remaining 5 units were each supplied with a different bulk carbon source, cellulose (unit 2), chitin (unit 3), lignin (unit 4), lignin & cellulose (unit 5) and lignin & chitin (unit 6). The two columns in each unit were treated differently. The larger 5 l column was illuminated on a 12 h day and 12 h night cycle for the first three month, called 'light' treatment. The smaller 3 l column was kept continually in the dark, called 'dark' treatment. The columns were filled with Bristol`s medium on the 22.08.1996 and starting material was added on the 30.08.1996. According to the CHN analysis of the starting materials a final concentration of 20 mg l^{-1} carbon was added as a bulk carbon source, assuming 1% conversion. Table 4.1 shows the actual amounts added to each column.

The columns were not specifically designed for this experiment, but for an enclosed ecosystem, thus they had several inlets and outlets that served no purpose for this study. The air was pumped through a frit located at the base of the column. While air was pumped through the inside column, the liquid inside was pushed upwards and descended between the inside and outside column. Thus the inlets, outlets and the area below the frit were dead volumes. To assure aerobic degradation silicon dioxide was added to the columns to settle in the dead volumes prior to the addition of the starting materials.

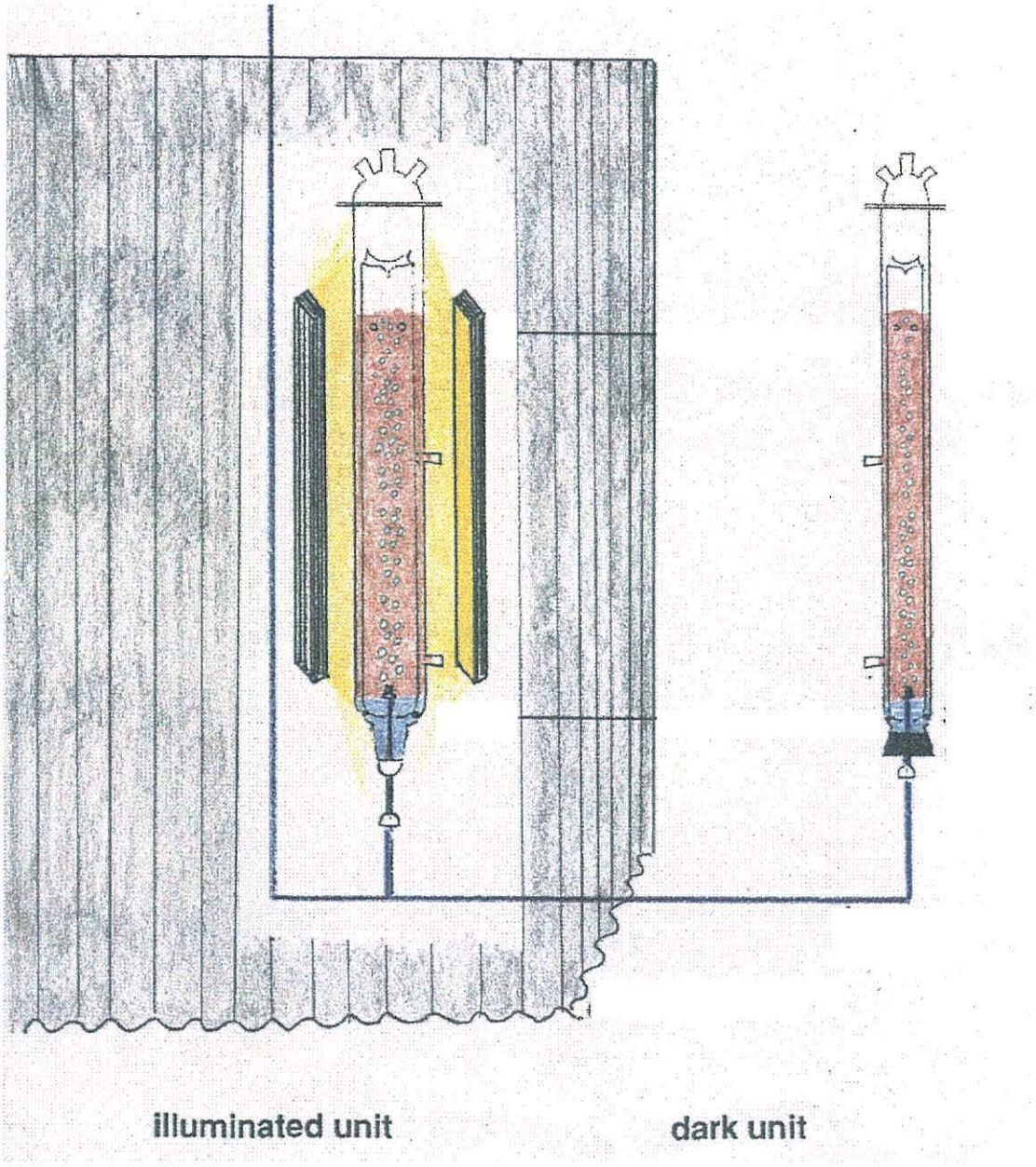


Figure 4.1: Experimental set-up for decomposition study. Shown is one unit, the column on the left received the light treatment, the column on the right received the dark treatment. Scale is 1 : 20.

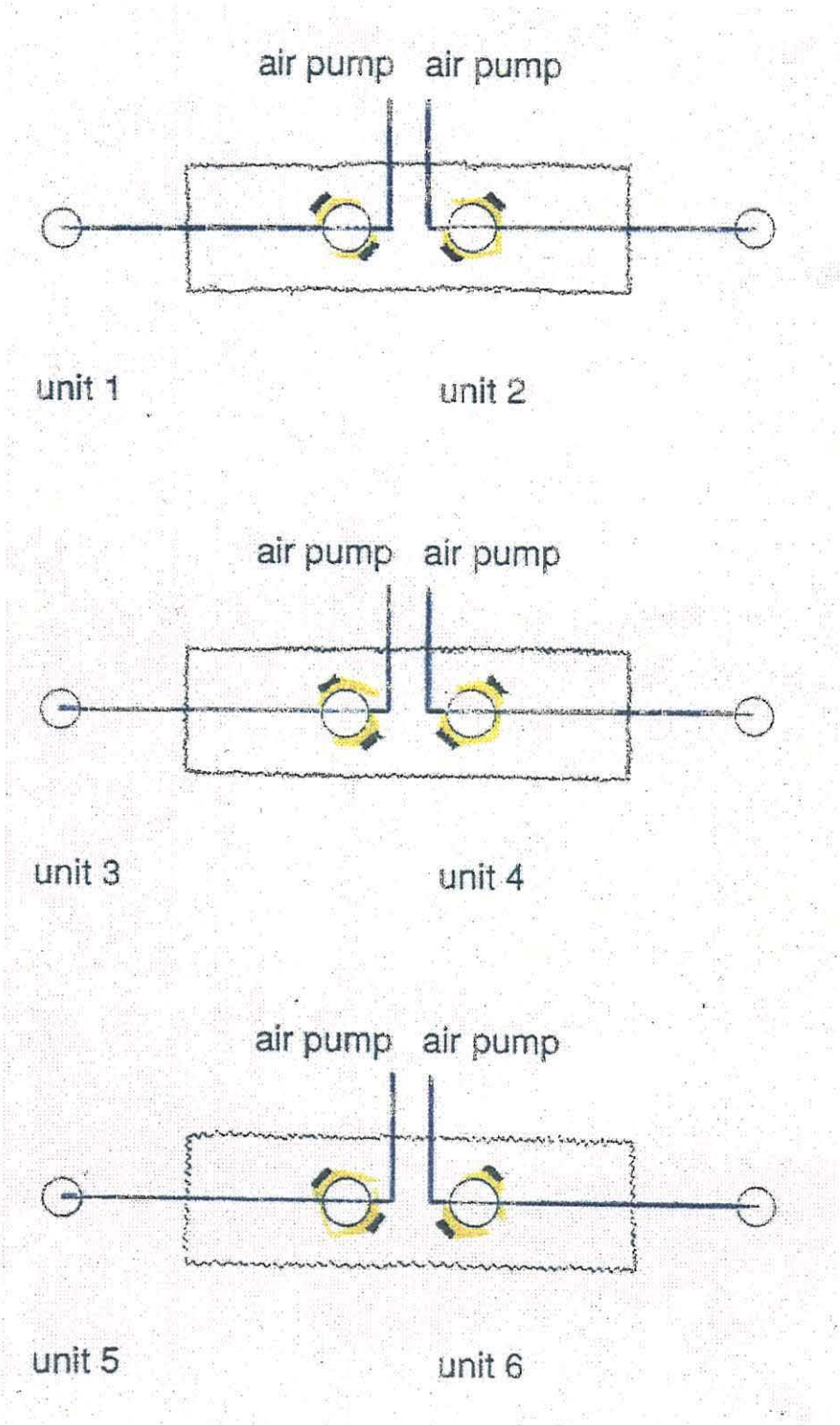


Figure 4.2: Experimental set-up for decomposition study. Shown are all six units. Scale is 1 : 10.

At the start of the decomposition study the TOC content (TOC-500 Total Organic Carbon Analyser) of the solution in each column, their total phenols content (Box 1983) and the UV absorbance at 300 nm was measured before addition of the inoculant and one week after. The experiment ran until December 1997 (15 month) when a fingerprint of the DOM was obtained. The temperature remained between 16 and 18 °C throughout the experiment.

The starting Materials: ALCELL® lignin (donated by Dr. M Lawther, Bio Composite Centre), chitin (from Sigma, practical grade from crab shells, ground to a fine powder with Heico Mill) and cellulose (from Mackerey and Nagel & Co, for thin layer chromatography, average particle size 10 μm) were used. A CHN analysis of the starting materials was obtained with a Carlo Erba CHNS-O EA 1108 Elemental Analyser. Samples were submitted for ^{13}C-NMR at the Department of Chemistry, University of Wales, Bangor. Mineral content was determined using a Spectrace 900 portable XRF.

A standard inorganic growth medium (Bristol's) was used for the matrix: Stock solutions A: 25 g l^{-1} $NaNO_3$, 7.5 g l^{-1} $MgSO_4$*7 H_2O, 2.5 g l^{-1} $CaCl_2$*2 H_2O, 17.5 g l^{-1} KH_2PO_4, 7.5 g l^{-1} K_2HPO_4, 2.5 g l^{-1} NaCl, stock solutions B: 50 g l^{-1} EDTA (Ethylene Diamine Tetra Acetic acid) and 31 g l^{-1} KOH, 4.98 g l^{-1} $FeSO_4$*7 H_2O in 0.1 % conc. H_2SO_4, 11.42 g l^{-1} H_3BO_3, 8.82 g l^{-1} $ZnSO_4$*7 H_2O and 1.44 g l^{-1} $MnCl_2$ and 0.71 g l^{-1} MoO_3 and 1.57 g l^{-1} $CuSO_4$*5 H_2O and 0.49 g l^{-1} $Co(NO_3)_2$*6 H_2O (all chemicals used were AnalAr grade obtained from Merck). The medium was made up of 1% stock solutions A and 0.1 % stock solutions B.

A broad spectrum inoculate was derived from oak leaf mulch, pine needle mulch, sycamore leaf mulch, a partially rotten crab, a mouldy Fe-Chitosan complex (donated by S. Runacres), newspaper mulch with associated fungi and two different compost heaps, one mostly grass cuttings (donated by M.A. Lock), the other mostly vegetable waste and animal manure. About 5 ml of each was mixed and macerated until a dark slurry was obtained. The

slurry was spread out in a petri dish and left in contact with room air until four days later, when 2 ml of it were added to each of the light columns and 1 ml to each of the dark columns.

Analysis of DOM after 15 month of controlled decomposition

Fingerprint of Products

The same methods were used as described in Section 3.2. Fresh samples were taken for each assay and filtered through Whatman GF/F.

Lipase Assay

Solutions used were **phosphate buffer** pH 7 (0.01 M), made up from 50 ml 0.1 M KH_2PO_4 and 29.1 ml 0.1 M NaOH in1000 ml. **Lipase solution** (0.1 gl^{-1}) made up from porcine pancreatic lipase (crude Type II from Sigma) in buffer. **Substrate solution** Fluorescein diacetate (from Sigma) was dissolved in warm acetone and diluted with buffer to final concentration of 10 µM. **Product solution** Fluorescein (from Sigma) was dissolved in acetone and diluted with buffer to concentrations of 40, 80 and 120 nM.

Procedure: For the calibration 1.5 ml of each sample, 0.75 ml product solution for each concentration and 0.75 ml lipase solution were used. For *lipase absent* 1.5 ml sample, 0.75 ml buffer and 0.75 ml substrate solution were used. For *lipase present* 1.5 ml sample, 0.75 ml substrate solution and 0.75 ml lipase solution were used. Buffer solution was used as a blank. The cuvettes were inverted and left for 60 minutes. The fluorescence was measured at excitation 490 nm, slit width 5 nm, and emission 520 nm, slit width 15 nm.

The fluorescence measured after 60 minutes was converted into concentration according to the calibration for each sample. The amount of fluorescein produced, *lipase present* less *lipase absent*, for the sample was divided by the value for the buffer blank to obtain a ratio. Triplicate samples were run.

Statistical Analysis

Minitab release 11.21 was used for statistical analysis (2 sample t-test and cluster analysis). Cluster analysis was performed with standardised data using complete linkage and squared Pearson distance measure. The data set used for the cluster analysis encompassed only the results provided by the decomposition study.

Table 4.1: Amounts of starting material used in decomposition study.

	Big column (volume 5 l)	Small column (volume 3 l)
Cellulose	25 g	15 g
Chitin	25 g	15 g
Lignin	15 g	10 g
Lignin & Cellulose	8 g lignin + 12 g cellulose	6 g lignin + 8 g cellulose
Lignin & Chitin	8 g lignin + 12 g chitin	6 g lignin + 8 g chitin

Table 4.2: CHN content of starting materials used in decomposition study.

	Nitrogen (%)	Carbon (%)	Hydrogen (%)
Cellulose	0.000	41.686	5.993
Chitin	8.721	42.152	6.808
Lignin	0.000	66.188	6.527

Table 4.3: Mineral content of starting materials used in decomposition study.

