NATURAL ORGANIC MATTER: ISOLATION AND BIOAVAILABILITY

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Natural Organic Matter: Isolation and Bioavailability

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LIST OF ABBREVIATIONS

CZE	Capillary Zone Electrophoresis
DOM	Dissolved Organic Matter
ED	Electrodialysis
ESI-MS	Electro-Spray Ionization Mass Spectrometry
HS	Humic Substances
NMR	Nuclear Magnetic Resonance spectroscopy
NOM	Natural Organic Matter
RO	Reverse Osmosis
TOC	Total Organic Carbon
UF	Ultrafiltration

SUMMARY

Electrodialysis (ED) experiments were conducted on synthetic freshwater samples whose chemical compositions most closely resemble those of un-desalted freshwater samples that have been concentrated by reverse osmosis (RO). ED experiments were also conducted on RO-concentrated solutions of natural organic matter (NOM) from six rivers. The ED processes successfully recovered 88% ± 11% of total organic carbon (TOC), and removed 83% ± 19% of SO_4^{2-} and 67% ± 18% of H_4SiO_4. More importantly, the molar ratios of SO_4^{2-} /TOC and H_4SiO_4 /TOC were reduced to a mean value of 0.0046 and 0.032, respectively, surpassing the goal of 0.008 for removal of SO_4^{2-} and nearly achieving the goal of 0.021 for removal of H_4SiO_4. The ED process can lower the SO_4^{2-} /TOC ratio in samples whose initial SO_4^{2-} /TOC ratios are already far below the limit of 0.008 used in this study. The coupled RO/ED process that has been described in this study offers a fast, simple, chemically mild (relative to other methods), and reproducible method for the isolation of large quantities of relatively un-fractionated, low-ash NOM from freshwaters.

RO/ED was also successfully used for isolating and concentrating marine DOM. The effort successfully recovered a median of 72% of the TOC from 200 L samples within six to nine hours of processing through a combination of ED and RO, greatly exceeding the current norm of 30%. The relatively high recovery of DOM implies that classes of DOM previously missing are included in these samples which should yield new insight into the chemistry of marine DOM.

The bioavailability of freshwater NOM from six diverse rivers was determined. It was possible to distinguish between the bioavailability of river NOM, linking bioavailability to bulk elemental composition: the H/C and N/C molar ratios are positively and strongly correlated with bioavailability.

Freshwater samples processed by electrodialysis were analyzed by capillary zone electrophoresis (CZE), nuclear magnetic resonance spectroscopy (NMR), and electro-spray ionization mass spectrometry (ESI-MS). The CZE, ^{1}H- and ^{13}C-NMR, and the ESI-MS data provide further evidence linking bioavailability of NOM to its bulk chemistry as described by the molar H/C ratio and aromatic and aliphatic content.

Using an independent dataset (STORET) of water quality parameters, calculated BOD/TOC ratios were found to be moderately correlated with measured bioavailability and can be used as a surrogate for bioavailability of geochemically diverse riverine DOM.

CHAPTER 1

OVERVIEW

1.1 Introduction

1.1.1 Why is Natural Organic Matter Important?

Natural organic matter (NOM) is present in the environment in dissolved, colloidal, and particulate forms in water, soil, sediment, rock, and the atmosphere. As part of the global carbon cycle, NOM is an important part of the earth's ecosystem. In its various forms, NOM is a carbon and energy source for micro-organisms in water and soil. It affects the movement of nutrients such as Ca^{2+}, Mg^{2+}, and Se and of toxic substances (anthropogenic and natural) in the environment by binding these; the presence of NOM may limit exposure of micro-organisms to toxic substances. In water, NOM imparts color, it attenuates light, and its constituent organic acids affect and buffer the pH. The importance of NOM for the environment and for human health rests on several key lines of evidence: the formation of disinfection by-products when chlorine is added to drinking water, the speciation and bioavailability (toxicity) of trace metals, as a nutrient for micro-organisms, and its part in the global carbon cycle (Perdue and Ritchie, 2003, and references therein). In non-aqueous environments, organic acids (constituents of NOM) are implicated in soil formation processes, surface weathering and generation of subsurface porosity (via their ability to dissolve minerals), and ore formation (via metal complexation) (Lewan and Pittman, 1994). Humic substances, the dominant fraction of NOM, are of commercial value as boiler scale inhibitors, plant growth

promoters, soil conditioners, drilling mud additives, and detoxifiers of soil and water (Steelink, 1999).

The chemistry of freshwater NOM depends on numerous factors including its source (allochthonous versus autochthonous), the pH and ionic strength of the water which the NOM in part determines, the major cation make-up of the water, fractionation by sorption to sediments, and photolytic and microbial processes (Leenheer and Croué, 2003). The hydrological conditions at the time of sampling will also impact the chemistry of freshwater NOM: a storm event prior to sampling resulting in a large input of surface and soil runoff will change the "average" NOM makeup in the water.

Given the many ways in which NOM interacts with the environment through biotic and abiotic processes, it is important to know not only at what concentrations NOM is present in various environmental compartments but also its physical and chemical characteristics and how these impact the conditions of the environment. Furthermore, changes due to climate and land-use changes may have an effect on the physical and chemical characteristics of NOM which in turn may affect a number of biotic and abiotic processes. Knowing how and how fast these changes take place is also essential.

1.1.2 Scope of this Research

This thesis will focus on two issues. The first part will explore the development, testing, and application of a new method to more efficiently isolate NOM from freshwater and seawater. Specifically, this work will optimize and evaluate electrodialysis as a method for the removal of sulfate and silica from solutions of

freshwater (river) NOM that were pre-concentrated by reverse osmosis. This work will also optimize and evaluate the same methods to isolate seawater NOM, in this case using electrodialysis first to remove most inorganic solutes, followed by reverse osmosis to concentrate the NOM. The differences in the inorganic composition of river water and seawater require different approaches to isolate the NOM. The aim of this research is to produce relatively ash-free, freeze-dried NOM that is useful for scientific investigations of its chemical properties and reactivity. The isolation and concentration of marine NOM is expected to be a major step toward a more complete characterization of its constituents.

For this part of the thesis, the hypotheses put forth are the following:

- The concentration and isolation of freshwater NOM by coupling reverse osmosis with electrodialysis will yield recoveries of NOM exceeding 80%.
- Recovery of NOM by electrodialysis is independent of the chemical make-up (pH, hardness, sulfate, silica, TOC) of the water from which the NOM is being isolated.
- Isolation by electrodialysis will yield NOM that is suitable for direct chemical and spectroscopic characterization by a number of methods to be used here, including elemental analysis, capillary zone electrophoresis, NMR spectroscopy, and electro-spray ionization mass spectrometry.
- The concentration and isolation of marine NOM by electrodialysis and reverse osmosis will yield recoveries of NOM exceeding the current state of the art of ~ 30%.

The second part of this thesis focuses on the bioavailability of river water NOM. Bioavailability is a measure of the quality of the NOM with regard to its usability within the aquatic bacterial cycle. The research questions put forth are the following:

- Does the bioavailability of river water NOM vary spatially in the southeastern United States?
- Can the ratio of BOD/TOC (biological oxygen demand / total organic carbon) be used to predict the bioavailability of NOM in geochemically diverse river waters?
- Can bulk elemental composition, the determination of which requires ash-free NOM, and aliphatic carbon content of river water NOM of varying chemical composition be used to predict its bioavailability?

In this part of the thesis, it will be shown that the properties inferred from capillary zone electrophoresis (CZE), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), and elemental analysis are related to the bioavailability of six river NOM samples from the south-eastern United States. The use of electrodialysis to isolate freshwater NOM from inorganic constituents is central to the ability to carry out these analyses, and ultimately to relate the chemical properties of the NOM to its bioavailability.

The hypotheses put forth for this part of the study are the following:

- The more bioavailable NOM will tend to exhibit distinctive peaks in CZE. These peaks are expected to have low mobility, reflecting a low charge-to-surface area ratio.
- NMR spectroscopy is expected to identify functional group differences between differentially bioavailable waters, whereas mass spectrometry is expected to identify molecular-level differences between these same waters. Such findings bolster the hypothesis that the bioavailability of NOM is related to its bulk chemistry, and that

aliphatic and relatively un-oxidized NOM is preferentially biodegraded in natural waters.

- The more bioavailable NOM will exhibit a larger aliphatic content and lower carbonyl and aromatic contents, as determined by NMR.
- Bioavailability is related to its bulk elemental chemistry. Elemental analysis and mass spectrometry data are expected to show that NOM of relatively greater bioavailability will have relatively larger H/C and N/C ratios and relatively smaller O/C ratios.

The impact of this research extends into the field of NOM chemistry, its characterization, and its compositional variability as related to environmental factors. The isolation of freshwater NOM by the chemically "soft" method of electrodialysis and its subsequent characterization by methods requiring low-ash NOM may yield further insight into the properties of NOM.

To date, marine NOM can at best be recovered with an efficiency of 30%. The unrecovered 70% of the marine NOM may not be represented in the fraction being collected thereby omitting entire classes of NOM in the analysis. A superior recovery of marine NOM may yield insights into the as yet uncharacterized fraction.

1.2 Literature Review

The literature reviewed here covers only those topics not included in the literature reviews of subsequent chapters. The list of topics reviewed is not exhaustive; only those topics deemed relevant to the research presented here are covered. These include

terminology, classification, isolation methods, characterization, and the use of UV absorption.

1.2.1 Terminology

By its very name, NOM implies the exclusion of organic contaminants. In natural waters, organic contaminants are relatively insignificant (Leenheer and Croué, 2003). The samples used in the current study were taken from isolated sites judged to be natural and mostly free of organic contaminants.

The literature on aquatic organic matter contains references to dissolved organic matter (DOM), natural organic matter (NOM), humic substances, humic and fulvic acids, particulate organic matter, and other fractions. DOM specifically refers to organic matter that passes through a 0.45 um filter, but DOM and NOM are commonly used interchangeably. Humic substances refer to a biogenic organic fraction in water that is colored, of high molecular weight, and refractory (Aiken et al., 1985). Humic substances (HS) themselves consist of humic acids, fulvic acids, and humin (Aiken et al., 1985). Typically, surface water consists of ~50% HS whereas the other 50% consists of low molecular weight acids, neutral compounds such as carbohydrates, amino acids, proteins, lipids, and waxes, bases, and contaminants (Schnitzer and Khan, 1972; Drever, 1997). As a result, surface water NOM is a complex mixture of low and high molecular weight molecules, and of humic and fulvic acids. The work presented in this thesis focuses on unfractionated NOM.