	Cellulose	Chitin	Lignin
Titnium	335 ± 74 mg kg^{-1}	393 ± 83 mg kg^{-1}	-----
Strontium	86 ± 13 mg kg^{-1}	211 ± 21 mg kg^{-1}	75 ± 12 mg kg^{-1}
Zirconium	26 ± 5 mg kg^{-1}	< LoD	25 ± 5 mg kg^{-1}
Molybdenum	20 ± 6 mg kg^{-1}	44 ± 8 mg kg^{-1}	< LoD
Lead	90 ± 27 mg kg^{-1}	< LoD	< LoD
Calcium	----	37927 ± 595 mg kg^{-1}	-----
Iron	< LoD	11522 ± 758 mg kg^{-1}	< LoD
Rubidium	< LoD	890 ± 51 mg kg^{-1}	----

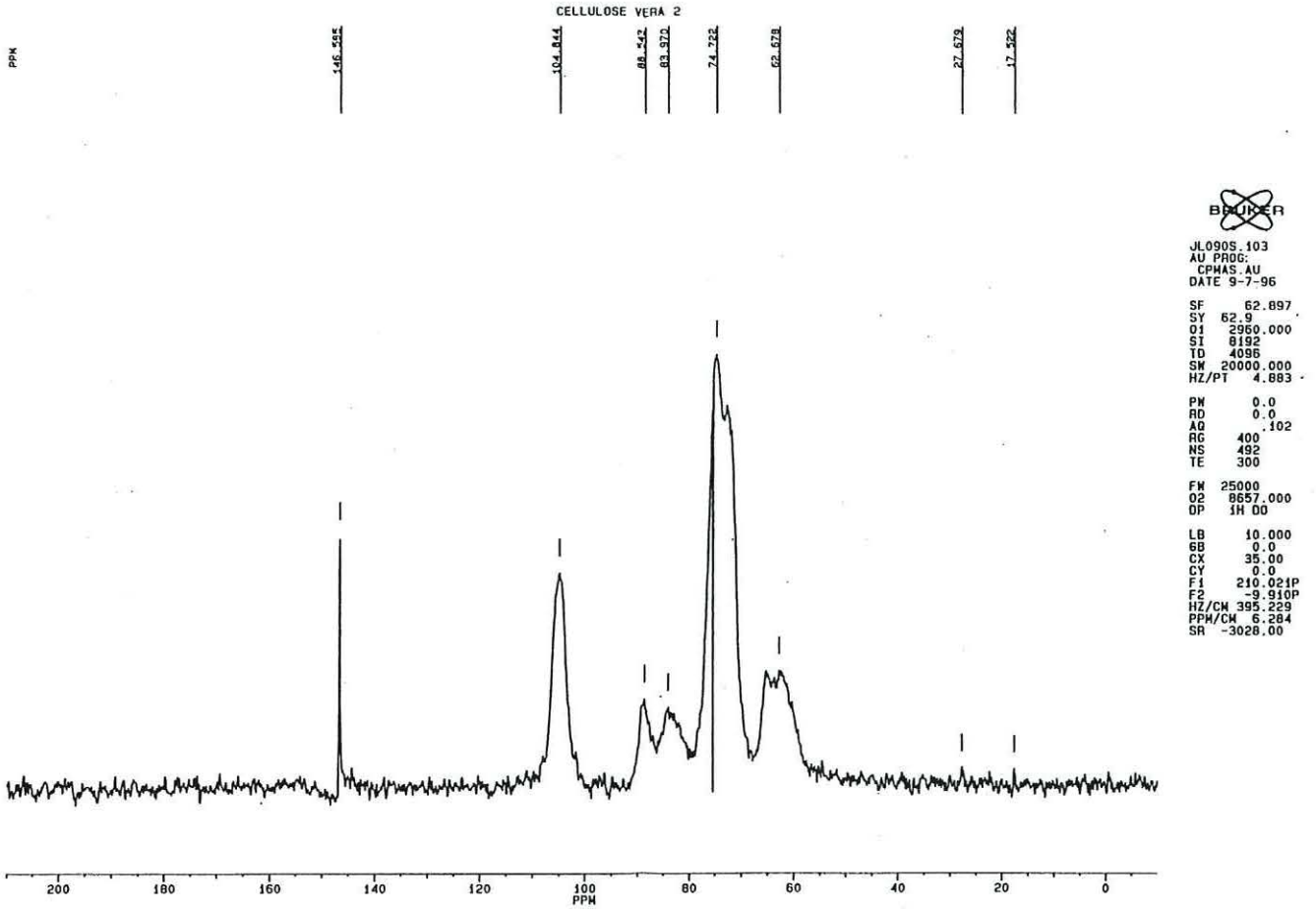


Figure 4.3a: ^{13}C-NMR of cellulose used as starting material for the decomposition study.

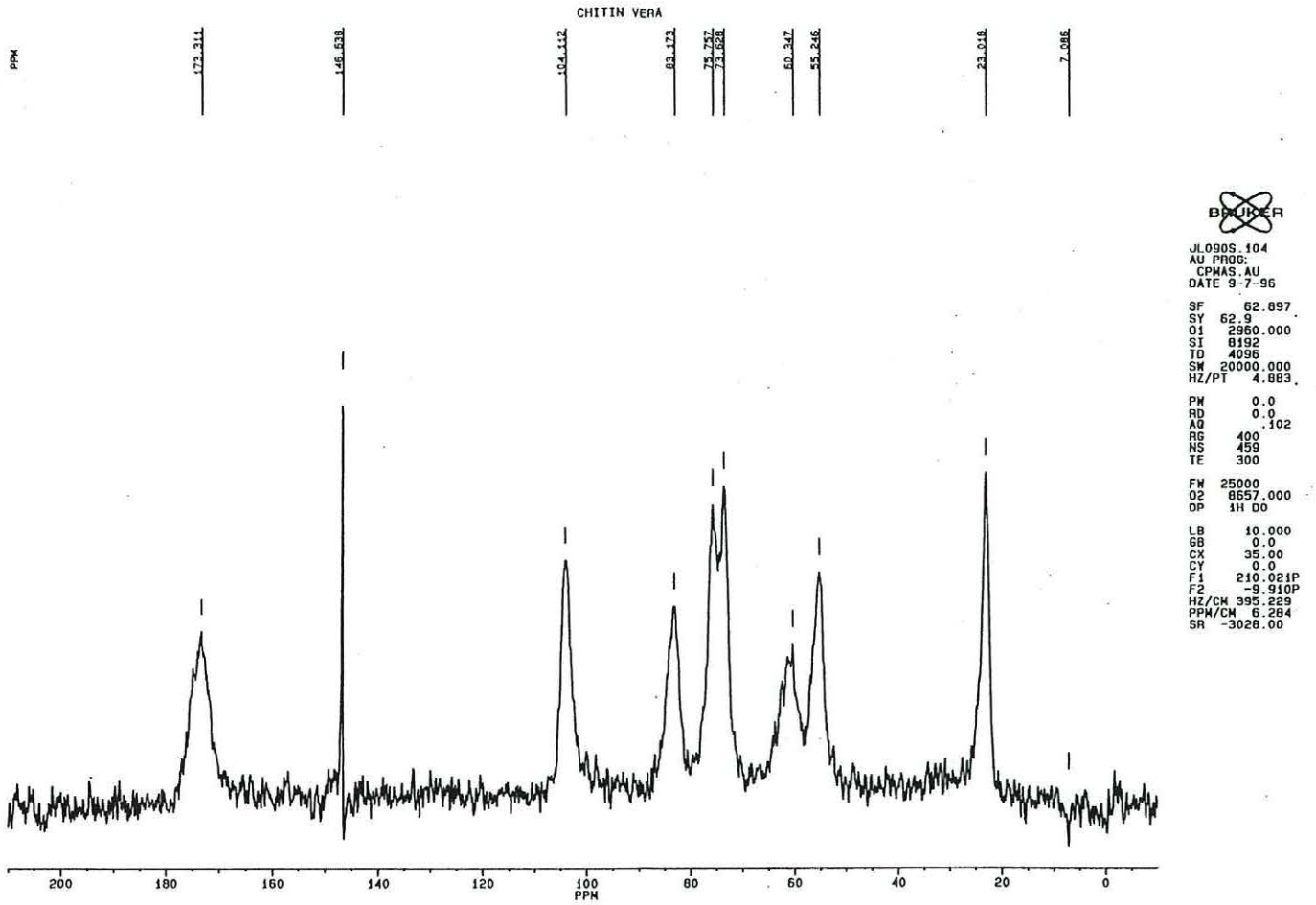


Figure 4.3b: ^{13}C-NMR of chitin used as starting material for the decomposition study.

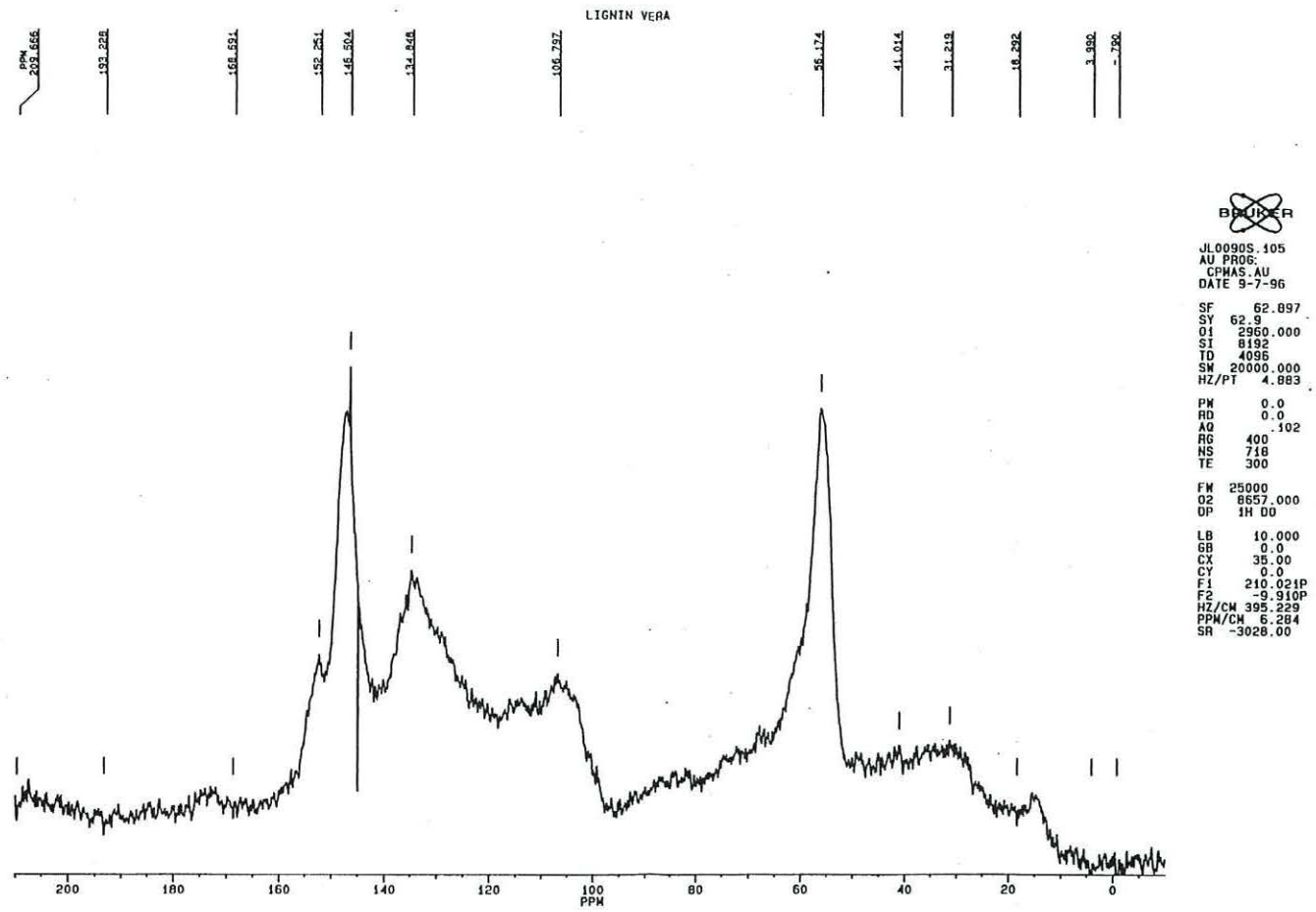


Figure 4.3c: ^{13}C-NMR of lignin used as starting material for the decomposition study.

4.3 Results

4.3.1 Analysis of Starting Materials

Cellulose did not contain nitrogen (Table 4.2). The mineral content was high in titanium, thought to originate from titanium dioxide used as a bleaching agent in its preparation. The content of the other detected elements, strontium, zirconium, molybdenum and lead was below 100 mg l^{-1} (Table 4.3). Chitin did contain nitrogen and had a high carbon to nitrogen ratio (Table 4.2) in addition to a high mineral content, especially calcium and iron (Table 4.3). The chitin used was from ground crab shells and in comparison to the other two starting materials it was relatively impure. The lignin used in this study was relatively pure. No trace of nitrogen was detected (Table 4.2) and the mineral content was restricted to strontium and zirconium (Table 4.3).

In addition to CHN and mineral content a ^{13}C-NMR was obtained for the starting materials (Figure 4.3 a, b and c). According to Hayes (1997) aromatic carbons show resonances from 112 to 148 ppm. Lignin showed a broad spread between 106 to 152 ppm, while chitin and cellulose did not show any peaks in that region confirming their non-aromaticity. The aromaticity of lignin was equally inferred from the CHN analysis (Table 4.2), where the high carbon to hydrogen ratio was indicative of an aromatic nature. Additionally lignin showed a distinct methoxyl peak at 56 ppm. The region between 65 to 110 ppm was considered the saccharide region of the spectrum (Hayes 1997). Chitin and cellulose showed distinct peaks in those areas while lignin showed low resonances. In addition chitin showed a peak at 173 ppm originating from the carbonyl carbon of the amide functionality.

4.3.2 General Observations

At the onset, the columns were filled with clear nutrient solution, silicon dioxide was added to settle in the dead volumes and the columns were left for one week. The starting material was then added. Cellulose was a white

powder. Chitin had a crab shell colour, light orange. Lignin was chocolate brown. After one day only, differences between the starting materials could be seen in the solutions. The only aromatic substance, lignin, showed a brown colouring due to lignin dissolving partially in the nutrient solution. Cellulose and chitin were not water-soluble. The cellulose columns contained a white suspension, the chitin columns an orange suspension.

Foaming was a major problem first for the lignin containing columns, occurring within the first three days, and after about 2 weeks for the chitin containing columns as well. After three weeks foam collectors were attached to the top of the columns, but the exact loss of substrate was not determined. It was thus not possible to give an accurate mass balance of carbon. Because of the foaming, surface tension was measured after 15 months but no difference was found between any of the columns.

Three days after the addition of the starting materials, samples of the solution in each column were taken and TOC and total phenols content determined. The columns were then inoculated and samples were taken after another week. Although a time-series analysis of this study may have proved instructive, it was not possible on logistical grounds, because the 'fingerprinting' was developed during the decomposition study. A decision was taken to restrict sampling to about one year, a length of time considered to be significant in terms of annual pulses of dead plant material found in northern temperate zones. Once a week the columns were checked and deionized water added if necessary to keep the volume constant.

The large columns were illuminated for 12 hours each day for a period of about three months, called 'light' treatment. Algal growth was observed in the 'light' columns. Algae grew strongest in the control and the least in the lignin containing columns (visual observation) in the areas receiving direct illumination. Those were the same areas where biofilm development was noticed after a few months. The control showed brownish biofilms. Cellulose turned yellow in the biofilms and the lignin containing columns had dark

brown biofilms. After the 15 months, the structure of the lignin biofilms was lumpy as opposed to the lignin & chitin or lignin & cellulose biofilms, which showed a fine structure.

Chitin degraded so quickly that no visible biofilm development occurred. Overall the control, cellulose and chitin column gradually turned yellow while the lignin containing columns faded from dark brown to strong yellow.

On the 6.1.1997 half of the control dark and about two thirds of the control light were lost due to leakage. On the 17.3.1997, a third of cellulose dark was lost. On the 28.4.1997, the frit on lignin & chitin dark blocked and was unblocked using increased air pressure. On the 28.7.1997, the lignin & cellulose dark frit blocked. The frit could not be unblocked using increased air pressure and was taken out and cleaned with chromic acid. After reinsertion of the frit no proper connection was obtained and the contents were lost subsequently. The analysis of the decomposition study products began on 2.12.1997.

4.3.3 Chemical analysis before, after 1 week and after 15 months of the addition of the inoculant

TOC content

Chitin degraded the most rapidly as illustrated in Figure 4.4. The amount of TOC in the column increased tenfold in one week. Equally the lignin & chitin containing columns showed a significantly higher amount of TOC after one week if compared with the lignin & cellulose columns. Apart from chitin 'dark', all other units showed higher TOC levels for the 'light' treatment after 15 months. As was visually noticed by the darker colour of the solution, the lignin containing columns initially showed higher levels of TOC compared to control, cellulose and chitin. The degradation of lignin was the slowest of all starting materials. After 15 months, the TOC content was still 70.4 ± 12.1 mg l^{-1} for the 'dark' column and 79.0 ± 12.1 mg l^{-1} for the 'light' column. In the presence of a high carbon substrate, cellulose or chitin, the TOC levels were reduced to 43.0 ± 3.5 mg l^{-1} in the 'dark' and 50.9 ± 4.4 mg l^{-1} in the 'light'

in the presence of chitin. After 15 months, the lignin & cellulose column 'light' had 54.8 ± 9.1 mg l^{-1} TOC.

Total Phenolics content

Figure 4.5 shows the total phenolics content of the columns over time. While the levels of total phenolics for the lignin 'dark' treatment decreased, the level increased for the lignin 'light' over time. The chitin containing columns showed the same effect, more total phenolics were present after 15 months for the 'light' treatment and less total phenolics for the 'dark' treatment when compared to the levels determined after one week. The noticeable difference between the columns 'light' when compared to the columns 'dark' was algal growth.

UV absorbance at 300 nm

Figure 4.6 shows the UV absorbance at 300 nm for all the columns. Regarding the different response of the lignin containing columns for total phenolics, a similar pattern occurred for UV absorbance. In the 'dark' a higher absorbance was determined after 1 week than after 15 month, while in the 'light' the absorbance after 15 month was higher. Overall the lowest absorbance was seen in the cellulose dark column. This could be due to the loss of both half and about one third of the column during the experiment, dilution thus occurred. It was interesting to note the increase of absorbance for the lignin & cellulose column after 15 months, as opposed to the decrease for the lignin & chitin column after 15 months, when compared to the results after one week. This could be taken as evidence for the slower degradation of cellulose when compared to chitin.

Overall the initially expected pattern of degradation was followed: Cellulose was not fully degraded after 15 month. Chitin broke down rapidly, after one month no visual evidence of chitin was present. Lignin degraded slowly. Lignin with high quality carbon substrate added degraded more rapidly.

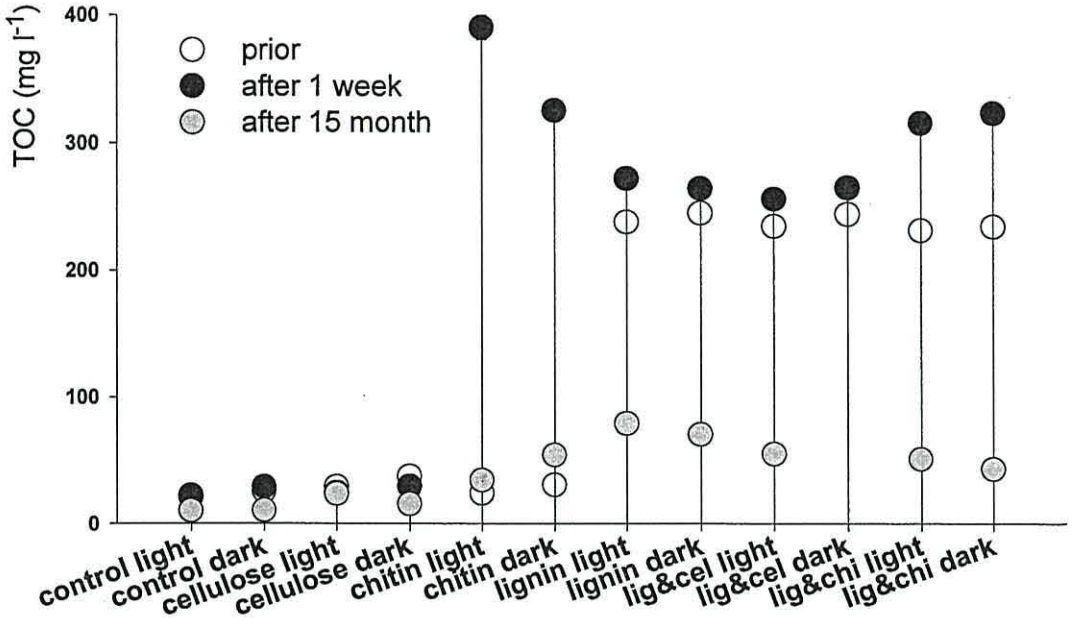


Figure 4.4: TOC (Total Organic Carbon) content in mg l^{-1} of columns over time. Shown are the TOC levels determined prior, one week and fifteen months after the addition of the broad-spectrum inoculant.

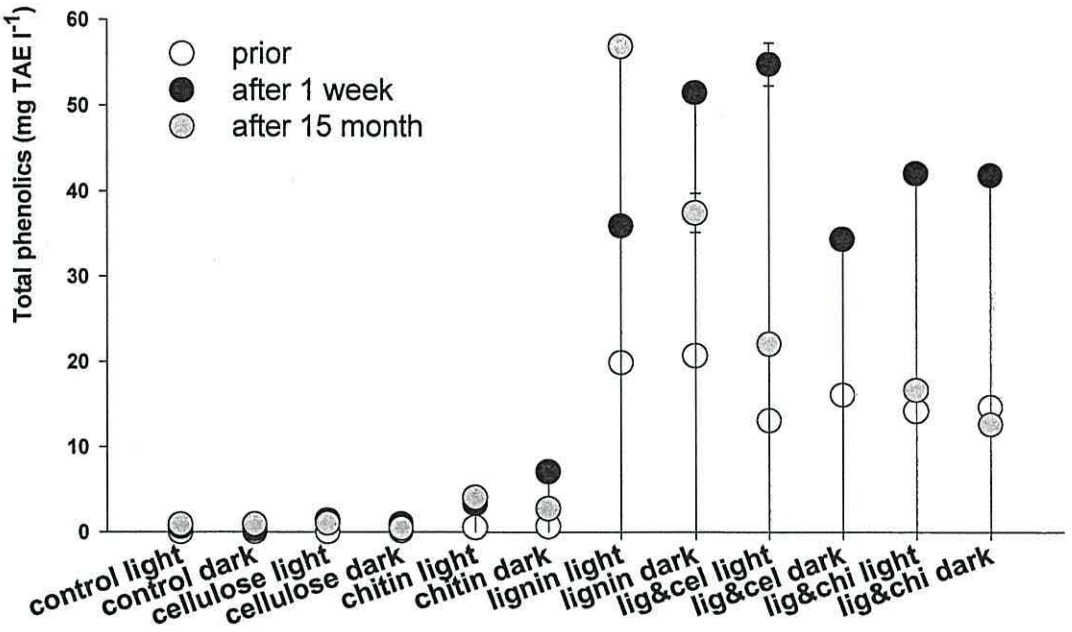


Figure 4.5: Total phenolics content of the columns over time. Shown are the TAE (Tannic Acid Equivalents) in mg l^{-1} as determined with the Folin-Ciocalteau assay prior to, one week after and fifteen months after the addition of the inoculant.

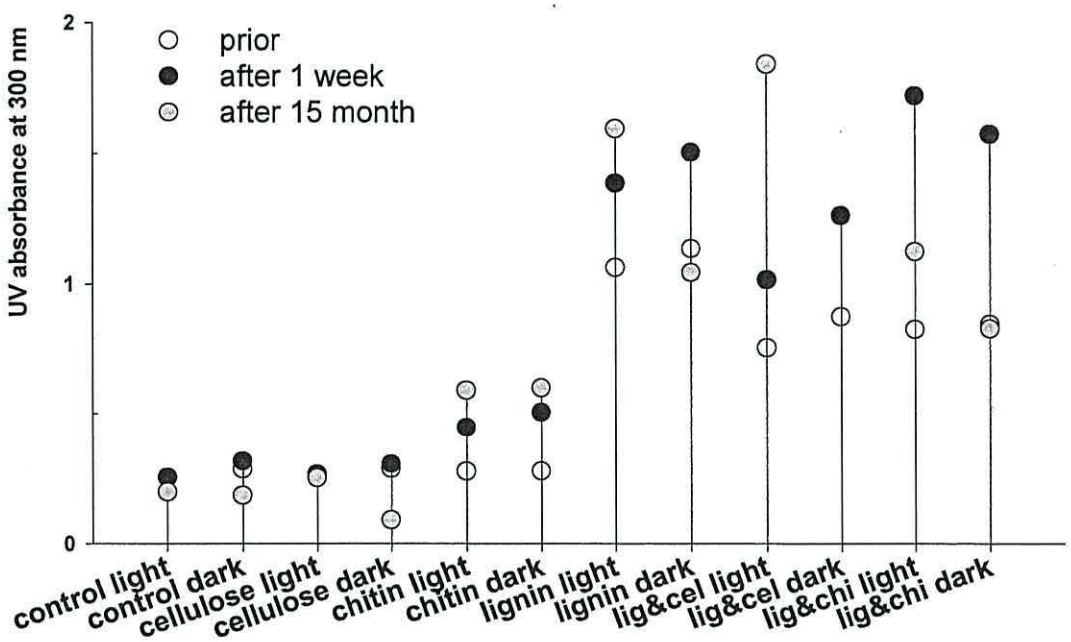


Figure 4.6: UV absorbance at 300 nm of the columns over time. Shown are the UV absorbance values prior to, one week and fifteen months after the addition of the inoculant.

4.3.4 'Fingerprints' of DOM after 15 Months of Decomposition

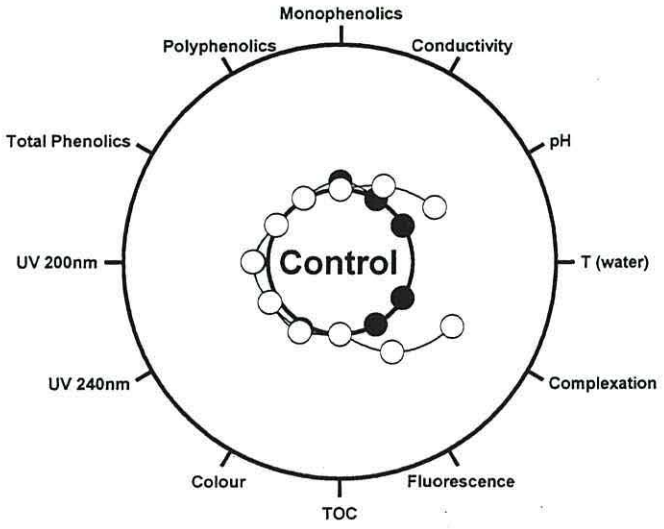
Figure 4.7 shows the fingerprints of DOM after 15 months while Table 4.4 shows the raw data. The graphics included 11 assays. The temperature was not included, because it was kept constant between 16 and 18 °C throughout the whole period. For the remaining assays the data was normalised based upon the data set presented in Table 4.4, meaning the lowest value was set at zero and the highest value was set at 1 for each individual assay. The results of the seasonal variation (Chapter 3) were not included in these results. A set of 'fingerprints' based upon the combined data sets will be given in Chapter 5.