1.2.2 History

The earliest references to humic substances, a major constituent of NOM, are from the 1760's, at which time Wallerius observed that they adsorb water and plant nutrients (Schnitzer and Khan, 1972). In the 1830's, Berzelius classified humic substances into three fractions: humic acids, humin, and crenic and apocrenic acids which were later renamed fulvic acids. These early HS scientists believed the three fractions to be different. However, the structural features of these fractions, as determined by chemical analysis, ^{13}C NMR, and mass spectrometry, are actually very similar (Schnitzer, 1999). Scientists have studied the nature of aqueous NOM since the early 1900's (see Shapiro (1957) for more details). Following Thurman's (1985) review book on the subject, a significant accumulation in the knowledge of NOM has accrued; see Perdue and Ritchie (2003) for a review.

1.2.3 Acidic Character and Makeup of Freshwater NOM

An important property of NOM is its acidic character. Shapiro (1957) made a detailed analysis of isolated yellow organic acids from lakes. Subsequent research on the acids of NOM found that they consist of a mixture of many acids with a continuum of pK_as for carboxyl and phenolic groups (e.g., Beck et al., 1974, for nine south-eastern Georgia coastal plain rivers; Perdue and Lytle, 1983). Numerous studies have modeled the acidic functional groups of humic substances (see Perdue and Ritchie, 2003, for a review). The main functional groups of NOM are carboxyls and phenols, but other groups are present to a lesser extent including alcohols, "active methylene" (-CO-CH_2-CO-), thiols (S-H), and sulphonic acids (R-SO_2OH) (Perdue, 1985).

Many researchers use fractions of NOM instead of whole NOM. NOM has been divided into four broad classes based on size and polarity: hydrophobic, hydrophilic, transphilic, and colloidal (Leenheer and Croué, 2003). The hydrophilic and hydrophobic fractions have further been fractionated into acids, bases, and neutrals. Knowledge of the makeup of freshwater NOM includes compound classes and a few specific compounds, namely carbohydrates, carboxylic acids, amino acids, and hydrocarbons (Thurman, 1985; Perdue and Ritchie, 2003). Multi-stage mass spectrometry coupled with electro-spray ionization has recently yielded details about molecules possibly present in freshwater samples. It is postulated that relatively few compounds act as precursors for the observed complex mass spectra (Leenheer and Croué, 2003).

1.2.4 Concentration and Isolation of NOM

Strictly speaking, isolation implies that the NOM is separated from the inorganic matrix; the NOM may or may not be concentrated in the process. Concentration, however, does not necessarily imply isolation. The RO method concentrates NOM along with most of the inorganic ions present in water. As a result, the RO method does not isolate the NOM. Use of an H^+-saturated cation exchange resin removes a large portion of the cations. There is also a loss of low molecular weight neutral compounds through the RO membrane. Ultrafiltration (UF) and adsorption by XAD resins isolate and concentrate the NOM.

One of the main problems in the study of NOM has always been its isolation, whereby a sufficient quantity of NOM free of inorganic constituents can be made available. A number of methods have been used to isolate NOM and its fractions,

including Sephadex gels in gel permeation chromatography (Gjessing and Lee, 1967; Wershaw et al., 1970; Wershaw and Pickney, 1971), anion exchange resins (Weber and Wilson, 1975; Fu and James, 1990), precipitation with metals (Weber and Wilson, 1975), and adsorption by XAD resins (Mantoura and Riley, 1975). After Thurman and Malcolm (1981) showed that aquatic humic substances could be isolated in relatively large quantities using XAD-8 resins, Malcolm and MacCarthy (1992) incorporated the use of XAD-4 resins to isolate an even larger fraction of the original NOM. This last method yields 85% of the original NOM. Given the operational nature of XAD resins - only hydrophobic compounds are bound to the XAD resin and NOM is irreversibly lost on both XAD-8 and XAD-4 resins - it is apparent that whole classes of NOM compounds are missing from the isolate.

It has been postulated by Aiken and Malcolm (1987) that the XAD isolation procedure used to obtain fulvic acids, which requires a pH of 2, destroys naturally existing interactions between fulvic acids and other chemical constituents in the water such as dissolved silica and dissolved and colloidal iron. Such interactions could yield larger species. Furthermore, the isolation procedure may result in chemical alteration of the fulvic acids themselves, such as hydrolysis of ester and ether linkages (Abbt-Braun et al., 2004).

The current work avoids the problems associated with the fractionation of NOM. Instead, whole NOM, not only certain fractions, is isolated from its inorganic constituents.

Of all the methods used to concentrate NOM from freshwater in the past 30 years, reverse osmosis (RO) has been superior in terms of percent recovery of NOM (see Perdue

and Ritchie (2003) for a review). Serkiz and Perdue (1990) and Sun *et al.* (1995) first introduced and perfected reverse osmosis (RO) as a clean and relatively chemically mild method of concentrating NOM from freshwater, yielding average recoveries of 88% or more. The process of RO relies on high pressure to push clean water through micro-porous membranes, while retaining most NOM molecules as well as inorganic constituents. Small, uncharged organic molecules that penetrate the RO membrane are lost. This loss, however, amounts to no more than 20%, and on average 12%.

In a study of nine lakes of widely differing chemistries, Gjessing et al. (1999) concentrated NOM by RO and by evaporation and compared the concentrates. Relative to the original samples, RO concentrated NOM was deficient in conductivity (19 % loss on average), color (7 %), UV absorbance (254 nm; 8 %), and DOC (13 %). The loss of NOM was speculated to be low molecular size molecules passing through the RO membrane and precipitation of NOM during the filtering step, though they did not quantify the relative importance of these.

The study by Gjessing et al. (1999) also highlighted the fact that even with a cation exchange resin at the front end of the RO unit, none of the cations can be fully removed. Consideration of the process shows that the water is continuously recirculated through the cation exchange resin so that there is a batch equilibrium between the sample and the resin: the ions in the sample are in equilibrium with those adsorbed to the resin. With this setup it is not possible to completely remove non-complexed cations such as Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} from the sample. Other cations cannot be removed because they are complexed to the NOM (e.g., Fe^{3+}, Al^{3+}, Cu^{2+}).

Ash is the residue remaining after organic matter is burned in O_2. Ca^{2+}, Mg^{2+}, K^+, Na^+, and other cations bound to organic material may form compounds during the ashing process that affect the result obtained (Huffman and Stuber, 1985). For example, ashing organic material having Ca bound to it would produce not only CO_2 and H_2O but also $CaCO_3$, CaO, and $CaSO_4$. As a result the O and the S would be counted as ash and as organic, if measured separately. The determination of O, usually done by difference, would be in error because the ash would contain the O present in sulfates and carbonates such as $CaCO_3$ and $CaSO_4$.

The co-concentration of inorganic constituents by RO has been a problem for a number of reasons. First, several methods used in the characterization of NOM require that the NOM be relatively ash-free. For example, elemental analysis, whereby the C, H, O, N, and S contents of an NOM sample are determined, will be biased low in H or O if significant amounts of the oxides of Na (Ca, Mg) are present. The presence of amorphous silica (SiO_2), however, will not affect the elemental analysis because silica does not change during the oxidation process used to convert organic compounds to CO_2 and H_2O. On the other hand, the 1^{st} pK_a of silicic acid (H_4SiO_4) of 9.60 is within the range of the pK_a values for phenolic hydroxyl groups ($pK_a > 8$) present in NOM, so the presence of silicic acid in a sample, if not taken into account, can lead to an over-estimation of phenolic content. The presence of SO_4^{2-} as H_2SO_4 will result in an overestimation of carboxyl content, if the latter is measured by titrations.

The isolation of NOM by XAD resins yields a low-ash product, but the conditions for isolating the NOM are relatively chemically harsh (see above). Methods for the removal of silica (a major constituent of ash) exist, but all require relatively harsh

chemical conditions that result in significant losses of NOM (Serkiz and Perdue, 1990; Sun et al., 1995). Harsh chemical conditions may also adversely affect the chemistry of the NOM being isolated.

Ultra-filtration (UF) and nano-filtration (NF) are also used to isolate NOM. As Perdue and Ritchie (2003) demonstrate, the minimum molecular weight for NOM calculated using UF data is much greater than the generally accepted value. UF is subject to solute-membrane and solute-gel interactions resulting in higher molecular weights (Aiken and Malcolm, 1987). These interactions also appear to be related to the ionic strength of the solution. Aoustin et al. (2001) found that the rejection of humic acid by UF membranes is highest at low ionic strength when the functional groups on HS are ionized creating charge repulsion and a more open structure. As ionic strength increases, cations become associated with the ionized groups thereby lowering the charge repulsion and resulting in a smaller molecular size. The smaller molecules have an increased ability to pass through the UF membrane.

1.2.5 Characterization of NOM

Aquatic natural organic matter is made up of thousands of different molecules making it impractical to separate out each molecule for analysis. As such, the characterization of a complex mixture such as NOM results in values representing an average for the entire mixture. There are numerous ways to characterize NOM (Schnitzer and Khan, 1972; Abbt-Braun et al., 2004). The methods of characterization can be grouped into four categories: (i) elemental and molecular: electrospray ionization Fourier transform ion cyclotron resonance mass spectroscopy – ESI FT ICR MS, elemental

composition; (ii) functional group: IR and NMR spectroscopy, electrometric titrations - potentiometric, conductometric, non-aqueous, and high-frequency titrations to characterize acidic functional groups; (iii) mass/size/charge - capillary zone electrophoresis (CZE), molecular weight by vapor pressure osmometry, freezing point depression, ultra-filtration, gel permeation chromatography, size-exclusion chromatography, muti-angle laserlight scattering, laser desorption mass spectrometry, flow-field flow fractionation, small angle X-ray scattering, ultracentrifugation; and (iv) other (spectroscopy - UV-visible, fluorescence, electron spin resonance, X-ray analysis, thermal analysis, radiocarbon dating). Probably the most powerful technique to date is ESI-FT-ICR-MS which can separate NOM molecules according to molecular weight (see Chapter 8).

1.2.6 Ultra-Violet and Visible Light Absorption by NOM

A common method to characterize NOM is based on its ability to absorb UV and visible radiation. UV–visible light absorption has been used since the 1930's for characterization of wastewater and natural water. A wavelength of 254 nm was chosen for absorption studies because of the intense beam emitted by a mercury lamp at that wavelength (Pons et al., 2004). A significant fraction of NOM adsorbs at 254 nm, but not all NOM molecules do. Chromophores, groups of atoms containing unsaturated bonds such as C=C and C=O as well as unshared electron pairs, will adsorb UV-visible radiation (Schnitzer and Khan, 1972; Bloom and Leenheer, 1989). The wavelength (and energy) at which light is absorbed depends on the energy difference between the highest occupied molecular orbital and the lowest unoccupied molecular orbital (Solomons,

1980). The energy difference is greatest for molecules having unconjugated pi-bonds and less for those containing conjugated pi-bonds. The general rule is that the greater the number of conjugated multiple bonds in a molecule, the longer the wavelength of light the compounds absorb (Solomons, 1980). Humic substances, which make up ~50% of a typical surface water sample (Drever, 1997), contain chromophores. Auxochromes, mostly hydroxyl (C-OH) and amine ($C\text{-}NH_2$) groups, do not themselves absorb radiation but increase the absorptivity of chromophores.

A decrease in absorbance by NOM and its fractions with increasing wavelength has been noted in many studies (e.g., Shapiro, 1957; Schnitzer and Khan, 1972; Chen et al., 1977; Bricaud et al., 1981; Zepp and Schlotzhauer, 1981; Davis-Colley and Vant, 1987; Green and Blough, 1994; Blough and Green, 1995). Light at longer wavelengths carries less energy and thus imparts less energy to electrons; at longer wavelengths the observed decrease in absorbance is attributed to the decreased movement of pi electrons over aromatic C rings and over unsaturated bonds because these have less energy (Schnitzer and Khan, 1972).

There are elements that may adsorb in the UV range. Rare earths and heavy metals adsorb UV radiation (Clark et al., 1993), but these are expected to be either absent or present at insignificant concentrations in the samples used in this study.

Care must be taken when comparing UV-visible absorption from different NOM samples. The pH of the solution will affect the protonation of carboxyl and hydroxyl groups of NOM, and in turn affect the energy required to excite an electron in an unsaturated bond to the next level. The pH and ionic strength of the solution may also affect the macromolecular structure of NOM (see discussion above related to UF) (Swift,

1989). To avoid differing pH and ionic strength effects, the dissolution of NOM samples in a 0.05 M $NaHCO_3$ buffer is suggested (Chen et al., 1977).

The effect of pH on humic substances was noted early on. Shapiro (1957) discovered that increasing the pH of isolated yellow aquatic organic acids from 5 to 11 resulted in increased color, implying increased absorbance. Similarly, Schnitzer and Khan (1972) and Zepp and Schlotzhauer (1981) found that the specific absorption coefficients for humic substances increased with increased pH. The latter group speculate that this may be due to the ionization of phenolic and/or carboxyl groups. Schnitzer and Khan (1972) also noted an upward shift in the wavelength of maximum absorption with increased pH.

A commonly used term denoting UV-adsorption is $SUVA_\lambda$ which stands for the "specific UV absorbance at wavelength λ" normalized for the TOC concentration. ^{13}C-NMR work indicates that $SUVA_\lambda$ is strongly correlated to the aromatic carbon content of the sample (Croué et al., 1999).

1.2.7 Elemental Composition

Huffman and Stuber (1985) describe methods for determination of C, H, O, and N composition of an NOM sample. Prior to analysis of elemental composition, the sample must be dried to remove moisture or the water content must be directly measured.

Elemental composition must be determined on a sample that is relatively free of inorganic ions. Cations such as Na^+ and Ca^{2+} will result in the formation of oxides and carbonates during the oxidation of the sample during analysis, with the result that the O

and C in the oxides and carbonates are counted as part of the ash and not the organic content of the sample, from which they originate.

The elemental composition of an NOM sample can be calculated from the average oxidation state of the organic carbon (see Chapter 4). The elemental composition can also be an indicator of the source of the carbon in the NOM. A high H/C ratio indicates an aliphatic character of the NOM, whereas a low H/C ratio may be indicative of a more aromatic character and the presence of double bonds. A high O/C ratio indicates the possible presence of carbohydrates and a higher content of COOH groups.

1.3 Concluding Remarks

The work presented here is done in the context of what has come before. The relevance of NOM in the natural environment and its impact require that its physical and chemical characteristics be elucidated. To this end, its isolation must be improved. NOM must be relatively free of inorganic ions for a number of important physical and chemical characterizations. Electrodialysis is a new and more efficient method for the isolation of NOM from freshwater and sea water. The NOM from a diverse set of rivers is processed by electrodialysis and characterized by bioavailability, elemental analysis, capillary zone electrophoresis, nuclear magnetic spectroscopy, and electro-spray ionization mass spectrometry. The results of the characterization of the NOM will elucidate the spatial variability of its bulk elemental chemistry and it bioavailability. It will also be demonstrated that bioavailability and organic carbon measurements, taken together, can be used to make general observations of the bulk chemistry of NOM.

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CHAPTER 2

COUPLING REVERSE OSMOSIS WITH ELECTRODIALYSIS TO ISOLATE NATURAL ORGANIC MATTER FROM FRESHWATERS

2.1 Introduction

Whenever scientific investigations of the chemical properties and reactivity of natural organic matter (NOM) require the collection of significant quantities of NOM from a natural water at a single point in time, the NOM is often converted to a freeze-dried powder. In this state, the likelihood of chemical and/or biological changes in the NOM can be minimized, and its storage and distribution are facilitated. Depending on the intended use of the NOM, it may also be purified by removal of as much inorganic matter as possible before it is freeze-fried. Dry samples are relatively stable for decades and are easily distributed to laboratories around the world, where they are often used for inter-calibration of experimental methods.

2.1.1 Concentration and Isolation Methods

In the past thirty years, several methods have been used to concentrate large quantities of NOM from many types of freshwaters. The most widely applied methods are adsorption of NOM on XAD resins and membrane filtration using ultrafiltration (UF) and reverse osmosis (RO) membranes. General overviews of these methods, their respective advantages and disadvantages, and detailed statistical summaries of the recoveries of NOM by each method have been recently examined by Perdue and Ritchie

(2003). Adsorption on XAD resins has the advantage of yielding low-ash products from which most known biomolecules have been largely removed. Its disadvantages include a median recovery of only about 56% of total organic carbon (TOC) from freshwaters and the exposure of NOM to conditions of extremely acidic and alkaline pH. UF and RO concentrate both NOM and inorganic solutes by selective removal of water across a semi-permeable membrane under very mild chemical conditions (i.e., neither acid nor base is used). The median recoveries of NOM from freshwaters using 100 kD, 10 kD, and 1 kD UF membranes and RO membranes are 31%, 53%, 71%, and 90%, respectively (Perdue and Ritchie, 2003). Only 1 kD UF membranes and RO membranes give median recoveries of NOM that exceed those obtained using XAD resins.

Of all the methods of concentration, reverse osmosis (RO) is clearly superior in terms of the recovery of NOM. On the negative side, some inorganic solutes are also co-concentrated by RO. The most notable exception is borate, which occurs in freshwaters mainly as the uncharged $B(OH)_3$ molecule and is very poorly rejected by RO membranes. Most cations are efficiently removed during the RO process by continuously circulating the sample through an H^+-saturated cation exchange resin. This prevents polyvalent cations such as Ca^{2+}, Al^{3+}, and Fe^{3+} from accumulating and the solubility of $CaCO_3(s)$, $Al(OH)_3(s)$, and $Fe(OH)_3(s)$ from being exceeded. If those solid materials precipitate on the surface of the RO membrane, its performance will be greatly diminished. The decrease in pH resulting from exchange of cations for H^+ also causes inorganic carbon to be largely removed as $CO_2(g)$.

Other anions that produce relatively volatile acids (Cl^- and NO_3^-) are largely eliminated during the freeze-drying process. Of the major solutes in freshwaters, only

sulfate (SO_4^{2-}) and silicic acid (H_4SiO_4) are strongly co-concentrated in freeze-dried samples of NOM (Serkiz and Perdue, 1990; Sun *et al.*, 1995). Minor solutes such as phosphate (PO_4^{3-}) are quantitatively insignificant relative to NOM in most freshwaters, and their removal is not addressed in this research.

2.1.2 Effect of Reverse Osmosis on NOM

The concentration of NOM by RO does not alter its reactivity toward Cu^{2+} and Zn^{2+} (De Schamphelaere *et al.*, 2005), its trihalomethane formation potential (Kitis *et al.*, 2001), or its UV/visible absorptivity (Gjessing *et al.*, 1998). Kilduff *et al.* (2004) examined the effects of concentration by RO on a range of properties of NOM, and they concluded that "RO isolation preserves DOM properties such as size, polarity, charge density, and isoelectric point". Interestingly, if RO-concentrated solutions of NOM are freeze-dried, minor changes have been noted in its molecular weight distribution (Maurice *et al.*, 2002). Given that freeze-drying is generally used, regardless of the method by which NOM is concentrated from waters, its effects of the chemical properties of NOM may warrant further investigation.

2.1.3 Ash Content – Sulfate and Silicic Acid

Solid samples of NOM that are obtained by freeze-drying solutions that have been desalted using an H^+-saturated cation-exchange resin will contain finely dispersed sulfuric acid. The reactions of sulfuric acid with a wide spectrum of organic compounds are very well documented in any textbook of organic chemistry. Whether by condensation, dehydration, polymerization, isomerization, or sulfonation reactions, NOM

may be strongly and irreversibly altered by reactions with sulfuric acid. Any unreacted sulfuric acid in the NOM is a form of titratable acidity that, if not taken into account, will cause the carboxyl content and total acidity of NOM to be over-estimated.

Freeze-dried samples of NOM will also contain silicic acid; however, this very weak acid is not expected to cause chemical modifications of NOM during the freeze-drying process. Nonetheless, the pK_a of silicic acid is in the same range as pK_a values of phenolic hydroxyl groups, so its presence in a sample, if not taken into account, can lead to an over-estimation of phenolic content. Furthermore, some highly useful means of characterization of NOM (e.g., elemental analysis) require low-ash samples and yield erroneous results when ash contents are higher than about five percent.

For the reasons addressed above, it is highly desirable to remove both SO_4^{2-} and H_4SiO_4 from concentrated solutions of NOM before they are freeze-dried. The removal of inorganic solutes from concentrated (by RO) freshwater has previously been addressed. Crum et al. (1996) used RO followed by a series of UF membranes to separate NOM into size fractions. They found that the inorganic solutes, as well as 34% of the total dissolved organic carbon, remained in the low molecular weight fraction. Cantrell (1989), Serkiz and Perdue (1990), and Sun et al. (1997) used an ion retardation resin with a high affinity for strong acids to remove silica (after reaction with HF to convert H_4SiO_4 to SiF_6^{2-}) and SO_4^{2-}. They found that approximately 20% of NOM was lost, especially the more strongly acidic compounds. Mopper (1977) also found that a weak anion exchange resin (HCO_3^- form) can be used to remove SO_4^{2-} from water.

It is also possible to remove silicic acid in the form of amorphous silica, $SiO_2(s)$, which is sparingly soluble in water. If a freeze-dried NOM sample containing $SiO_2(s)$ is

re-dissolved in a minimal amount of water with sufficient added NaOH to maintain circumneutral pH, most, if not all, of the NOM will re-dissolve, but only about 0.002 mol L^{-1} of the $SiO_2(s)$ will re-dissolve. The undissolved $SiO_2(s)$ can be removed by filtration (Maurice et al., 2002). After desalting to remove added Na^+, the solution can be freeze-dried. Although this time-consuming method partially accomplishes the goal of removal of H_4SiO_4, it has the potential to cause fractionation and loss of NOM, if only partial re-dissolution of the NOM is achieved.