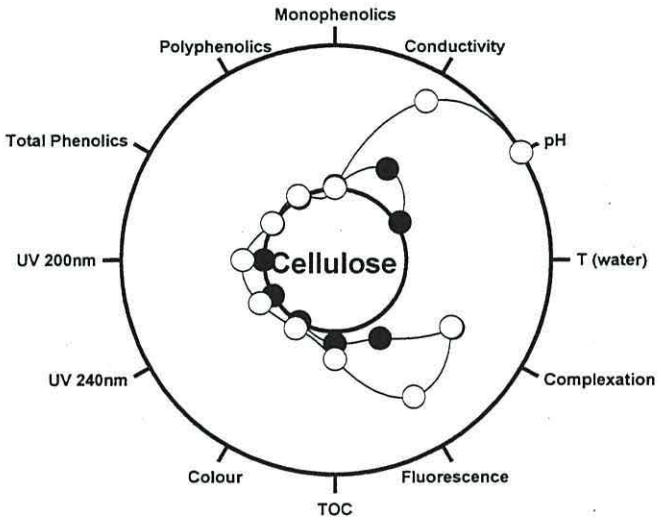
Figure 4.7 shows the 'fingerprints' for each unit. The columns kept in the 'dark' were given black circles while the results for the columns receiving the 'light' treatment were given hollow circles. The control 'dark' showed low values throughout. For the control 'light' pH, conductivity, complexation and fluorescence showed higher values when compared to the 'dark'. Due to initial illumination algal growth appeared in the column, thus a potentially microbially labile organic carbon source was generated during the experiment.

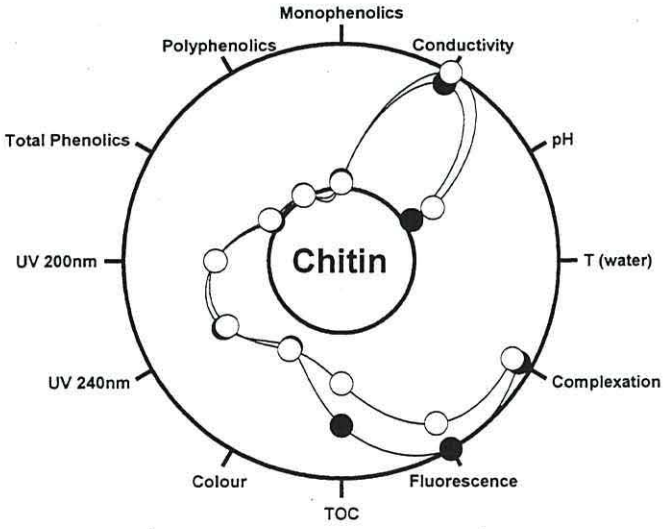
The 'fingerprints' can be described by looking at the range of values displayed, points close to the outer circle have high values, while points close to the inner circle show low values. There were four quadrants, matrix related, bulk carbon characteristics, UV absorbance and phenolics quadrant.

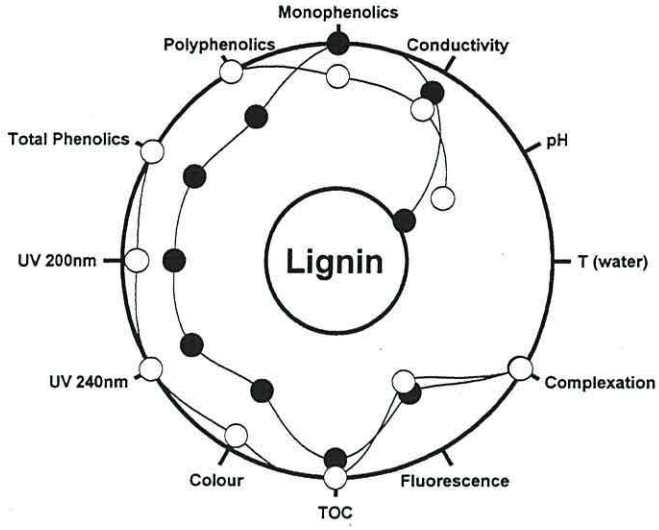
As described for the control, the columns receiving the 'light' treatment showed higher values, with the exception of TOC and fluorescence for chitin and monophenolics and conductivity for lignin. The generation of a more microbially labile organic carbon source increased the values obtained for most assays.

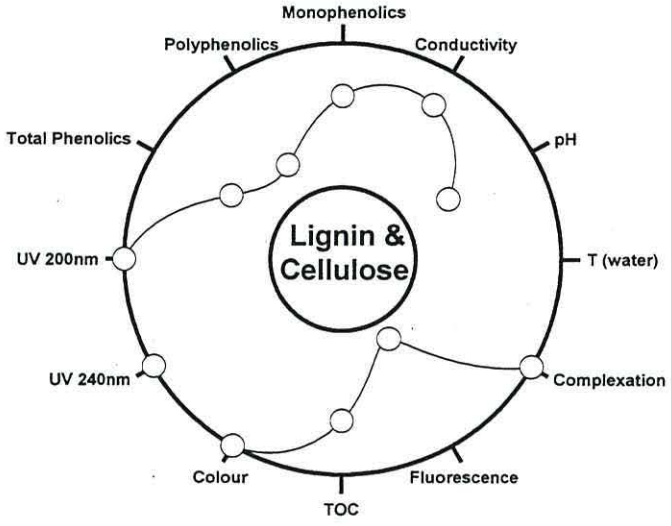
Decomposition Study











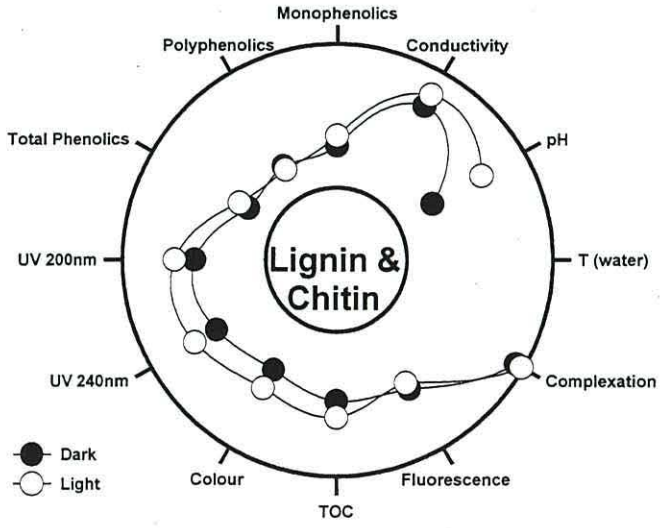


Figure 4.7: 'Fingerprints' for decomposition study. The filled circles indicate results for the columns that received the dark treatment, while the hollow circles refer to the columns that received the light treatment.

Table 4.4: Results of assays for 'fingerprint' from decomposition study. Units, where applicable, were given at the top column (PE = Phenol Equivalents, TAE = Tannic Acid Equivalents). Standard error was given in brackets.

Treatment		pH	Conductivity (µS)	Monophenolics (± se, mg l^{-1} PE)	Polyphenolics (± se, mg l^{-1} TAE)	Total Phenolics (± se, mg l^{-1} TAE)
Control	Dark	3.8	208	0.083 (0.030)	0.239 (0.108)	0.878 (0.033)
	Light	4.7	297	0.021 (0.036)	0.341 (0.118)	0.951 (0.063)
Cellulose	Dark	3.9	350	0.051 (0.015)	0.116 (0.062)	0.404 (0.014)
	Light	7.4	573	0.028 (0.089)	0.480 (0.062)	1.094 (0.012)
Chitin	Dark	4.0	1800	0.094 (0.022)	0.463 (0.072)	3.484 (0.092)
	Light	4.6	850	0.066 (0.000)	0.425 (0.124)	3.488 (0.107)
Lignin	Dark	4.0	807	1.210 (0.044)	19.41 (1.75)	35.22 (0.35)
	Light	5.1	765	1.736 (0.252)	32.06 (3.03)	48.32 (3.18)
Lignin & Cellulose	Light	6.2	719	0.632 (0.037)	7.92 (1.00)	22.88 (2.13)
Lignin & Chitin	Dark	4.8	807	0.390 (0.024)	7.31 (0.55)	13.98 (0.68)
	Light	5.0	710	0.426 (0.013)	6.55 (1.32)	17.59 (0.72)

Table 4.4: Results of assays for 'fingerprint' from decomposition study. Units, where applicable, were given at the top. Standard error was given in brackets.

	Treatment	UV Abs. 200 nm	UV Abs. 240 nm	Colour (Grade Hazen)	Total Organic Carbon (mg C l^{-1})	Fluorescence	Complexed Phenol (%)
Control	Dark	2.726	0.428	20	10.267	18.21	0.217
	Light	2.742	0.419	29	9.984	80.06	0.522
Cellulose	Dark	2.572	0.226	5	15.733	55.43	0.568
	Light	2.814	0.578	25	23.307	187.7	0.557
Chitin	Dark	3.174	1.654	82	54.088	297.9	0.922
	Light	3.171	1.556	89	34.103	241.4	0.881
Lignin	Dark	3.64	2.437	220	70.395	172.2	0.973
	Light	4.071	3.542	364	78.993	148.4	0.982
Lignin & Cellulose	Light	4.237	3.51	404	53.161	57.8	0.992
Lignin & Chitin	Dark	3.401	1.793	157	43.048	167.1	0.948
	Light	3.635	2.38	215	50.856	154.2	0.981

The different substrates supplied for the decomposition study resulted in different chemical characteristics of the DOM after 15 months, recognisable as distinct 'fingerprints' in Figure 4.7. The slow degradation of cellulose resulted in low UV absorbance and phenolics values, but high conductivity and pH. The rapidly degraded chitin showed high conductivity, complexation, fluorescence and UV absorbance at 200 and 240 nm. The aromatic substrate lignin showed high values for all assays apart from pH and midrange fluorescence. The addition of cellulose did reduce total and polyphenolics and resulted in low fluorescence. The addition of chitin to lignin showed a stronger reduction, with low values for the phenolics quadrant and less than midrange values for UV absorbance. Fluorescence increased when compared to lignin on its own.

Summarising the 'fingerprints' gained from the decomposition study, a pattern was obtained depending on the carbon source used (as starting material) and was independent of the treatment given.

4.3.5 Influence of DOM towards Lipase Activity

The activity of porcine pancreatic lipase in the presence of DOM from the decomposition study was measured. Figure 4.8 shows the results as a ratio when compared to the blank, organic carbon free water. Table 4.5 shows the raw data. Of the eleven columns, only the chitin column that received the 'dark' treatment showed a 28% stimulation of lipase activity ($p=0.031$, two sample t-test). Inhibiting for lipase activity were the lignin 'light' (21%), lignin & chitin 'light' (10%) and 'dark' (20%) samples ($p=0.002$, $p=0.005$ and $p=0.009$ respectively).

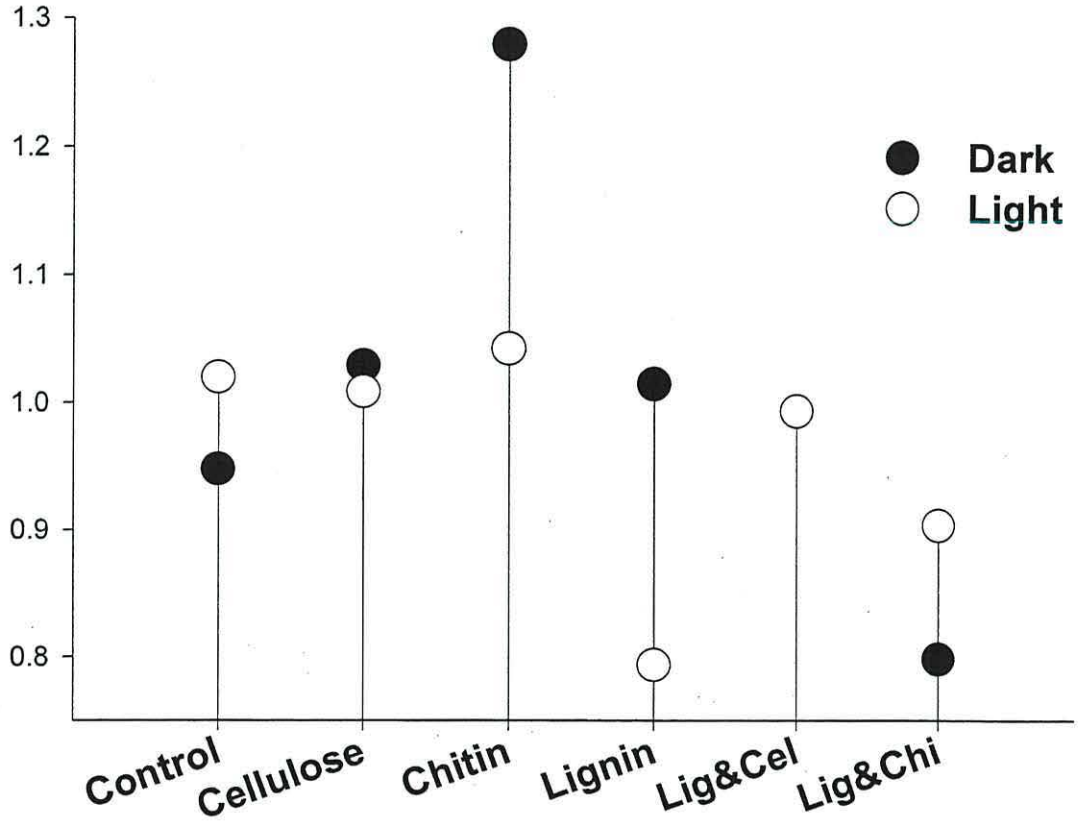


Figure 4.8: Result of lipase assay for samples from decomposition study. Shown are the ratios of product converted after 60 minutes in the presence of DOM divided by product converted in the absence of organic carbon. The dark circles refer to the columns that received the dark treatment, while the hollow circles refer to the columns that received the light treatment.

Table 4.5: Results of lipase assay. The calibration of fluorescein was determined in the presence of buffer as blank and the sample, intercept, slope and r^2 values are given. The concentration of fluorescein (nM) converted from fluorescein diacetate after 60 min in the absence and presence of lipase is given with standard error.