2.1.4 Electrodialysis (ED)

In ED, a sample to be desalted and a solution to receive the salts are circulated through adjacent channels of a membrane stack consisting of alternating anion and cation exchange membranes (Figure 2.1). A DC electrical current is directed through this membrane stack (Korngold, 1984; Baker, 2000). The ion exchange membranes are non-porous above the size of the interstitial spaces between the polymer chains in the cross-linked hydrated ion exchange resin.

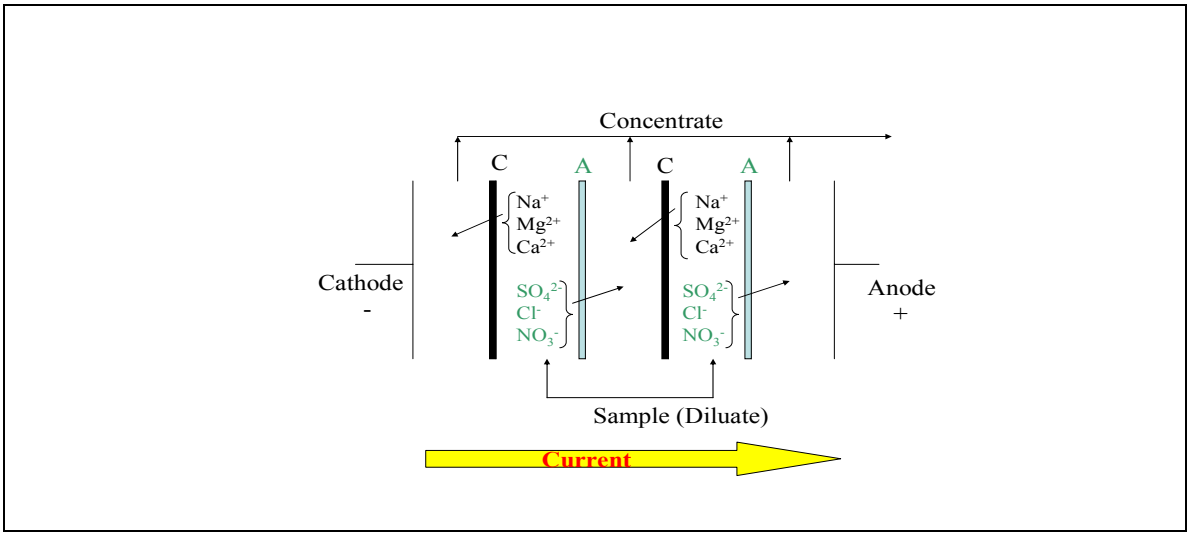

Figure 2.1: Schematic of the electrodialysis process. C and A are the cation and anion exchange membranes, respectively.

Cations can pass only through the cation exchange membrane, and anions can pass only through the anion exchange membrane (commercially available membranes have permselectivities of 98% or higher). The polarity of the applied electric field is chosen to facilitate movement of ions toward the membrane through which they can pass. ED can be used to desalt feed solutions until their conductivities are around 100 μS/cm, which is about two-thirds the conductivity of the world average river.

Jeffrey and Hood (1958) used a method that they termed ED, which was actually conventional dialysis using (non-ion selective) cellulose acetate membranes in the presence of an applied electrical field. As such, that earlier method is not comparable to modern ED, which uses advanced ion exchange membranes that were not available at the time of their study. Schnitzer and Desjardins (1962) used ED to purify soil organic matter. No details of the ED process were given other than the fact that they electro-dialysed the sample until the current fell to less than 25 mA at a potential of 500 V.

Both cations and anions such as SO_4^{2-} can be removed from aqueous solutions by ED, a process in which dialysis of the ions is accelerated by an applied electric field. Electrodialysis rather non-specifically removes ions from a feed solution, although singly-charged ions are removed more readily than are polyvalent ions. Consequently, both major and minor solutes in RO-concentrated solutions of NOM (Na^+, Cl^-, NO_3^-, HPO_4^{2-}, $B(OH)_3$, etc.) will be removed by ED. $B(OH)_3$ (pK_a 9.24) and H_4SiO_4 (pK_a 9.6) will be removed by ED only if the pH of the sample is raised above 9.0 by addition of NaOH so that they may be ionized. Even in waters that contain unusually high concentrations of NO_3^-, HPO_4^{2-}, or $B(OH)_3$, the removal of these solutes by ED is

expected to be straightforward and comparable to the removal of SO_4^{2-} and H_4SiO_4 under the conditions used in this research.

In two recent studies, RO and ED have been combined to isolate NOM from surface waters. Drewes et al. (2002) used monovalent-selective anion exchange membranes for ED, which retained both organic solutes and SO_4^{2-}. No specific attempt was made to remove SO_4^{2-}, and the issue of removing silica was not addressed. Rybacki et al. (1998) applied RO and ED to surface water, and they were able to remove 65% of SO_4^{2-} while recovering 94% of total organic carbon (TOC). Again, the issue of removing silica was not addressed. Many studies of NOM indicate that its number-average molecular weight is around 10^3 and that 50% or more of its acidic functional groups are dissociated at pH > 4 (Perdue and Ritchie, 2003). This combination of large size and high negative charge probably accounts for its selective retention by ED membranes.

2.1.5 Endpoints for Ash Removal

To what extent should SO_4^{2-} and H_4SiO_4 be eliminated from an NOM sample? A practical goal for removal of SO_4^{2-} should be based on the ratio of SO_4^{2-} to NOM, rather than simply on the concentration of SO_4^{2-}, because the undesirable effects of sulfuric acid on NOM should logically depend on the relative proportions of the two in the freeze-dried product. The rationale for establishing this goal depends on the objectives of the research in which the sample of NOM will later be used. Research in this laboratory often deals with the acidic functional groups of NOM, so the goal is defined here in those terms. The sum of the median concentrations of carboxyl and phenolic groups for NOM is 6.7 mmol / g NOM (Perdue and Ritchie, 2003). The arbitrarily chosen goal is to

remove SO_4^{2-} until the H^+ from H_2SO_4 is less than 10% of the total acidity of NOM (6.7 mmol/g). The goal for removal of H_4SiO_4 is to obtain a freeze-dried product containing no more than 5% ash as amorphous silica – SiO_2(s). When expressed on a molar basis, assuming that NOM contains 50% carbon, the SO_4^{2-}/TOC ratio should be less than 0.008, and the H_4SiO_4/TOC ratio should not exceed 0.021. In the context of these goals, the final SO_4^{2-}/TOC ratio of 0.046 that was achieved by Rybacki et al. (1998) using RO and ED is considerably greater than the goal of this research.

It is insightful to apply these goals to the world-average river, which contains 0.11 mM SO_4^{2-}, 0.17 mM H_4SiO_4 (Berner and Berner, 1996), and 0.80 mM TOC (Perdue and Ritchie, 2003). The average molar SO_4^{2-}/TOC ratio is 0.14, and the average molar H_4SiO_4/TOC ratio is 0.21. The operational goals of this study could be achieved in the world-average river, if 94% of SO_4^{2-} and 90% of H_4SiO_4 were removed without any loss of TOC.

In the present study, an experimental protocol for removal of SO_4^{2-} and retention of NOM is developed and tested using concentrated synthetic river waters and applied to NOM samples that were concentrated from freshwaters by reverse osmosis. Because the potential chemical consequences of SO_4^{2-} (through reactions of H_2SO_4 with NOM) are deleterious and irreversible, the major focus of the experiments described herein is the removal of SO_4^{2-}. Nonetheless, experiments are also described in which the potential of ED for removal of H_4SiO_4 is evaluated. In all experiments, the efficacy of the ED process is evaluated based on the recovery of total organic carbon (TOC) rather than on the chemical properties of NOM.

The method of RO/ED is then applied for the concentration and isolation of NOM from five southeastern rivers, demonstrating the applicability of the ED method to remove ash from a wide range of waters. These NOM samples were being used for another project detailed in Chapter 4, but required ED for the project to succeed.

The next chapter will explore the application of ED and RO for the isolation and concentration of marine NOM.

2.2 Methods

2.2.1 Reverse Osmosis

A RealSoft PROS/2S portable RO system, which uses Filmtec TW30-4021 membranes and compatible pressure vessels, was used in this research. In the RO method, a feed solution consisting of water and aqueous solutes is placed under pressure and passed across a semi-permeable membrane, where the feed solution is separated into a permeate solution (relatively lower concentrations of solutes) and a retentate solution (relatively higher concentrations of solutes). As the feed solution is processed, the retentate solution is recycled back to the sample reservoir and the permeate solution is discarded. As more feed solution is added either continuously or discontinuously to the sample reservoir, the concentrations of all solutes that are well rejected by the membrane gradually increase in the sample reservoir. Further details regarding the use of RO to concentrate NOM can be found in Sun et al. (1995) and in Serkiz and Perdue (1990). Perdue and Ritchie (2003) provide a summary of the application of RO for concentration of NOM from freshwaters.

2.2.2 Electrodialysis (ED)

In this research, five pairs of polyvalent CMX (cation exchange) and AMX (anion exchange) membranes (Ameridia) were used. In all experiments, the initial concentrate solution (which receives the cations and anions) was 5.70×10^{-3} M NaCl, and the initial electrode rinse solution was 3.86×10^{-2} M $NaH_2PO_4 \cdot H_2O$. During electrodialysis of a sample, pH and conductivity were measured periodically, and aliquots of the sample (diluate), concentrate, and electrode rinse solutions were collected periodically for analyses of major cations, anions, H_4SiO_4, and TOC. In some of the ED experiments described herein, pH was adjusted at irregular intervals to pH 6 or to pH > 9 by addition of NaOH, and conductivity was likewise adjusted to >1 mS cm^{-1} by addition of NaCl.

Most of the experiments reported here were conducted using a power source that was limited to a maximum of 1 A and a potential of 18.7 V. Three experiments, which will be identified as such when they are discussed, were conducted using a power source capable of generating 3 A and a potential of 50 V. Initial short-duration experiments (< 80 min) were conducted with little effort to maintain constant current in the ED system. For longer-term experiments, the current was adjusted so that the applied voltage was 10 – 15% less than the maximum voltage required to maintain constant current.

During the ED process, the temperature of the sample increased from 20ºC to 31ºC due to the action of the pumps and the resistance of the membrane stack to the applied current (i.e., heat dissipation). This was not considered a problem, because the environmental temperature of NOM is within that range during the summer months.