		Calibration			Lipase absent	Lipase present
Column	Treatment	Intercept	Slope	r^2	[Fluor.] ± se	[Fluor.] ± se
Blank	Dark	0.38	17.3	1.000	.69 ± 0.04 nM	76.3 ± 0.9 nM
Blank	Light	3.31	17.0	0.999	.48 ± 0.18 nM	71.2 ± 1.1 nM
Control	Dark	0.91	16.4	1.000	.15 ± 0.03 nM	72.3 ± 2.8 nM
Control	Light	6.26	17.5	0.999	.09 ± 0.11 nM	72.6 ± 1.4 nM
Cellulose	Dark	3.21	15.7	1.000	1.12 ± 0.04 nM	78.5 ± 2.6 nM
Cellulose	Light	46.73	16.0	0.994	0.33 ± 0.05 nM	71.8 ± 1.4 nM
Chitin	Dark	7.04	10.7	1.000	1.56 ± 0.40 nM	97.6 ± 6.6 nM
Chitin	Light	5.74	13.3	0.999	1.09 ± 0.21 nM	74.2 ± 0.9 nM
Lignin	Dark	5.81	13.6	1.000	3.38 ± 0.15 nM	77.4 ± 0.7 nM
Lignin	Light	9.13	13.0	0.999	0.71 ± 0.20 nM	56.5 ± 0.4 nM
Lignin & Cellulose	Light	13.79	11.6	0.999	1.17 ± 0.23 nM	70.7 ± 1.3 nM
Lignin & Chitin	Dark	7.33	13.7	1.000	1.10 ± 0.02 nM	60.9 ± 2.4 nM
Lignin & Chitin	Light	9.32	13.1	0.999	0.94 ± 0.09 nM	64.3 ± 1.2 nM

4.3.6 Statistical Analysis

Cluster analysis was employed for the statistical evaluation of the fingerprints. Cluster analysis is a multivariate statistical technique that assumes independence of the assays. Each datum for each column for each assay is placed in a cluster. The clusters are then joined via linking the furthest data apart. The similarity of the columns is thus recognised by the linkages between the clusters. As the dendrogram in Figure 4.9 shows, there were three 'roots' that were more similar to each. One 'root' included the control and the cellulose units, the second 'root' included the chitin and the lignin & chitin units while the third 'root' included the lignin and lignin & cellulose units.

4.4 Discussion

The aim of the decomposition study was to investigate the relationship between the quality of the carbon source and the chemical characteristics of the resulting DOM. The fingerprints obtained from the study showed a distinct pattern regarding the quality of DOM for each unit (Figure 4.7). This was confirmed in the cluster analysis where three distinct clusters were obtained (Figure 4.9). Lignin and cellulose were recalcitrant substrates, requiring more time for complete degradation than employed in the study. The lignin-containing columns receiving either treatment were found to give similar responses, being joined by the lignin & cellulose light column, while dissimilarity to the other columns was found.

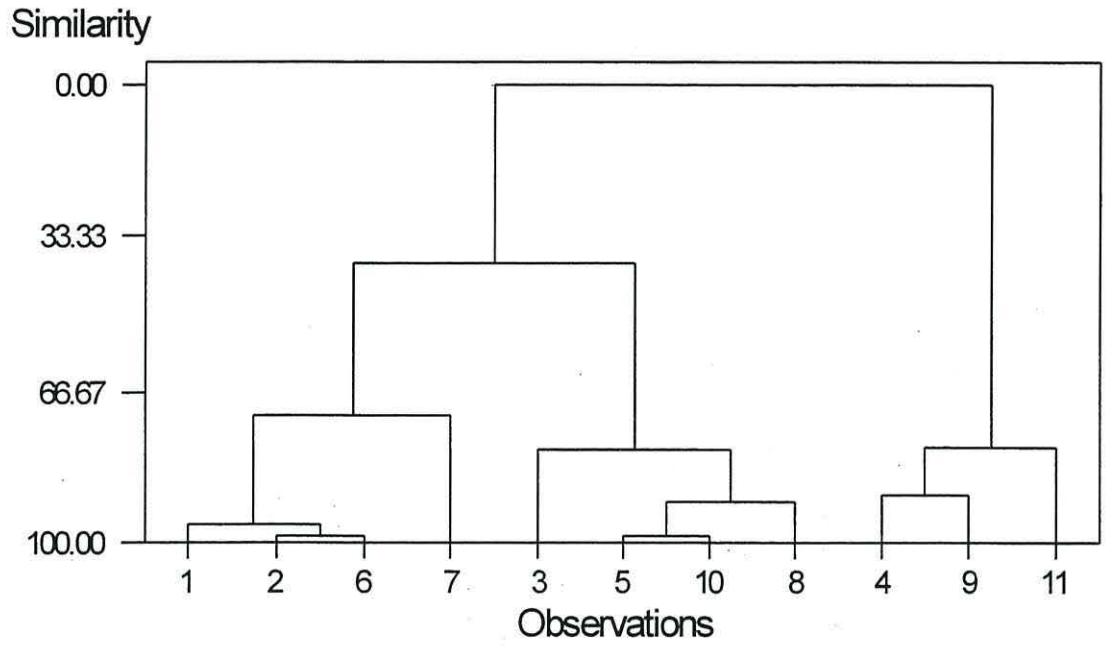


Figure 4.9: Result of cluster analysis, complete linkage and squared Pearson distance measure on standardised variables from the fingerprints, for samples from decomposition study. Numbering: 1 control dark, 2 cellulose dark, 6 control light, 7 cellulose light, 3 chitin dark, 5 lignin & chitin dark, 10 lignin & chitin light, 8 chitin light, 4 lignin dark, 9 lignin light and 11 lignin & cellulose light.

In contrast to lignin, cellulose was not aromatic and neither UV absorbance nor colour contributed to the DOM generated during the decomposition study. As seen in Figure 4.7, the fingerprints of the cellulose columns were most similar to the control column, which was confirmed in the cluster analysis (Figure 4.9). Both 'light' treatments showed increased levels of conductivity, pH and fluorescence. The absence of light with cellulose provided and the absence of a substrate provided made those three columns most similar to each other (Figure 4.9). Although the cellulose 'light' column joined that 'root', the similarity was reduced to 70 % because of conductivity and pH being much higher. The third 'root' in the dendrogram of Figure 4.9 included the chitin and lignin & chitin containing columns. The fingerprints shown in Figure 4.7 confirm the result of the cluster analysis, because they give a similar shape for all four columns.

Regarding the polysaccharides, cellulose and chitin, the main difference in the quality of the substrate was the presence of intramolecular nitrogen in chitin. Jones (1999) found the biodegradation of amino acids, also compounds containing intramolecular nitrogen, to be independent of soil type. On average 34 % of ^{14}C-labelled amino acids were respired as carbon dioxide, while the remainder was incorporated into microbial biomass.

Chitin showed a distinct fingerprint thought to illustrate high microbial activity, although no assay for microbial activity was performed. However, the conditions for the decomposition study were designed to allow high microbial activity with the provision of sufficient nutrients and a high amount of carbon substrate. Visual observation showed no biofilm development in the columns, thus all substrate provided was degraded rapidly. From the results obtained conductivity, complexation, fluorescence and UV absorbance at 200 and 240 nm were suspected to be indicators for high microbial activity, although the origin of the high fluorescence and high conductivity are uncertain. The DOM obtained from the chitin columns was low in colour and low in phenolics content, although a large amount of the carbon initially

provided was found as TOC.

The lignin containing columns showed a fingerprint at the high end of the range due to lignin being a phenolic polymer and was thus difficult to degrade (Aber and Mellilo 1995). The fluorescence values obtained from both treatments did not support the theory by Larson and Weber (1994) who suspected the fluorescence of humic substances to derive from lignin degradation products. Although the fluorescence could have been quenched by complexation, it could not explain the high fluorescence value obtained for the chitin containing columns, where overall small levels of phenolics were detected.

For the columns containing the mixtures of lignin and carbohydrate the addition of a high quality carbon substrate increased lignin degradation (Aber and Mellilo 1995). Lignin & chitin and lignin & cellulose both showed lower concentration for all phenolics assays when compared with lignin. Chitin increased lignin degradation further as illustrated by the lower amount of TOC and UV absorbance values obtained when compared with the lignin & cellulose containing columns. Unfortunately the lignin & cellulose containing column that received the 'dark' treatment was lost during the experiment. For the cluster analysis the lignin & chitin columns fell into the same cluster as the chitin column, while the lignin & cellulose column formed part of the lignin cluster, additional evidence for the higher substrate quality of chitin and the importance in providing intramolecular nitrogen for decomposition.

The 'fingerprints' obtained for the lignin & chitin columns were an overlay of the fingerprints of the substrates themselves, indicating that the chemical characteristics of DOM derived from the starting materials for decomposition.

Regarding the biochemical activity of the DOM, its influence upon lipase activity was investigated. The lignin 'light' and both lignin & chitin containing columns inhibited lipase activity. According to existing theories on the inhibition of extracellular enzymes, the phenolic character of the DOM was thought to be responsible (Wetzel 1991). Phenolics complex enzymes and

were suspected to be able to alter their tertiary structure inducing inhibition (Figure 1.4). The neutral response of the lignin & cellulose 'light' and lignin 'dark' containing column did not support this theory. However, additional supporting evidence was found in the stimulation of lipase activity for the chitin 'dark' column, because although the DOM showed strong complexation towards phenol, it contained the lowest levels of phenolics. The 'fingerprinting' methodology applied onto the products of decomposition seems to be able to distinguish between a physicochemical (complexation) and a toxicological phenomenon (phenolics content).

With regards to theories on humification the importance of lignin was substantiated. Box (1993) used the determination of phenolic groups as indicator for the presence of humic compounds in natural waters. The assay developed by Box for that purpose is the total phenolics assay and the results presented here substantiate the need for a phenolic precursor to obtain phenolic DOM. The sugar-amine-condensation pathway for humification (Figure 1.3) was not substantiated as being of importance for humification, because the cellulose-containing columns were most similar to the control columns, where no carbon source was provided.

4.5 Conclusions

A decomposition study was performed based upon the biopolymers most abundantly produced in nature. The 'fingerprints' obtained for the DOM found in the columns after 15 months of degradation showed different patterns depending upon the chemical characteristics of the starting materials. Indicators for biological activity, UV absorbance at 200 and 240 nm, fluorescence and conductivity were identified based upon the chitin containing columns and the columns receiving the 'light' treatment. However, the origins of high conductivity and fluorescence require further investigation. Existing theories about the inhibition of lipase originating from the phenolic content of DOM were substantiated. For current theories on humification the pathways including polyphenolics were found to be more important than the sugar amine condensation.

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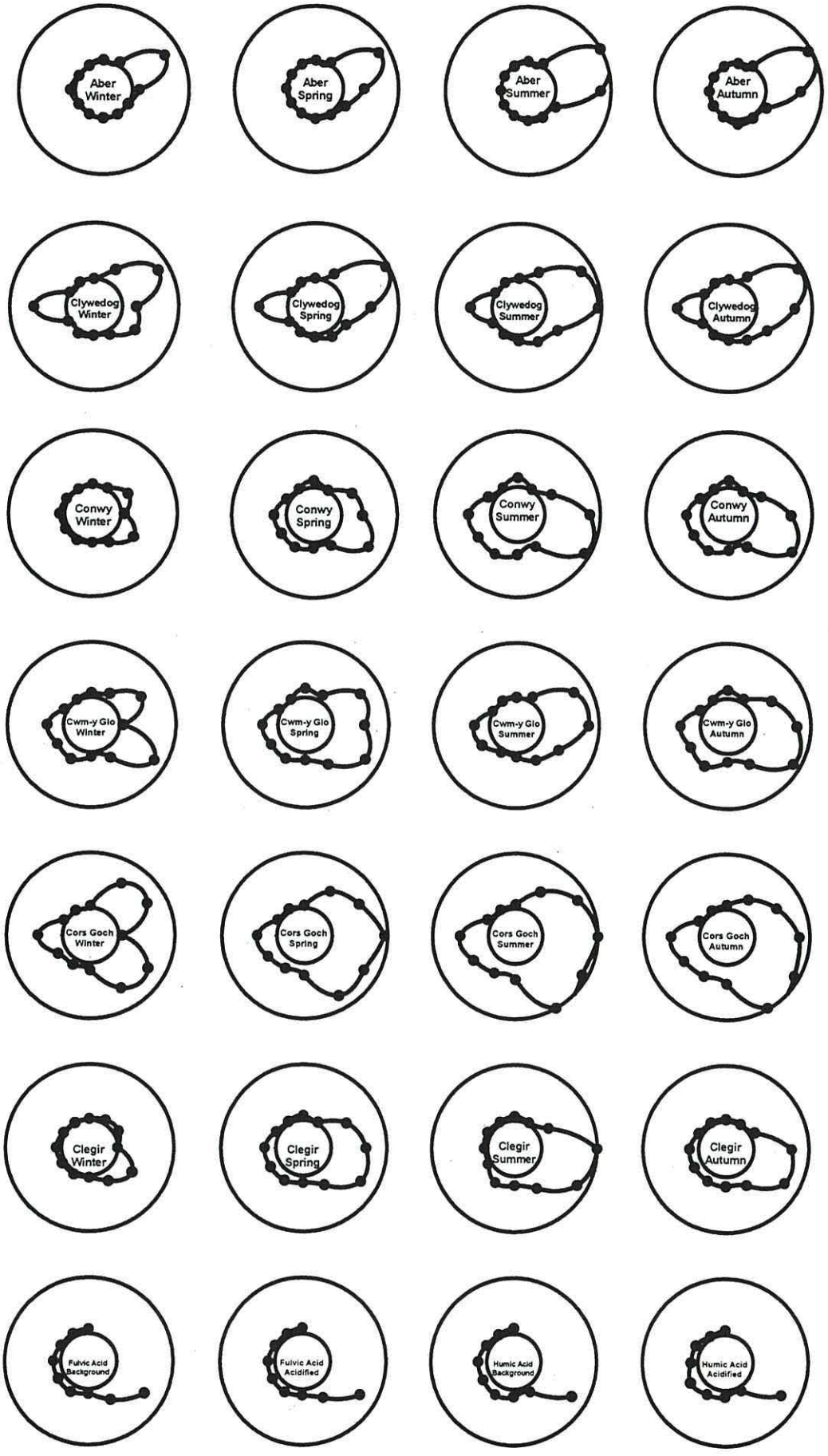
Chapter 5: Conclusions

5.1 Introduction

During this study a new methodology for the analysis of DOM (Dissolved Organic Matter) was introduced. As described in Chapter 1 the analysis was based upon the reaction mechanisms occurring during humification. Chapter 2 detailed the development of the analysis. In Chapters 3 and 4 the new methodology was used in the study of the seasonal variation of DOM on three rivers and three wetland sites and in a controlled decomposition study. In this Chapter the results from both applications will be combined into one data set and multivariate statistics will be applied.

The 'fingerprinting' methodology was designed for expanding data sets. Either for the cluster analysis or for the graphical display, the normalisation of the data was a prerequisite. Normalisation implied each datum to have a value between 0 and 1. The two separate data sets in Chapters 3 and 4 were normalised separately; thus up to this point it was not possible to compare the results. It was the intention to keep the results separate, the seasonal variation was a field study, while the controlled decomposition was a laboratory experiment. The seasonal variation study analysed DOM as it was produced under environmental conditions in different ecosystems with varying vegetation, pH and mineral content. Additionally humic and fulvic acids from the humic lake 'Skjervajern' were included into the study to compare the alternative methodology of processing water samples into humic substances. The controlled decomposition study kept the conditions for degradation, temperature and mineral content, constant while the starting materials varied. Thus both parts investigated different aspects of degradation. Their combination for the chemical characteristics of DOM was expected to show the major trends and to help interpret the assays and the contribution of each pathway towards humification.


Aber Winter
Aber Spring
Aber Summer
Aber Autumn
Clywedog Winter
Clywedog Spring
Clywedog Summer
Clywedog Autumn
Conwy Winter
Conwy Spring
Conwy Summer
Conwy Autumn
Cwm-y Glo Winter
Cwm-y Glo Spring
Cwm-y Glo Summer
Cwm-y Glo Autumn
Cors Goch Winter
Cors Goch Spring
Cors Goch Summer
Cors Goch Autumn
Clegir Winter
Clegir Spring
Clegir Summer
Clegir Autumn
Fulvic Acid Background
Fulvic Acid Acidified
Humic Acid Background
Humic Acid Acidified

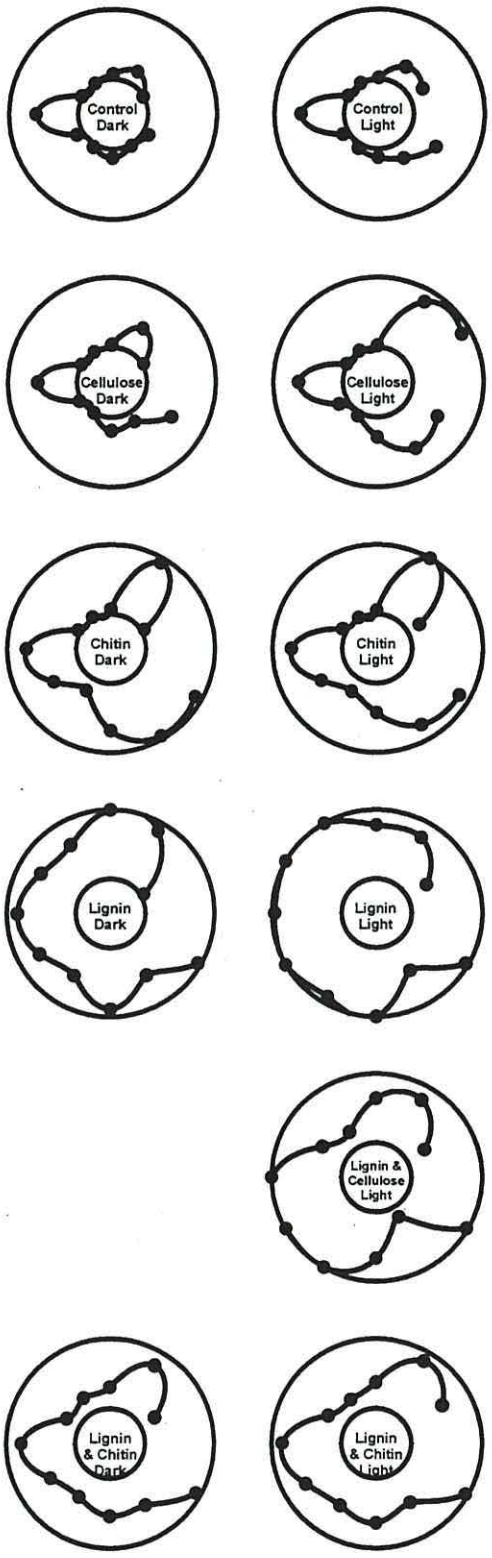


Figure 5.1: Fingerprints of all data from seasonal variation and controlled decomposition study.

5.2 Materials and Methods

The data set incorporated the data given in Tables 3.1 and 4.4. The data was normalised, multivariate statistics applied and displayed as 'fingerprints' as described in Section 2.6.2.

5.3 Results

Figure 5.1 shows the 'fingerprint' for each sample. The joining of the data sets altered the range for some assays. Temperature related only to the wetland sites. pH values were not obtained for the humic and fulvic acid samples, Aber summer had the highest pH with 7.9 while the control dark showed the lowest value with 3.8. The initial assumption of the pH range for natural water samples between 4 and 8 was thus verified. Conductivity measurements were equally not obtained for the fulvic and humic acid samples. For the other samples a discrepancy was found, because the wetland sites encompassed a range from 12 to 511 μS, while the decomposition study ranged from 180 to 800 μS. For the 'fingerprints the shapes obtained previously will thus be skewed.

The phenolics quadrant of the 'fingerprints' for the wetland sites was low for poly- and total phenolics for all sites and monophenolics for most sites. The increase in range originated from the lignin containing samples, lignin 'light' with 1 mg l^{-1} monophenolics, 30 mg l^{-1} polyphenolics and 57 mg l^{-1} total phenolics, exceeded the highest wetland value by a factor of 5, 15 and 10, respectively.

A similar effect for the UV absorbance quadrant was obtained. However, the range was doubled and it is interesting to note the strong absorbance at 200 nm of all Clywedog, Cors Goch and controlled decomposition study samples. These support the earlier hypothesis of UV absorbance at 200 nm being an indicator for high microbial activity, because all sites with high UV absorbance at 200 nm provided appropriate conditions.

The quadrant containing bulk carbon characteristics was skewed for TOC, while the highest value for the seasonal variation was Cors Goch autumn with 28 mg C l^{-1}, the lignin 'light' sample contained 79 mg C l^{-1}. The ranges for fluorescence and complexing strength towards phenol overlapped.

Although the 'fingerprints' were skewed when compared to the individual data sets as presented in Chapters 3 and 4, the shapes obtained for each site in the seasonal variation, the humic and fulvic acid samples and the samples from each unit in the controlled decomposition study remained similar to each other. The application of Principle Component Analysis (PCA) gave information about the contribution of each assay to the overall result (Table 5.1). For the PCA temperature was omitted. In order to include pH and conductivity the humic and fulvic acid samples were equally omitted.

The result of the PCA shows pH as being the leading variable for Eigenvalue 1. Being a logarithmic value, pH extends over a wider range than the other assays. Apart from fluorescence and pH all other assays contributed to a similar extent, about -0.3, to the first Eigenvalue, explaining 65.5 % of the variation within the data. TOC gave the highest contribution, closely followed by UV absorbance at 240 nm. Colour, mono- and total phenolics gave slightly lower contributions (-0.33). UV absorbance at 200 nm (-0.3), complexing strength towards phenol and conductivity followed. Eigenvalue 2 had the largest contribution from fluorescence, followed by conductivity, all phenolics assays and complexation. Eigenvalue 2 explained 13.2 % of the overall variation within the data. While the PCA performed in Section 3.3.1 seemed to give an Eigenvalue 1 relating to the chemical characteristics of DOM and Eigenvalue 2, 3 and 4 relating to the water chemistry, the PCA performed on the combined results did not support those earlier findings. Rather it showed all assays contributing to a similar extent indicating the balanced design of the methodology.

Figure 5.2 shows the result of the cluster analysis; depicted is a dendrogram in which four 'roots' can be identified. These 'roots' represent groups of

samples that were similar to each other. Starting on the left-hand side of the dendrogram, the first 'root' contained Cors Goch summer and autumn samples and all the chitin containing samples from the decomposition study. These samples were thought to have provided conditions for high microbial activity. Cors Goch was an alkaline fen, thus calcium was provided and the pH was circumneutral. The two samples in this 'root' summer and autumn had favourable conditions for degradation with higher temperatures and input of plant debris. Chitin, due to the presence of intramolecular nitrogen, was rapidly degraded during the controlled decomposition, thus providing conditions for high microbial activity.

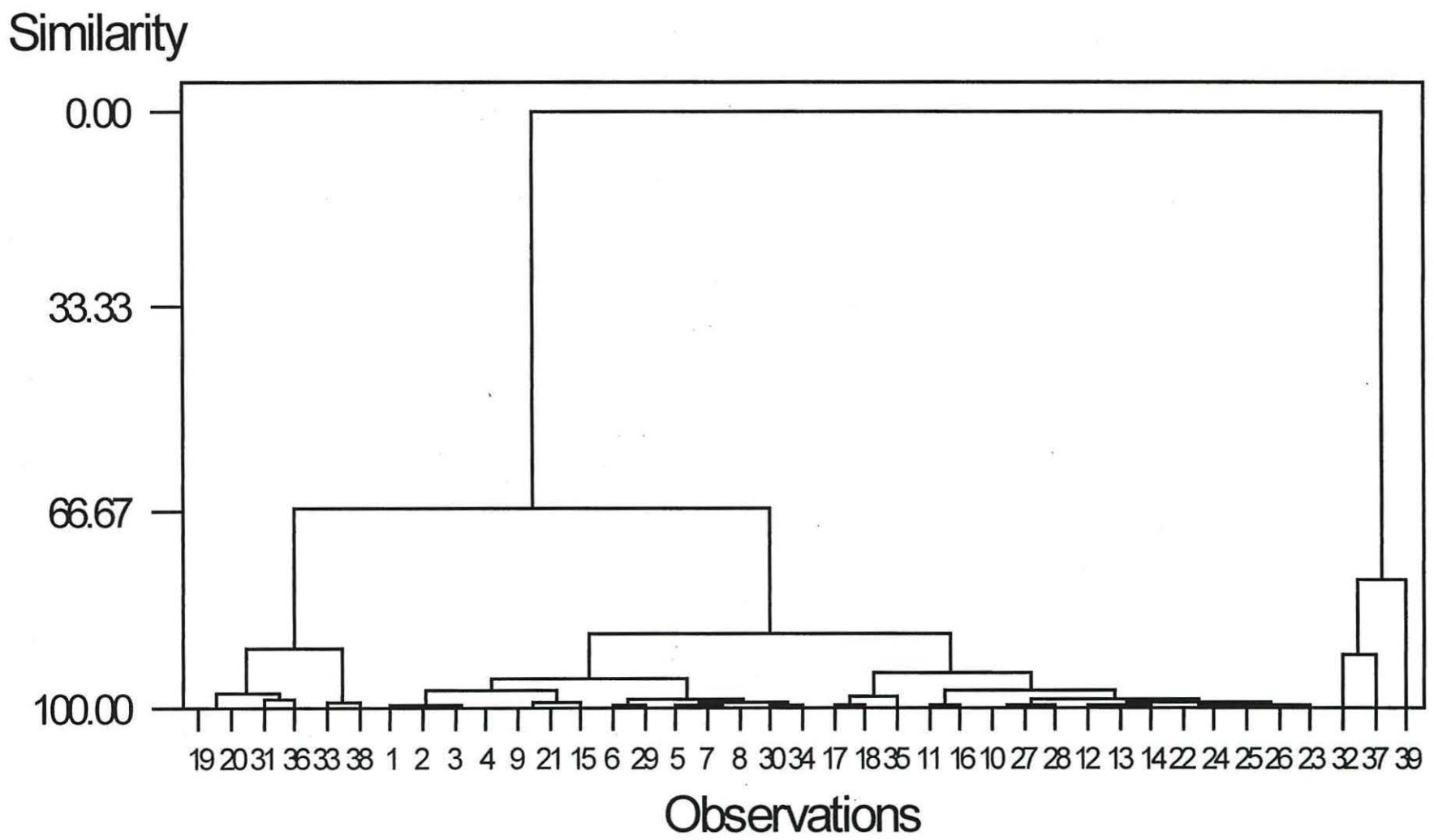
The first 'root' was linked at a similarity level of 67 % with the next two 'roots', which split at a similarity level of 79 %. The left hand 'root' encompassed all Aber and Clywedog samples, Conwy and Clegir winter samples, both control samples from the decomposition study and Cwm-y Glo summer. This group of samples could be described as showing low values for most assays (For more detail see Chapter 3).

The third 'root' contained the high in carbon seasonal samples and the humic and fulvic acid samples. As before (Chapter 3) the humic acid samples were most similar to Conwy spring, while the fulvic acid samples were most similar to the Clegir autumn, spring and summer samples. From the decomposition study the cellulose 'light' sample was equally found in this third 'root' being most similar to Cors Goch winter and spring.

The last 'root' was not similar to the first three 'roots'. In this 'root' the lignin 'light' and 'dark' samples were joined by the lignin & cellulose 'light' sample. The lack of intramolecular nitrogen in the substrate for all three samples resulted in DOM that showed no similarity with the sites or the humic substances.

Table 5.1: Result of principle component analysis for the 11 assays comprising the fingerprint as calculated by Minitab version 11. Shown are the 2 eigenvalues contributing 783 % of the overall variation amongst the data (seasonl variation and controlled decomposition study).