2.2.3 Cleaning of the Membrane Stack

The cation- and anion-exchange membranes used in the ED system are functionally equivalent to ion exchange resins, in that they bind cations and anions, respectively. During operation of the ED system, a fraction of the cations and anions from the diluate and concentrate are adsorbed to the ion exchange sites in the membranes. The only way to effectively clean the membranes of NOM anions, SO_4^{2-}, and residual cations (Ca^{2+}, Mg^{2+}, K^+) is to use a multi-step cleaning procedure. After a completed ED run, the tanks and membrane stack were drained and rinsed thoroughly with DI water. To solubilize any NOM adhering to the anion exchange membranes, a 0.1 M NaOH solution was added to the sample and concentrate tanks and allowed to flow through the system for a few minutes. After the NaOH solution was drained, a 0.1 M HCl solution was added to the sample and concentrate tanks and allowed to flow into the membrane stack to solubilize any precipitated $CaCO_3$(s) or $MgCO_3$(s) that might have formed. After a few minutes the HCl solution was drained from the stack which was then rinsed with DI water. Finally, to release all residual cations and anions from inside the membranes, a solution of 0.028 M NaCl was added to all three tanks, and ED was performed for a few minutes. This procedure pushed out all the other anions and cations from inside the membranes, replacing them with Na^+ and Cl^-. Failure to do so will result in increases in the concentrations of ions such as Ca^{2+} and Mg^{2+} in the sample and concentrate solutions during the initial phase of ED.

2.2.4 ED Membrane Stack Configuration

The membrane configuration of [cathode – $(CMX – AMX)_5$ – anode] was used in this study, so the electrode rinse was, in effect, a receiving solution for ions from the

sample. This resulted in a slight increase in pH, conductivity, and concentrations of ions in the rinse solutions as the ED proceeded. Although there was no harm in setting up the stack in this manner, it may be a concern if there was interest in preventing any of the rinse anions from migrating into the sample, as might have occurred in the present set up. Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_20$) was used in this study as the rinse solution, so it is at least possible that PO_4^{3-} ions could seep into the sample. This possibility could be eliminated by using an extra cation exchange membrane, having monovalent selectivity, at each end of the membrane stack.

2.2.5 Limiting Current Density

The limiting current density is the amperage per unit area of membrane where ions are depleted at the membrane interface and mass transfer of ions by diffusion occurs (Korngold, 1984). This occurs at the anion exchange membrane, and a concurrent drop in sample pH and rise in concentrate pH are observed. These changes are due to water splitting in the absence of sufficient ions to maintain the current (Spiegler, 1962). A further consequence is that scaling (precipitation) by $CaCO_3(s)$ and $MgCO_3(s)$ may occur on the concentrate side of the anion exchange membranes. To prevent such phenomena, the ED system was operated at 10 –15% below the limiting current density.

2.2.6 Analysis of TOC and Inorganic Solutes

TOC concentrations were determined by using either UV-persulfate oxidation (Sievers 800 TOC analyzer) or high temperature catalytic oxidation (Shimadzu TOC-VCSN). Concentrations of Ca^{2+}, Mg^{2+}, K^+, and Na^+ were measured by flame atomic absorption spectrometry (Perkin-Elmer 3100). Concentrations of SO_4^{2-} were measured

by ion chromatography (Metrohm Compact 761) or spectrophotometrically, using the dimethlysulfonazone III (DMS) method (Reijnders et al., 1979). Concentrations of Cl^- were measured by ion chromatography (Metrohm Compact 761) or spectrophotometrically using the thiocyanate method (Standard Method 4500–Cl^- E, modified to be done manually). Silica was measured by the Heteropoly Blue Method (Standard Method 4500 - SiO_2 D) (Standards Methods, 1989).

2.2.7 Preparation of Concentrated Synthetic River Water

Twelve *concentrated* synthetic river water samples (Table 2.1) were prepared by dissolving appropriate quantities of reagent-grade inorganic chemicals in deionized water and equilibrating with air. The chemical compositions of most of the concentrated samples are intended to represent several-fold concentrates of natural waters having realistic ranges of TOC, hardness (Ca, Mg), sulfate, silica, and pH. In some cases, Na_2SO_4(s) and NaCl(s) were used to prepare solutions, but, for simplicity, those cases are represented in this Table by appropriate additions of H_2SO_4, HCl, and NaOH. The chemical speciation model MINTEQA2 (Allison et al., 1990) was used to model the chemistry in the final solutions. Some of the solutions were predicted to be supersaturated with respect to one or more of the minerals calcite, magnesite, and amorphous silica. In practice, measured concentrations of Ca^{2+}, Mg^{2+}, and H_4SiO_4 were only slightly lower than expected, and concentrations of Ca^{2+} and Mg^{2+} were quickly lowered by the ED process.

Table 2.1: Chemical recipes for all solutions used to develop and optimize the ED system[a]

Experiment	$CaCO_3$(s)	$MgCO_3$(s)	SiO_2(s)	SRNOM	H_2SO_4	NaOH(aq)	HCl(aq)	pH
1	5.98E-03	2.97E-03	1.98E-03	3.73E-03	2.78E-04	1.03E-02	2.08E-02	8.0
2	5.99E-03	2.99E-03	4.74E-04	3.80E-03	6.51E-04	1.25E-02	2.25E-02	8.0
3	6.00E-03	2.99E-03	1.98E-03	3.70E-03	9.30E-04	9.39E-03	1.88E-02	8.0
4	6.00E-03	2.99E-03	9.82E-04	1.85E-03	6.51E-04	2.15E-03	1.88E-02	4.0
5	9.99E-03	4.99E-03	1.98E-03	3.70E-03	2.78E-04	1.62E-02	3.13E-02	8.0
6	9.99E-03	4.99E-03	1.98E-03	3.70E-03	6.51E-04	1.68E-02	3.13E-02	8.0
7	9.99E-03	4.99E-03	1.98E-03	3.70E-03	9.30E-04	1.73E-02	3.13E-02	8.0
8	0	0	0	2.19E-03	8.80E-04	3.07E-02	2.80E-02	7.8
9	0	0	0	1.84E-03	3.50E-04	5.69E-03	1.85E-03	8.7
10	0	0	0	2.34E-03	8.80E-04	2.60E-02	2.39E-02	7.3
11	6.45E-03	3.66E-03	1.14E-03	4.83E-03	1.47E-03	5.26E-03	1.80E-02	8.5
12	6.59E-03	3.74E-03	2.33E-03	9.86E-03	1.50E-03	7.74E-03	1.84E-02	8.8

[a] All solutions were equilibrated with atmospheric CO_2(g), and all concentrations are in molar units.

2.2.8 Sampling

Two freshwaters in south Georgia (Suwannee and Withlacoochee rivers) were selected for evaluation of the RO/ED methodology on real freshwaters. Not only are these rivers readily accessible and familiar to this research group but their chemical compositions are also quite distinct from one another, insofar as most of the relevant water quality parameters are concerned. The Suwannee River was sampled on March 31, 2005 at the first sill on the southwestern edge of the Okefenokee National Wildlife Refuge. A 500-L sample was concentrated by RO to a final volume of 24.7 L. The Withlacoochee River was sampled on May 4, 2005 at a site located beside Georgia highway 31 at the Georgia-Florida border. At this site, a 501-L sample was concentrated by RO to a final volume of 19.6 L. In each case, after the retentate solution from the RO process was removed from the sample reservoir and drained as well as possible from the RO system, the RO system was rinsed with dilute NaOH (~ 0.01 M) to recover NOM that

was adsorbed on the RO membrane. This alkaline rinse was combined with the retentate solution to obtain the final concentrated NOM solution. Samples were transported back to the laboratory on ice and immediately stored at 4ºC.

Six south-eastern rivers were sampled in July and August of 2001, using the same protocol as described in the previous paragraph. Five of the samples were also used as further proof of the applicability of the ED method to remove ash from a wide range of river waters. Details of the samples can be found in Chapter 4 section 4.3.2.

2.2.9 Charge Balance

From the beginning to the end of an ED experiment, cations and anions are removed in opposite directions within the ED stack. Ions in the sample undergo ion exchange with ions already residing in the exchange sites of the cation and anion exchange membranes, solutions are withdrawn for chemical analyses, and solutions are added to adjust pH and/or conductivity. Depending on the timing of such events, the concentrations of cations and anions in the sample may increase or decrease with time. In either case, solutions must remain charge-balanced at all times. In some of the experiments that follow, the quality of analytical data was evaluated by this criterion – the sum of positive charges must equal the sum of negative charges. All concentrations of ions were obtained experimentally, except for the charge residing on the NOM. NOM was measured in the form of TOC, and then the bimodal Gaussian distribution model (Perdue and Lytle, 1983; Perdue *et al.*, 1984) was used to calculate the pH-dependent charge (in eq/L) that can be attributed to the ionized acidic functional groups of NOM.

The model presumes the existence of two broad symmetrical distributions with respect to their log K values for proton binding.

$$\sum[Org^-] = [TOC]\left[\frac{Q_1}{\sigma_1\sqrt{2\pi}}\int_{-\infty}^{+\infty}\left(\frac{1}{1+K\{H^+\}}\right)\exp\left(-\frac{1}{2}\left(\frac{\mu_1-\log K}{\sigma_1}\right)^2\right)d\log K + \right.$$
$$\left.[TOC]\left[\frac{Q_2}{\sigma_2\sqrt{2\pi}}\int_{-\infty}^{+\infty}\left(\frac{1}{1+K\{H^+\}}\right)\exp\left(-\frac{1}{2}\left(\frac{\mu_2-\log K}{\sigma_2}\right)^2\right)d\log K\right]\right]$$

where [TOC] is the analytical concentration of total organic carbon (in g C/L), Q_1 and Q_2 are the maximum concentrations of carboxyl and phenolic groups in NOM (in eq/g C), respectively, μ_1 and μ_2 are their respective mean proton binding constants, and σ_1 and σ_2 are fitting parameters that increase with increasing width of the distribution of log K values for proton binding. The values of Q_1, Q_2, μ_1 and μ_2, σ_1 and σ_2 for the Suwannee River NOM are 0.01016 eq/g C, 0.00314 eq/g C, 3.72, 9.67, 2.24, and 0.65, respectively. The same fitting parameters were used for NOM samples from both the Suwannee and Withlacoochee rivers.

An analysis of charge balance in the sample (diluate) at the beginning and end of each experiment not only evaluates the quality of analytical measurements but also provides a more comprehensive assessment of the overall performance of the ED system for removal of common cations and anions. The electro neutrality condition must be satisfied in any aqueous solution, and, in this study, the electro neutrality equation is:

$$[H^+] + [Na^+] + 2[Ca^{2+}] + 2[Mg^{2+}] + [K^+] = [OH^-] + 2[SO_4^{2-}] + [Cl^-] + [HCO_3^-] + \Sigma[Org^-]$$

where $\Sigma[Org^-]$ is the pH-dependent charge of organic anions in NOM for each sample.

2.2.10 Recoveries of TOC and Sulfate

During the course of an experiment, aliquots of the diluate, concentrate, and rinse solutions were collected periodically for chemical analysis. A proper mass balance for the process must consider those quantities of water, TOC, and inorganic solutes that were sacrificed for these analyses. Any water or solutes that were added to adjust pH (with NaOH or HCl) or conductivity (with NaCl) must also be considered. Accordingly, recoveries of TOC and SO_4^{2-} that are reported subsequently are the result of such corrections.

2.3 Results and Discussion

2.3.1 Concentrated Synthetic River Water

The initial phase of this research was designed to optimize a method of purification of NOM using ED, which would normally be used to process NOM solutions that have been concentrated in the field by RO. During the RO process, major cations are necessarily removed (to avoid fouling of the RO membrane) by cation exchange for H^+, which also causes HCO_3^- to be removed as $CO_2(g)$. The concentrated synthetic river water samples may be considered to be hypothetical RO concentrates in which not only NOM but also all inorganic solutes have been concentrated. As such, they represent a worst-case scenario that might be encountered when ED is used to desalt NOM samples that have been pre-concentrated by RO.

The first seven experiments (Experiments 1-7 in Table 2.1), which were of short-duration (< 80 min), were conducted to obtain rough estimates of the rate of removal of SO_4^{2-} and TOC and to assess the overall extent of mass and charge balance in the entire

ED system (diluate, concentrate, and rinse solutions). At the beginning of each experiment, only the sample (diluate) contained SO_4^{2-}; however, the sample, concentrate, and rinse solutions contained measurable quantities of SO_4^{2-} at the end of each experiment. Table 2.2 contains a summary of analytical results for these experiments. Most of the 6% loss of TOC was due to adsorption on the membranes.

Table 2.2: Summary of results of ED experiments on concentrated synthetic river water samples [a]

Parameter [b]	Expts. 1-7	Expts. 8 - 9	Expts. 10 - 12
pH	6.1 ± 1.8	7.1 ± 2.1	5.5 ± 1.4
Run Time (hr)	1.08	2.50	8.33
Initial Ratios:			
SO_4^{2-}/TOC	0.19	0.30	0.28
H_4SiO_4/TOC	0.48	0	0.26[c]
Yields (%):			
SO_4^{2-}	49	41	10
TOC	94	95	92
H_4SiO_4	87	n.d.	n.d.
Final Ratios:			
SO_4^{2-}/TOC	0.12	0.15	0.032
H_4SiO_4/TOC	0.38	n.d.	n.d.
Mass balance (%):			
SO_4^{2-}	96	n.d.	n.d.
TOC	94	n.d.	n.d.
H_4SiO_4	90	n.d.	n.d.
Charge balance:			
meq L^{-1}	0.4 [d]	n.d.	n.d.

[a] Results are average values within each group of experiments. Average mass balances were calculated by combining analytical data for the diluate, concentrate, and rinse solutions. Average yields were calculated from the concentrations of the solutes in the diluate solutions.
[b] SO_4^{2-}/TOC and H_4SiO_4/TOC are molar ratios.
[c] For experiments 11 and 12 only.
[d] Root mean square error.

As an indication that all parameters were considered for these experiments and that they were conducted rigorously, a mass balance of charge was calculated for Experiments 1-7 (Table 2.3). For all solutions, the root mean square error in the charge balance is 0.5 meq L^{-1}.

Table 2.3: Analysis of initial and final charge balance in ED test solutions of concentrated synthetic river water. The root mean square error in the charge balance is 0.5 meq L^{-1}.

	Initial Charge Balance (meq L^{-1})			Final Charge Balance (meq L^{-1})		
Experiment	**Cations**	**Anions**	**Diff.**	**Cations**	**Anions**	**Diff.**
1	21.4	21.1	0.3	5.7	5.6	0.1
2	23.0	23.9	-0.9	5.9	4.8	1.1
3	20.5	21.2	-0.7	6.4	6.7	-0.3
4	19.2	19.3	-0.1	4.7	4.6	0.1
5	30.1	30.3	-0.2	5.2	5.1	0.1
6	31.1	31.4	-0.3	7.2	7.2	0.0
7	32.0	32.1	-0.1	7.0	7.2	-0.2

The concentrations of TOC and SO_4^{2-} in the diluate solutions at the end of each experiment are plotted (as % remaining) versus time in Figure 2.2 (shaded symbols). The results are comparable for all seven solutions, even though the solutions initially contained significantly different concentrations and proportions of all solutes. This observation suggests that ED may be a suitable treatment for a wide range of freshwaters. The average molar SO_4^{2-}/TOC ratio in these short-duration experiments was only reduced from 0.19 to 0.12, which falls considerably short of the goal of this research, 0.008.

In preliminary attempts to fine-tune the ED process (not shown), it was determined that efficient removal of SO_4^{2-} and retention of TOC could both be achieved, if the pH of the sample was maintained above pH 6 by occasional additions of NaOH and

conductivity was maintained above 1 mS cm^{-1} by occasional additions of NaCl. To further optimize the ED protocol for removal of SO_4^{2-}, ED was performed on another series of concentrated synthetic river water samples (Experiments 8 – 12 in Table 2.1) while changing the conditions of amperage, current, and flow rate of the ED process and the pH and conductivity of the sample. The first three experiments (8 – 10) only contained TOC, SO_4^{2-}, Na^+, and Cl^-; however, the last two experiments (11-12) include the full suite of major solutes that are commonly found in RO-concentrated river water. Selected analytical results for Experiments 8 – 12 are presented in Table 2.2.

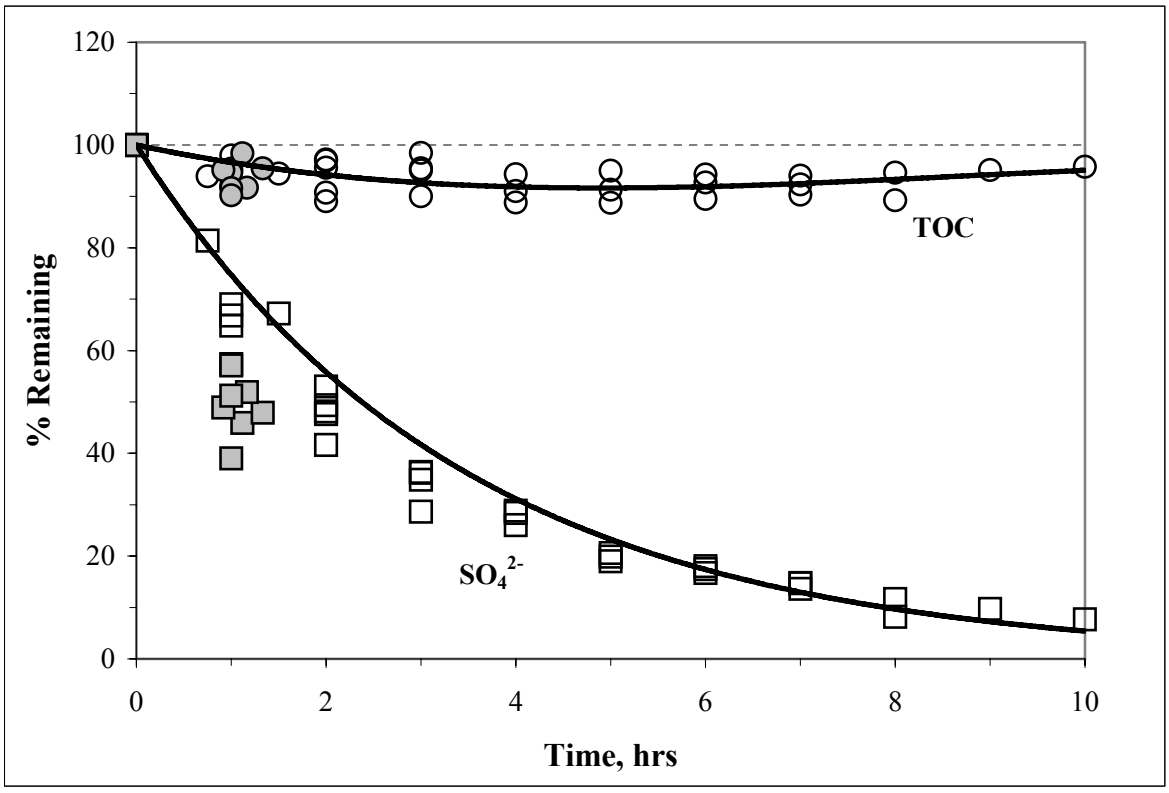

Figure 2.2: ED removal of SO_4^{2-} and recovery of TOC from concentrated artificial river water. Shaded symbols are for the seven initial short-duration experiments (Experiments 1 – 7) and open symbols are for the optimized experiments (Experiments 8 -12).

The time dependence of the removal of SO_4^{2-} and the recovery of TOC (Figure 2.2 – open symbols) was not strongly dependent on the initial compositions of the samples. Both processes were non-linear, and the rate of removal of SO_4^{2-}, in particular, appears to follow pseudo first-order kinetics. The fitted curves in Figure 2.2 indicate that 95% of SO_4^{2-} is removed and 95% of TOC is recovered when samples are processed by ED for 10 hours. Based on the average concentrations of SO_4^{2-} and TOC in these five samples, the molar SO_4^{2-}/TOC ratio is reduced from 0.29 to 0.013 after 10 hours of processing by ED, which is considerably better than achieved in the short-term experiments but still somewhat short of the goal of this research (0.008).

As was the case in the short-duration experiments, a small percentage (5 – 8%) of TOC was lost in the longer-term experiments (8 – 12), presumably by adsorption to the membranes. Most of this loss occurred within the first hour of ED (see Figure 2.2). Rybacki et al. (1998) reported an average loss of 9.5 ± 5% of TOC when using ED to desalt nine samples of freshwater that had been pre-concentrated by RO. Lee et al. (2002), also using AMX (anion exchange) membranes, lost 9.1% of TOC from the sample. Kim et al. (2002) examined the effects of pH and ionic strength on adsorption of NOM to an AMX anion exchange membrane, and they found that adsorption is greater at high pH and low ionic strength. They concluded that, at higher ionic strength, Cl^- ions in the background electrolyte out-compete NOM for anion exchange sites. They interpreted the greater adsorption of NOM at higher pH as evidence for high concentrations of phenolic hydroxyl groups in the NOM, even greater than the carboxyl content of the NOM. This explanation is inconsistent with most published data on the acidic functional groups of NOM and related materials (see Ritchie and Perdue, 2003).

2.3.2 Isolation of NOM from the Suwannee and Withlacoochee Rivers

Concentrated solutions of NOM were collected using reverse osmosis from the Suwannee and Withlacoochee rivers in south Georgia. Selected parameters of the original river waters and their RO concentrates are given in Table 2.4. As anticipated, TOC was recovered by RO in relatively high yield for both the Suwannee (92%) and Withlacoochee (82%) rivers. Both results are close to the reported median recovery of TOC by RO of 88% (Perdue and Ritchie, 2003). Inorganic anions and silicic acid were also recovered in relatively high yield. The lower recoveries of major cations are the result of using an H^+-saturated cation exchange resin to partially remove those ions during the RO process, so that $CaCO_3$(s) and related inorganic precipitates would not form and foul the RO membrane. The concentrated NOM solution from the Withlacoochee River was again desalted in the laboratory to more completely remove major cations. Prior to ED, the pH values of concentrated NOM solutions were adjusted to circumneutral pH (see earlier discussion), either by addition of NaOH or HCl.