	PC1	PC2
pH	0.147	0.016
Conductivity	-0.285	0.368
Monophenolics	-0.331	-0.310
Polyphenolics	-0.317	-0.338
Total Phenolics	-0.331	-0.327
UV 200nm	-0.302	0.198
UV 240nm	-0.358	-0.010
Colour	-0.338	-0.154
TOC	-0.361	0.054
Fluorescence	-0.184	0.642
complexed Ph.	-0.281	0.262
Eigenvalue	7.2035	1.452
Proportion	0.655	0.132
Cumulative	0.655	0.787


Similarity
0.00
33.33
66.67
100.00
19 20 31 36 33 38 1 2 3 4 9 21 15 6 29 5 7 8 30 34 17 18 35 11 16 10 27 28 12 13 14 22 24 25 26 23 32 37 39
Observations

Legend:

19 Cors Goch Summer	20 Cors Goch Autumn	31 Chitin 'dark'
36 Chitin 'light'	33 Lignin & Chitin 'dark'	38 Lignin & Chitin 'light'
1 Aber Winter	2 Aber Spring	3 Aber Summer
4 Aber Summer	9 Conwy Winter	21 Clegir Winter
15 Cwm-y Glo Summer		
6 Clywedog Spring	29 Control 'dark'	5 Clywedog Winter
7 Clywedog Summer	8 Clywedog Autumn	30 Cellulose 'dark'
34 Control 'light'		
17 CorsGoch Winter	18 Cors Goch Spring	35 Cellulose 'light'
11 Conwy Summer	16 Cwm-y Glo Autumn	10 Conwy Spring
27 Humic Acid Background	28 Humic Acid Acidified	12 Conwy Autumn
13 Cwm-y Glo Winter	14 Cwm-y Glo Spring	22 Clegir Spring
24 Clegir Autumn	25 Fulvic Acid Background	26 Fulvic Acid Acidified
23 Clegir Summer		
32 Lignin 'dark'	37 Lignin 'light	39 Lignin & Cellulose 'light'

Figure 5.2: Result of cluster analysis for 9 assays (monophenolics, polyphenolics, total phenolics, UV absorbance at 200 and 240 nm, colour, TOC, fluorescence and complexing strength towards phenol) as calculated by Minitab version 11 for the data from the seasonal variation, the humic and fulvic acid samples and the controlled decomposition study.

5.4 Discussion

In Figure 1.3 (Chapter 1) the four different pathways for humification are illustrated. The results of both experiments, seasonal variation and the controlled decomposition study, were designed to investigate the contribution of each pathway towards humification. Pathway 1 related to the sugar-amine-condensation and was represented by the Conwy and Clegir sites and cellulose and chitin as substrates.

Cellulose was the only 'chemically pure sugar' investigated in this study. The DOM resulting from its decomposition was low in TOC, phenolics and fluorescence. The solution had a yellow colour, the UV absorbance was high at 200 nm, perhaps indicative of high microbial activity, while lessening towards higher wavelengths (Figure 5.1). In the cluster analysis cellulose 'dark' was closely related to the control 'dark' and the Clywedog samples, which equally showed high absorbance at 200 nm. Cellulose 'light' fell into a cluster with Cors Goch winter and spring. This could have originated from the known slow degradation of cellulose that occurred naturally, after leaf fall. The high pH of the cellulose 'light' sample made it most similar to the alkaline fen Cors Goch. Overall the evidence gathered for cellulose did not support it contributing significantly to humification, rather if cellulose is broken down enzymatically, it yields the high energy substrate glucose that is metabolised easily.

The high UV absorbance at 200 nm could indicate a high protein content, which so far was explained as high microbial activity, without any supporting evidence. In the context of humification pathways a high absorbance at 200 nm in comparison to the UV absorbance at 240 nm could also indicated a small degree of polymerisation. UV spectra of humic substances, high molecular weight DOM with extensive polymerisation, show a gradual decline towards longer wavelength. The appearance of a peak between 200 and 240 nm, as was found for Clywedog, Cors Goch and all controlled decomposition samples, could indicate 'new' DOM with a high proportion of

small molecular weight substances.

Chitin degradation occurred rapidly, presumably aided by its intramolecular nitrogen. Chitin is an aliphatic substrate that provides energy and nitrogen when broken down (Aber and Mellilo 1991). The analysis of the DOM resulting from chitin degradation was performed at least 12 months after all the substrate was degraded, thus it potentially represented an example of a prolonged sugar-amine-condensation. In the cluster analysis, chitin 'light' and 'dark' were similar to Cors Goch summer and autumn. Degradation is most active during summer because of the high temperature and autumn when a high input in dead matter occurred, implying the conversion of most of the substrate into microbial biomass and its subsequent transformations during humification. Both sugar containing columns related to Cors Goch, chitin the faster degrading substrate to summer and autumn, cellulose the slower degrading substrate to winter and spring. The 'fingerprints' obtained for Cors Goch were similar in shape, but differed with regard to their concentrations. As for all other sites, the concentrations found during winter and spring were smaller in comparisons to the concentration found during summer and autumn. Relating this to the chitin and cellulose units in the controlled decomposition study, substantiates the slower degradation of cellulose.

Conwy and Clegir contained sphagnum moss as a large part of their vegetation on the catchment. As explained in Section 1.2, peat bogs represent environments low in polyphenolics but high in the production of a keto-sugar: sphagnan (Painter 1995). Historically the sugar-amine-condensation was incorporated to account for those environments (Stevenson 1994). However, a keto-sugar is chemically different from, e. g., glucose. Sphagnan, like lignin and tannins, is a plant defence agent, synthesised to prevent microbial decay. The additional keto group bonds with amino groups and thus builds a sort of molecular 'spiders web' (more details in Section 1.3). As opposed to lignin and tannins sphagnan is aliphatic and more water-soluble, thus the correlation between percentage

peat cover on the watershed and DOC concentration in rivers as found by Aitkenhead et al (1999), could be explained with sphagnan's higher water solubility. The increase in size of the 'spiders web' leads to the brown colour of the water found in bogs, commonly described as humic. The humic and fulvic acid samples in the cluster analysis were most similar to Conwy spring and Clegir, respectively.

The sugar-amine-condensation as described by Stevenson (1994) needs to be more refined in view of the results gained during this study. As outlined above it will depend upon the chemical quality of the sugar and to what extent it is humified. While cellulose contributes little to humification, sphagnan contributes strongly because of its reactivity with amino containing compounds in water.

Pathways 2 and 3 (Figure 1.3) both involve the microbial transformation of lignin or polyphenolics and their subsequent oxidation to quinones prior to polymerisation with amino containing compounds. The ratio of phenolics to quinones is difficult to assess (Davies and Ghabour 1999). It is also difficult to quantify oxidation occurring in natural waters (Hope et al. 1994). Two assays that measure poly- and total phenolics formed part of the 'fingerprint'. The polyphenolics assay oxidised phenolics to quinones in acid conditions, while the Folin-Ciocalteau reagent oxidised total phenolics at pH 10. The ratio of poly- to total phenolics gave some indication to the extent of phenolics oxidation: the lower the ratio, the less phenolics were present. Even though redox-based assays are not specific, there are indicative of the oxidation potential of the analyte. The fulvic acid samples had a ratio of 0.5 while the humic acids had a ratio of 0.4 (background) and 0.3 (acidified soil), indicating the higher oxidation of humic acids when compared to fulvic acids (Hayes 1997). A low ratio of 0.2 to 0.3 was obtained for the sites Conwy and Clegir, the sphagnum dominated environments that were expected to be low in phenolics. The lowest ratio of 0.15 was obtained for chitin 'light'. High ratios of 0.5 or more were obtained for the Aber in autumn and winter, Clywedog in summer and autumn, the Cwm-y Glo summer sample that was

taken from within a tree root (Materials and Methods in Chapter 3), and the lignin 'dark' and 'light' and lignin & chitin 'dark' samples. However, the ratio of poly- to total phenolics can only be used as an indicator, because the redox-based assays are not specific for phenolics. Overall, both pathways are thought to be important contributors to humification due to the bonding of phenolics, prior to the oxidation to quinones or afterwards, with protein.

The pathway 4 during humification relates to modified lignins. The result of the cluster analysis (Figure 5.2) showed the lignin 'light' and 'dark' and lignin & cellulose 'light' sample to be dissimilar to all other samples. In addition lignin is water insoluble in its native state and thus this pathway seemed not to contribute to humification.

The random structure of high molecular weight DOM (Stevenson 1994), or humic substances, is thought to be a key aspect for its recalcitrance. As described earlier, decomposition processes are enzymatically driven (Allan 1995). Enzymes act upon specific molecules or molecular fragments for degradation but, with few exceptions like ligninase (Evans and Bucke 1998), fail to break down random structures. During humification, varying pathways for the transformation of plant debris are each followed by complexation with amino containing compounds and subsequent polymerisation. The randomness of high molecular weigh DOM is introduced during the complexation / polymerisation sequences, because they depend upon chance and are thus entropically controlled.

The 'fingerprints' (Figure 5.1) show that the 'starting material' for humification, the vegetation on the watershed or the substrate in the controlled decomposition study, influence the chemical characteristics of the resulting DOM. The presence of polyphenolics as plant defence agents slows degradation because of their interaction with enzymes (Sections 1.2 and 1.3). Complexation by polyphenolics has already been described but additionally complexation was also found to occur with chitin as a substrate, where no phenolic precursor was present. Chitin 'light' and 'dark' both had

high concentrations of TOC, while no lipase inhibition occurred (Figure 4.8). The resulting DOM from chitin 'light' and 'dark' thus exemplified the importance of complexation for humification inducing random structure and illustrated that complexation was independent of the presence of phenolics.

The methodology of quick and simple assays, that target bulk characteristics based upon the reaction mechanisms occurring during humification, introduced in this work was devised with this random structure in mind. As explained earlier, the processing of water samples into humic substances was thought to lead to a loss of information for ecological research. Supporting evidence was presented in Chapter 3 where lipase activity was reduced by 90 % by humic and fulvic acids at a concentration of 20 mg l^{-1} in comparison to all other samples, which inhibited lipase by a maximum of 30 %. Even the lignin 'light' sample with a TOC of 79 mg l^{-1} only inhibited lipase by 21 %. Performing the 'fingerprint' and the biochemical assays required less time than the processing of natural water samples into humic substances. A novel methodology has thus been developed during this study, whose balanced design was substantiated with the application of multivariate statistics. The aim of the methodology was to match the reaction mechanisms of degradation processes onto the analysis of the resulting DOM. The same reaction mechanisms explained the ecological functioning of DOM as was described in Chapter 1. In addition to the known redox-based assays, two novel assay based upon the complexation behaviour of DOM have been introduced. The degree of polymerisation could be deduced from the UV absorbance and the colour in grade Hazen.

The methodology was not able to identify the occurrence of distinct compounds, like the suspected origin of esculetin for DOM fluorescence. To utilise enzymes and their general specificity for distinct substrates, as it is possible with biosensors, seems a way to further the approach of quick and simple on-site analysis. Two assays, UV absorbance at 200 nm and fluorescence occurred with samples that provided conditions for high microbial activity (Chapters 3 and 4). However, this remains a hypothesis,

because no microbial assay was performed. Primarily due to its simplicity, the methodology will be useful for DOM surveys (Hope et al 1994), because it can potentially quantify the chemical processes, oxidation and complexation, contributing to the carbon cycle where dead matter is oxidised back to carbon dioxide.

Overall, humification occurring under natural conditions and during the controlled decomposition study showed the main pathways to be primarily based upon the interaction of plant defence agents with amino containing compounds. Substrates containing intramolecular nitrogen were being turned over quickly. Polyphenolics based plant defence agents showed a strong tendency for complexation. They were thought to leach out less and travel smaller distances when compared with aliphatic keto sugars, which were more water-soluble and thus lead to 'humic' waters. Submitting humic and fulvic acids to the same methodology showed similar 'fingerprints' but displayed different biochemical behaviour, thought to reflect artefacts incurred during the processing of natural water samples into humic substances.

5.5 Review of study

The key area addressed in this study were the pathways occurring during humification and the underlying reaction mechanisms. The review of the literature showed the lack of an appropriate gentle analysis to investigate DOM as found in natural water samples. Accordingly, a methodology was developed for chemical ('fingerprint') and biochemical characteristics of DOM. Novel assays for the analysis of DOM were developed, that concentrated on its complexing ability. As it was confirmed with the assays measuring complexing strength towards phenol and protein precipitation ability, complexation varied and is implicated in the ecological functioning of DOM, as for example illustrated in the results gained for the lipase assay. The evaluation of the 'fingerprint' from both the field and the laboratory study

showed the experimental design to be balanced.

Application of the methodology onto three river and three wetland samples confirmed the heterogeneity of DOM and its dependency on the vegetation and underlying geology of the watershed, because distinct patterns were obtained for each site. The statistical evaluating of the results showed differences between sites but, surprisingly, only small differences between seasons.

A controlled decomposition study using the bulk terrestrial and marine carbon sources was conducted to investigate the dependency of DOM on the chemical characteristics of the starting materials. The results gained from the DOM after 15 month of decomposition indicated differences between the resulting DOM and its subsequent biochemical activity. The experimental design of the controlled decomposition study was valuable, however, considering how the experiment was conducted it could be improved with regard to a time series analysis, a mass balance and application of the protein precipitating assay.

The combination of the results gained from the field and the laboratory study were combined to evaluate the contribution of the four pathways of humification. The sugar-amine-condensation was found to contribute only to a small extent to the genesis of DOM, while the DOM derived from lignin on its own or combined with cellulose was found dissimilar to all other samples, implying that the modified lignin pathway equally only contributes to a small extent. The most important pathways for humification, as found during this study, were those concerned with plant defence agents, either polyphenolics or keto-sugars.

5.6 Future Work

During this study a methodology called 'fingerprinting' has been developed that can be applied to DOM in a water matrix. Future work using this methodology should concentrate on two key areas relating to changes of the

matrix:

- Application onto marine samples
- Application onto soil solutions

Additionally sites in different climate zones with similar matrix characteristics should be analysed in order to investigate the effect of temperature onto degradation reactions.

Considering the findings of this study three phenomena were encountered that require further investigation

- Occurrence of a peak in the UV absorption between 200 and 240 nm. When this peak occurred the samples were generated in conditions thought to sustain high microbial activity (Clywedog, Cors Goch and controlled decomposition samples). A hypothesis given for the occurrence of those peaks is high protein content.
- Occurrence of high conductivity in all samples from the controlled decomposition study, apart from the control. The origin of the high conductivity is unclear. A hypothesis is the decomposition of the starting material and protein from cell lysis into charged fragments.
- Occurrence of high fluorescence in the chitin containing columns of the controlled decomposition study. Earlier theories suspected a lignin degradation product, caffeic acid, to be converted into a fluorophore, however, the findings in this study do not support this theory, because lower fluorescence values were obtained from the lignin containing columns. Assuming the origin of the fluorescence to be an unknown, but identifiable compound(s), a pre-concentration approach followed by chromatographic separation with identification and quantification is required.

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Appendix: Pre-concentration of DOM using Solvent Extraction, Adsorption onto Active Charcoal and Solid Phase Extraction

1 Introduction

At the start of the study a variety of methods of pre-concentration were investigated using a Conwy river sample: active charcoal for adsorption, solvent and solid phase extraction. For the solvent extraction GC-MS (Gas Chromatography - Mass Spectrometry) was used for the analysis. For the charcoal adsorption and solid phase extraction TOC (Total Organic Carbon) content and UV absorption at 200 and 240 nm of the Conwy sample after the pre-concentration were used, to assess the effectiveness of the method.

2 Materials and Methods

Solvent extraction

A Conwy river sample (500 ml) was stirred with dichloromethane (500 ml) for 24 h. The mixture was transferred into a separating funnel. The organic phase was dried over $MgSO_4$ and most of the solvent was removed in a rotary evaporator. The remainder was reduced to a final volume of ca. 0.1 ml while passing a stream of nitrogen over the sample and submitted for GC-MS analysis.

Active charcoal treatment

Active charcoal (granular, 10 - 18 mesh from BDH) was dried at 130 °C for 24 h. Active charcoal (5 g) was stirred with either doubly distilled water (150 ml) or Conwy river water (150 ml) for 3.5 h. The charcoal was removed by filtration through Whatman GF/F. The doubly distilled water sample served as a blank. The filtrate from the river sample was termed 'Conwy after treatment'.

Solid Phase Extraction (Sep-pak cartridges)

The NH_2-cartridges (obtained from Waters) were conditioned using methanol (50 ml), equilibrated with doubly distilled water (100 ml). Either a Conwy sample (300 ml) or doubly distilled water (300 ml) was passed through the cartridge at a flow rate of 10 ml min^{-1}. The water fraction was used as a blank while the river sample was termed 'Conwy after treatment'.

3 Results and Discussion

Initially samples from the river Conwy were chosen to investigate alternative pre-concentration techniques due to them being high in DOM (up to 20 mg C l^{-1}). An untreated Conwy sample was submitted for GC-MS (Gas Chromatography - Mass Spectrometry) analysis (Figure A.1) and the concentration of DOM was found too low to justify interpretation of the spectrum. Thus a solvent extraction on Conwy water was performed with dichloromethane. Even with a 5000:1 pre-concentration of the dichloromethane extract the spectrum showed no distinct peaks (Figure A.2).

There were two aspects of solvent extraction to consider regarding the GC-MS result. During the extraction, only part of the DOM was transferred into the organic phase. Dichloromethane iss an apolar and aprotic solvent. Charged compounds like acids and proteins were thus less likely to partition into the organic phase. Apolar aromatic compounds were thought more likely to transfer into the organic phase. Regarding GC-MS it was a pre-requisite for successful separation that the compounds were volatile in order to be carried into the GC column and that they did not decompose while the temperature increased in the sample chamber. Overall only part of the DOM separated into dichloromethane, of which only part was separated using gas chromatography.

Two other pre-concentration methods were investigated, adsorption of DOM onto active charcoal and solid phase extraction. The Conwy river sample

was analysed after either removal of the charcoal or after passing through the NH_2-cartridge and TOC and UV absorption at 200 and 240 nm were studied. Again only partial extraction was achieved (Table A.1 and A.2). Adsorption did occur, as was verified by the reduction in the absorbance at 200 and 240 nm. In direct comparison to solid phase extraction, active charcoal showed stronger absorbance (Tables A.1 and A.2). However, both adsorbents increased the amount of TOC in the 'Conwy after treatment' sample.

Overall the three above observations confirmed the description of DOM (Thurman 1978, Stevenson 1994) regarding its heterogeneous nature. Each pre-concentration was shown to be applicable to part of DOM, but which part exactly could not be verified with the methodology employed.

Table A.1: Results of analysis on Conwy river samples after pre-concentration studies using granular active charcoal.

Assay	Charcoal blank	'Conwy after treat.'	'Conwy pre treat.'
TOC	80.1 mg l^{-1} C	7.7 mg l^{-1} C	0.5 mg l^{-1} C
UV at 200 nm	0.15 mg l^{-1} C	0.22 mg l^{-1} C	0.53 mg l^{-1} C
UV at 240 nm	0.06 mg l^{-1} C	0.11 mg l^{-1} C	0.31 mg l^{-1} C

Table A.2: Results of analysis on Conwy river samples after pre-concentration using NH_2 solid phase extraction cartridges

Assay	Charcoal blank	'Conwy after treat.'	'Conwy pre treat.'
TOC	79.5 mg l^{-1} C	50.9 mg l^{-1} C	5.5 mg l^{-1} C
UV at 200 nm	0.14 mg l^{-1} C	0.37 mg l^{-1} C	0.53 mg l^{-1} C
UV at 240 nm	0.06 mg l^{-1} C	0.15 mg l^{-1} C	0.31 mg l^{-1} C

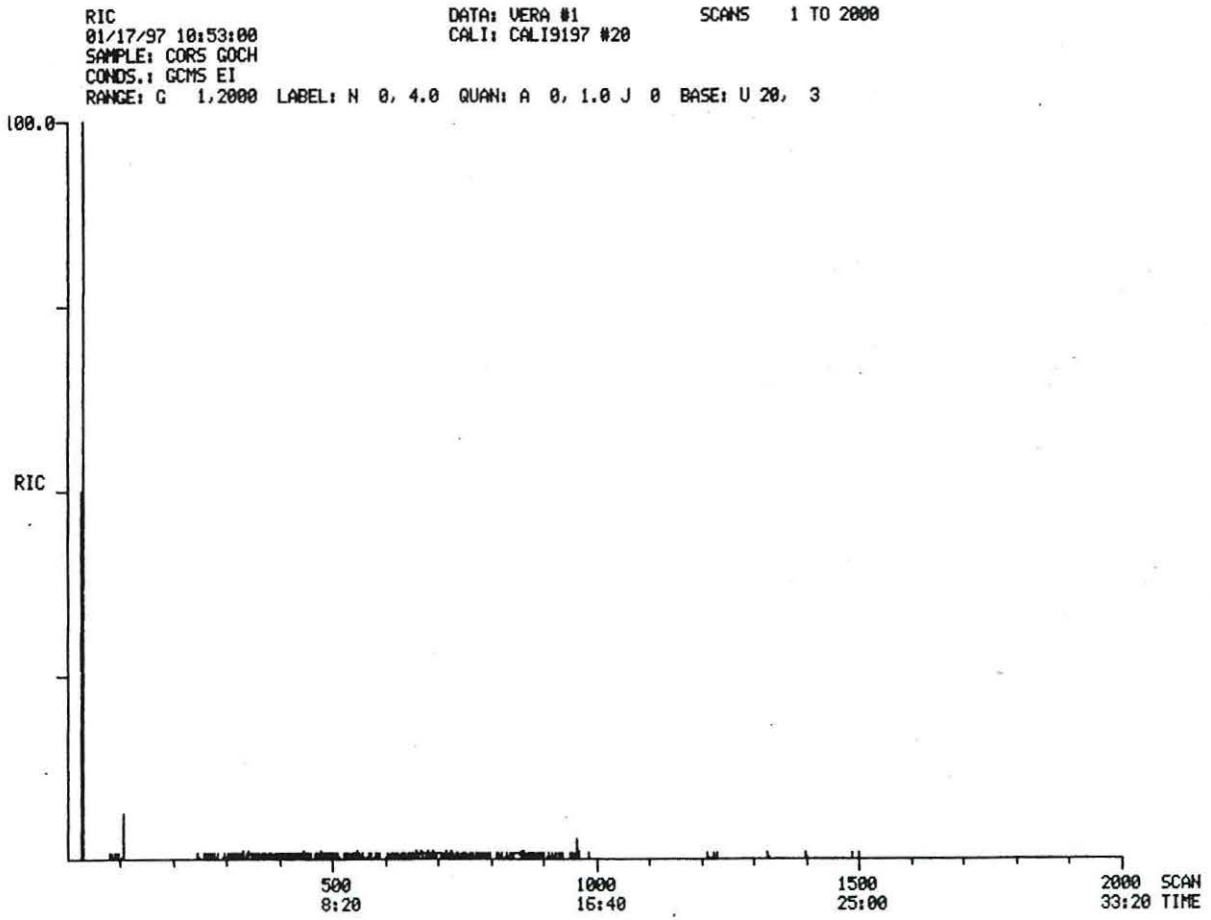

Figure A.1: GC-MS of Conwy sample.

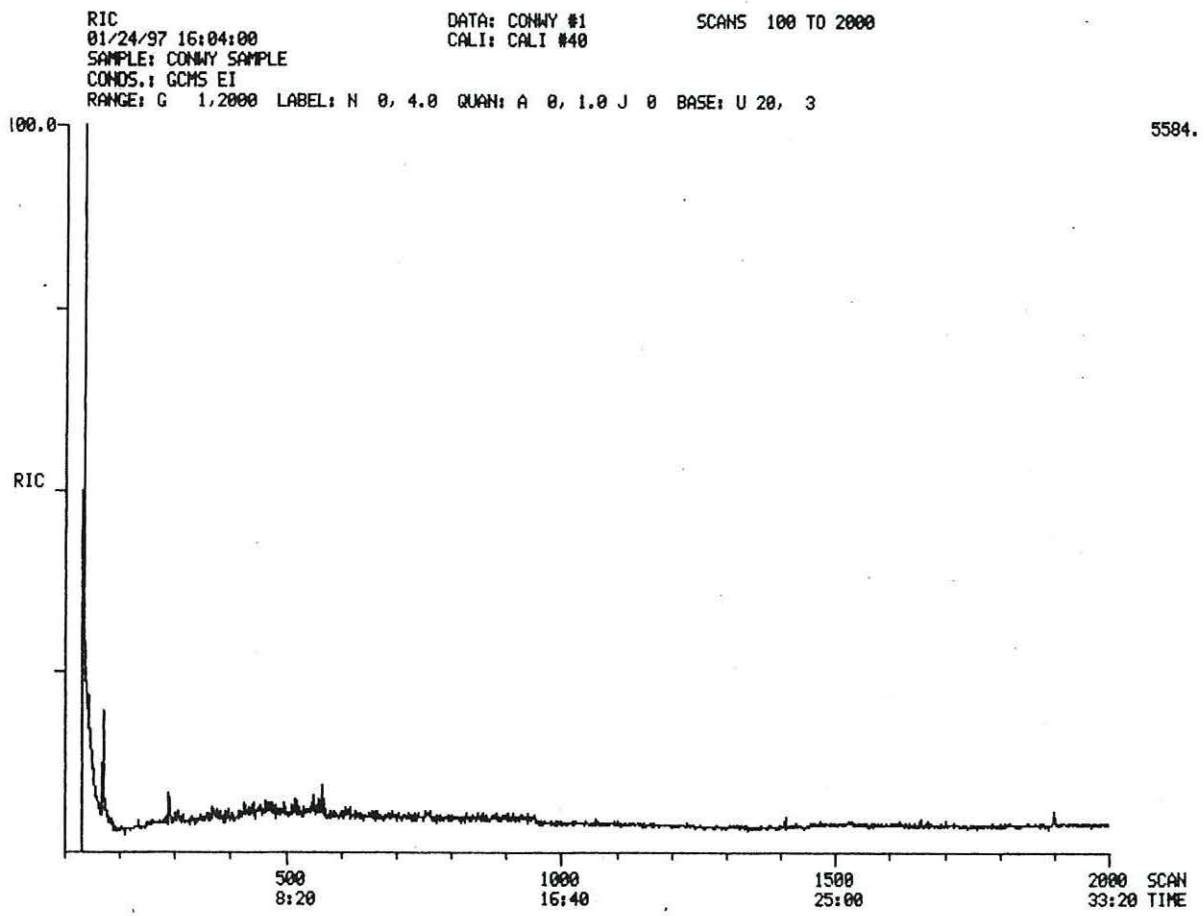

Figure A.2: GC-MS of Conwy sample after extraction into dichloromethane and pre-concentration by a factor 5000.

Table of Site Characteristics

Site	pH	T (water)	Conductivity	Colour	TOC
Aber	6.8 - 7.9	3 - 14 °C	47 - 68 μS	4 - 12 Hazen units	0.45 - 7.30 mg l^{-1}
Clywedog	7.1 - 7.7	5 - 16 °C	137 - 236 μS	13 - 25 Hazen units	1.93 - 7.78 mg l^{-1}
Conwy	4.5 -5.0	2 - 14 °C	12 - 40 μS	25 - 136 Hazen units	2.51 - 18.30 mg l^{-1}
Cwm-y Glo	5.5 - 6.4	2 - 14 °C	72 - 140 μS	27 - 138 Hazen units	6.78 - 12.36 mg l^{-1}
Cors Goch	6.0 - 7.1	2 - 17 °C	248 - 511 μS	50 - 132 Hazen units	10.41 - 22.96 mg l^{-1}
Clegir	4.2 - 5.2	1 - 17 °C	29 - 73 μS	24 - 88 Hazen units	7.06 - 10.12 mg l^{-1}