Table 2.4: Selected chemical properties of original river waters and RO-concentrated samples.[a]

	Suwannee River			Withlacoochee River		
Parameter	Original	Concentrate	RO Yield	Original	Concentrate	RO Yield
Volume (L)	500	24.7		501	19.6	
pH	4.22	3.26		8.08	9.93	
TOC	4.2E-03	7.8E-02	92	1.0E-03	2.1E-02	82
SO_4^{2-}	2.0E-06	4.2E-05	104	2.5E-05	5.9E-04	92
Cl^-	1.2E-04	1.4E-03	58	2.0E-04	3.4E-03	67
H_4SiO_4	7.0E-05	1.3E-03	92	7.1E-05	1.3E-03	72
Ca^{2+}	2.2E-05	5.8E-05	13	1.9E-04	1.3E-03	27
Mg^{2+}	1.6E-05	4.2E-05	13	7.2E-05	6.8E-04	37
Na^+	1.2E-04	n.d.	n.d.	1.5E-04	n.d.	n.d.
K^+	0	1.1E-05	----	5.3E-05	6.2E-04	46

[a] Concentrations are in mole/L, and yields are percentages.

2.3.3 ED of the Concentrated NOM Solutions

The molar SO_4^{2-}/TOC and H_4SiO_4/TOC ratios in the concentrated Suwannee NOM solution (see Table 2.4) are calculated to be only 0.00054 and 0.017, respectively, both of which are already below the stated goals of this study, 0.008 and 0.021, respectively. Even though no further processing of this sample was actually necessary, ED was performed on this sample. The average molar SO_4^{2-}/TOC and H_4SiO_4/TOC ratios in the concentrated Withlacoochee NOM solution are calculated to be 0.028 and 0.062, respectively, so ED was definitely required for that sample.

Results from five experiments on the concentrated NOM samples are given in Table 2.5. The recoveries of TOC and SO_4^{2-} in the samples through the course of these experiments are shown in Figure 2.3. These results are similar to those in Figure 2.2 for the synthetic samples that have already been discussed. After 10 hours of processing the sample of Suwannee NOM, 41% of the already low SO_4^{2-} was removed, and 94% of the TOC was recovered. The final molar SO_4^{2-}/TOC ratio was 0.00030. After nearly 15 hours of processing the sample of Withlacoochee NOM, 74% of the SO_4^{2-} was removed, and 88% of the TOC was recovered. The final molar SO_4^{2-}/TOC ratio was 0.010.

The last three experiments on Withlacoochee NOM were conducted using the more powerful power supply. These experiments differ from one another in processing time and pH. Run #2 was conducted in the same pH range as the previously discussed experiments on the Suwannee and Withlacoochee NOM samples. In Run #3 and Run #4, however, pH was adjusted to pH 9.2 – 9.6, so that H_4SiO_4 can be partially converted into its conjugate base $H_3SiO_4^-$. It was anticipated that this adjustment would allow at least partial removal of silica by ED.

Table 2.5: Summary of results of ED experiments on RO-concentrated NOM samples from the Suwannee and Withlacoochee rivers [a]

Parameter [b]	Suwannee	Withlacoochee			
	Run #1	Run #1	Run #2	Run #3	Run #4
pH	6.0 ± 0.3	5.8 ± 0.8	6.0 ± 0.6	9.2 ± 0.3	9.6 ± 0.2
Run Time (hr)	9.97	14.83	5.83	4.67	9.00
Initial Ratios:					
SO_4^{2-}/TOC	0.00054	0.028	0.028	0.028	0.028
H_4SiO_4/TOC	0.017	0.062	0.062	0.062	0.062
Yields (%):					
TOC	94	88	101	102	102
SO_4^{2-}	59	26	26	27	21
H_4SiO_4	n.d.	n.d.	n.d.	56	35
Final Ratios:					
SO_4^{2-}/TOC	0.00030	0.0100	0.0082	0.0094	0.0067
H_4SiO_4/TOC	n.d.	n.d.	n.d.	0.038	0.025
Mass balance:					
SO_4^{2-}	n.d.	n.d.	n.d.	n.d.	n.d.
TOC	95%	93%	107%	n.d.	n.d.
Charge balance:					
meq L^{-1}	0.5[c]	1.7	3.6	n.d.	n.d.

[a] Results are average values within each group of experiments. Average mass balances were calculated by combining analytical data for the diluate, concentrate, and rinse solutions. Average yields were calculated from the concentrations of the solutes in the diluate solutions. A more powerful power supply was used in Run #2 – Run #4.

[b] SO_4^{2-}/TOC and H_4SiO_4/TOC are molar ratios. Removal of H_4SiO_4 was only attempted in Run #3 and Run #4 for the Withlacoochee NOM sample.

[c] Root mean square error.

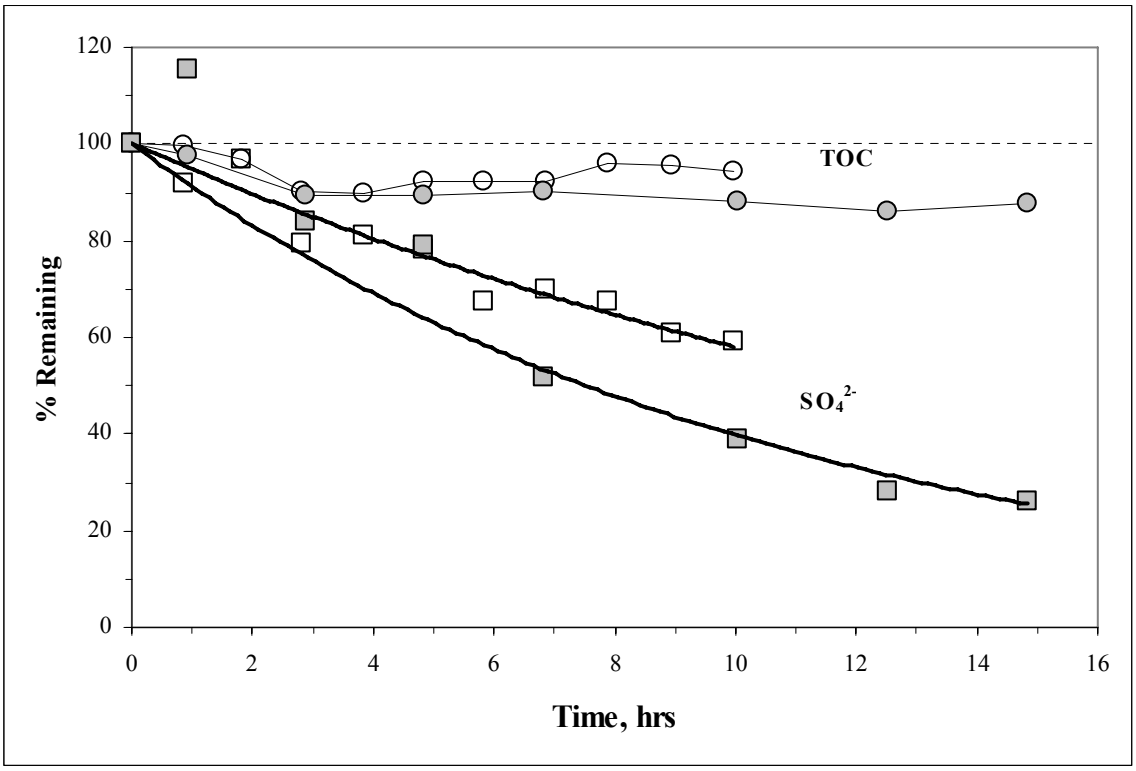

Figure 2.3: Recoveries of TOC and SO_4^{2-} during ED of the Suwannee (open symbols) and Withlacoochee (shaded symbols) NOM solutions.

The final molar SO_4^{2-}/TOC ratio for Run #2 was 0.0082. It was noted in this run that the minimum recovery of TOC after 1 hour of processing corresponds to the minimum Cl^- concentration in this experiment (data not shown). This observation supports the hypothesis of Kim et al. (2002) that Cl^- and TOC anions are in a competitive equilibrium for anion exchange sites in the anion exchange membranes. In Run #3 and Run #4, the final molar SO_4^{2-}/TOC ratios were 0.0094 and 0.0067, respectively. For the four experiments on Withlacoochee NOM, the average removal of SO_4^{2-} was 75%, the average recovery of TOC was 98%, and the average final molar SO_4^{2-}/TOC ratio was 0.0086, which is only slightly greater than the goal of this study. The goal could easily

be reached by increasing the number of ED cells in the membrane stack, the current, the processing time, or almost any combination thereof.

The main purpose of Run #3 and Run #4 was to examine the feasibility of using ED at high pH to remove silica from concentrated NOM solutions. The results reported here show that 44% of H_4SiO_4 was removed in 4.67 hr of ED in Run #3, and 65% of H_4SiO_4 was removed in 9.00 hr of ED in Run #4. The final molar H_4SiO_4/TOC ratios in Run #3 and Run #4 were 0.038 and 0.025, respectively, both of which are slightly greater than the goal of 0.021. It is believed that possible presence of organo-silica complexes (Marely et al., 1989) will hinder the formation and removal of $H_3SiO_4^-$ across the anion exchange membranes; however, Beck et al. (1974) did not detect such complexes in southeastern rivers. Again, minor improvements in operating parameters and a longer processing time should be adequate to obtain the goal of this study.

This research has demonstrated that the goals for removal of SO_4^{2-}, removal of H_4SiO_4, and recovery of TOC are all attainable, albeit with longer-than-desired processing times using the experimental apparatus described herein. The rate of desalting by ED is a function of chemistry (pH, conductivity), power (voltage, current), and total area of ion exchange membranes (the number of membrane pairs). To a significant degree, the chemical parameters have been optimized in this study. Future development of the coupled RO/ED method will address the optimization of the non-chemical parameters for ED. At constant chemical conditions and constant current, the rate of desalting can be increased by increasing the number of membrane pairs in the stack. Because resistance of the membrane stack increases more-or-less linearly as more membrane pairs are added, the growth of the stack must be coupled with a more powerful

source of electricity. With such enhancements, it is fully anticipated that the results of this study for desalting of NOM from the Suwannee and Withlacoochee rivers can be achieved for most other freshwaters.

2.3.4 ED of Five South-eastern River NOM Samples

In a separate project detailed in Chapter 4, the RO-concentrated NOM from five south-eastern rivers was also subjected to ED. All five samples initially had ash contents > 49% by weight (Table 2.6). The conditions of the ED process differed somewhat from the procedure used for the synthetic and concentrated NOM samples described above. For these five NOM samples, an ED stack with 25 membrane pairs was used, instead of 10 membrane pairs. The results of ED are in Table 2.7. The % of TOC recovered is lower for almost all the samples than that found previously. The % recovery of TOC ranged from 77 -92% (average = 81%) which is significantly less than the average of 96% recovered for earlier samples. It is believed that the cleaning protocol for the stack whereby adsorbed NOM is recovered needs to be improved; the procedure as described was suitable for a 10 membrane stack but requires more rigor and time to succeed for a 25 membrane stack where the surface area for adsorption of NOM is 2.5 times more. Visual inspection of the concentrate during these latter experiments leads us to believe that the membranes were slowly being compromised, requiring replacement. The final SO_4^{2-}/TOC ratios were all less than the goal of 0.008, with the exception of the Broad NOM at 0.011 (Figure 2.4). Silica removal was difficult: the operational goal of an H_4SiO_4/TOC ratio of < 0.021 was achieved for only two of the five samples and almost achieved for a third (Congaree). Removal of silicic acid is a slow process because only

40% of the silicic acid molecules (pK_a 9.6) are ionized at a pH of 9.2. Recovery of TOC was lower for the Congaree and New NOM as the ED stack washes are not included in the recoveries shown. It is not known why the %TOC recovery for the Broad NOM was also low. With the exception of the Broad NOM, the final SO_4^{2-}/TOC ratios were all < 0.008 and H_4SiO_4/TOC ratios were all < 0.028.

Table 2.6: Characteristics of five southeastern river NOM samples isolated by RO.

	pH	DOM (mg C L^{-1})	Concentrate DOM (mg C L^{-1})	% Recovery of DOM in Concentrate	% Ash*
Withlacoochee (GA)	7.48	10.7	104.7	93	49.6
Congaree (SC)	7.63	3.43	68.5	83	77.4
New (VA)	7.61	1.84	30.8	94	72.1
Ocmulgee (GA)	8.16	2.02	34.7	100	83.2
Broad (SC)	7.70	3.45	59.4	92	76.7

* Initial ash contents, determined by elemental composition of freeze-dried sample.

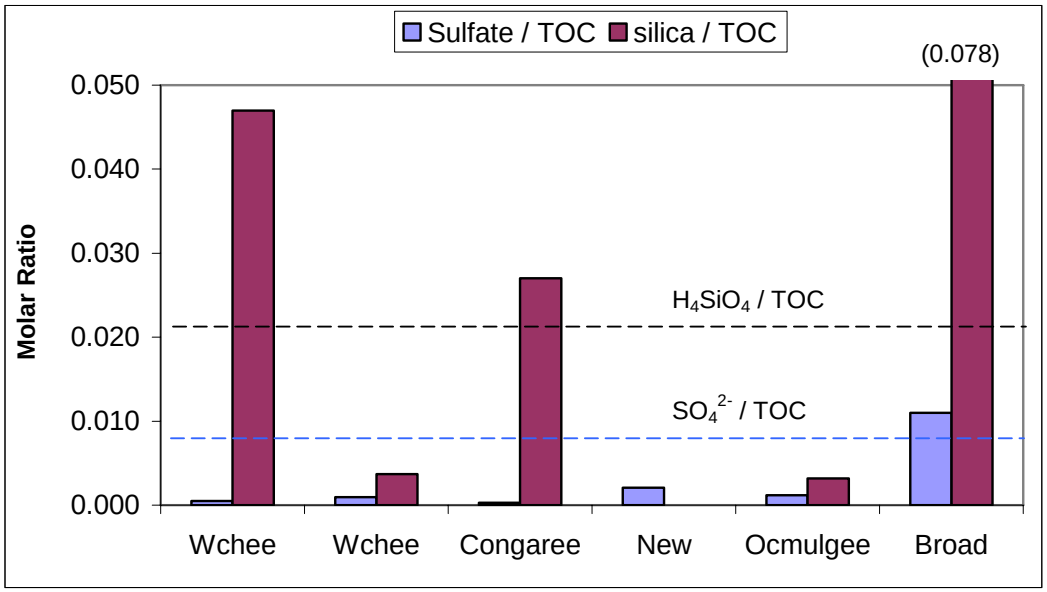

Figure 2.4: RO/ED of five south-eastern river NOM samples. The SO_4^{2-} / TOC goal was exceeded for all samples and almost achieved for the Broad NOM. Removal of silicic acid was more difficult (see text).

Table 2.7: Summary of results of ED experiments on RO-concentrated NOM samples from five southeastern rivers.

Parameter	Wchee[b]	Wchee[c]	Congaree	New	Ocmulgee	Broad
pH	8.0 ± 1.5	9.6 ± 0.3	9.2 ± 0.6	8.5 ± 1.5	9.2 ± 0.9	8.9 ± 1.1
Run Time (hr)	18.25	6.33	4.77	5.58	4.00	5.70
% Recovered:						
TOC	77	92	73	78	84	79[*]
Initial Ratios[a]:						
SO_4^{2-}/TOC	0.091	0.034	0.0022	N/A	0.65	0.48
H_4SiO_4/TOC	0.053	0.070	0.038	N/A	0.015	0.24
% Removed:						
SO_4^{2-}	99	99[d]	90	N/A	99	98
H_4SiO_4	30	89	49	N/A	82	74
Final Ratios[a]:						
SO_4^{2-}/TOC	0.0005	0.00096	0.00027	0.0021	0.0012	0.011
H_4SiO_4/TOC	0.047	0.0037	0.027	N/A[e]	0.0032	0.078

[a] Molar ratios; [b] Analyzed by CZE, NMR, MS; [c] Analyzed for elemental composition; [d] Includes a filtering step; [e] It appears that silica may have been desorbing from the ED membrane into the sample.
[*] Does not include washing of ED stack.
N/A: Not available.

2.4 Conclusions

ED experiments were conducted on synthetic freshwater samples whose chemical compositions most closely resemble those of un-desalted freshwater samples that have been concentrated by reverse osmosis. ED experiments were also conducted on RO-concentrated solutions of NOM from two rivers. These experiments have shown:

- The selective removal of SO_4^{2-} and retention of TOC were best achieved at pH > 6 and conductivity > 1 mS cm^{-1}. If the ED process was conducted at pH > 9, H_4SiO_4 could also be removed to a significant extent.
- When the RO and ED processes were applied in tandem to freshwaters using optimal conditions for ED, 88 ± 11% of TOC was recovered, 83 ± 19% of SO_4^{2-} was removed, and 67 ± 17% of H_4SiO_4 was removed. More importantly, the molar ratios of SO_4^{2-}/TOC and H_4SiO_4/TOC were reduced to 0.0046 and 0.032, respectively, thus surpassing the goal for removal of SO_4^{2-} and almost achieving the goal for removal of H_4SiO_4.
- The ED process can lower the SO_4^{2-}/TOC ratio in samples whose initial SO_4^{2-}/TOC ratios are already far below the limit of 0.008 used in this study.
- The degree to which SO_4^{2-} and H_4SiO_4 can be removed depends on the amount of time used for the ED process, on chemical conditions of pH and conductivity, and an adequate supply of electric current.
- Six southeastern rivers were sampled in July and August of 2001, using the same protocol as described in the previous paragraph. These samples were collected for another project but were also used as a proof of the applicability of the ED method to remove ash from a wide range of river waters.

Although some further enhancements are planned for the near future, the coupled RO/ED process that has been described in this study offers a fast, simple, chemically mild (relative to other methods), and reproducible method of isolation of large quantities of relatively unfractionated, low-ash NOM from freshwaters.

The next chapter will explore the application of ED and RO for the isolation and concentration of marine NOM.

2.5 Acknowledgments

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CHAPTER 3

COUPLING ELECTRODIALYSIS WITH REVERSE OSMOSIS TO ISOLATE NATURAL ORGANIC MATTER FROM SEAWATER

3.1 Introduction

3.1.1 Overview

This chapter explores the combined application of electrodialysis (ED) and reverse osmosis (RO) for the isolation and concentration of marine DOM. Due to the high inorganic content of seawater, the isolation of marine DOM requires that electrodialysis be used first to remove most inorganic solutes, followed by reverse osmosis to concentrate the NOM. The isolation and concentration of DOM from freshwater required RO followed by ED (see Chapter 2). Again, the aim of this research is to produce relatively ash-free, freeze-dried NOM that is useful for scientific investigations of its chemical properties and reactivity.

To date, marine DOM can at best be recovered with an efficiency of ~30%. The unrecovered 70% of the marine DOM may not be represented in the fraction being collected, thereby omitting entire classes of DOM.

Although the concentration of DOC in the oceans is relatively small (~40 to 80 uM), the size of the ocean reservoir means that the total DOC present is comparable to the quantity of organic carbon in terrestrial biomass. Given that oceanic DOM is part of the global carbon cycle, a better understanding of its chemistry and reactivity would provide valuable additional insight.

The hypothesis for this research is that the isolation and concentration of marine DOM by electrodialysis and reverse osmosis yields recoveries of DOM exceeding the current state of the art (~ 30%). The isolation and concentration of marine DOM constitutes a major step in a more complete characterization of its constituents.

3.1.2 Isolation of Marine DOM

To the author's knowledge, ED has not been used to isolate marine DOM. ED has been used on saltwater samples for different purposes. Jeffrey and Hood (1958) used dialysis membranes and an electric field to move ions across the membrane, but this method is not comparable to modern ED. The desalination of seawater using a combined RO/ED system was attempted by Schmoldt et al. (1981), but they were neither interested in the presence nor the fate of marine DOM. ED has been used to isolate specific classes of organic compounds, namely amino acids and carbohydrates, from highly saline solutions and from seawater. Montiel et al. (1998) and Resbeut et al. (1998) both recovered 85% of their target amino acids from phosphate and sulfate salt solutions. Josefsson (1970), Hirayama (1974), and Mopper et al. (1980) recovered 89% - 100% of added sugars to seawater samples. The results of these studies demonstrate that low molecular weight organic solutes are well retained by ED membranes – a prerequisite for successful application of ED for recovery of DOM from seawater.

The isolation and characterization of marine DOM represents a challenge due to its relatively low concentrations: ~ 80 uM for surface and ~ 40 uM for deep ocean water (Sharp et al., 1993, 1995) as well as the high salt content of the water. To date, the most effective method for concentrating marine DOM has been tangential flow UF, but this

method only recovers 30 – 35% of the DOM (Benner et al., 1992; Perdue and Ritchie, 2003) while losing the entire low molecular weight fraction (<1000 D) (Benner 1992). XAD, another commonly used method, isolates 5-25% of the NOM (Benner, 1998).

RO which very successfully concentrates freshwater NOM with an average recovery of ~88% also co-concentrates inorganic solutes. The co-concentration of inorganic solutes means that the use of RO to concentrate seawater NOM is not feasible. The high concentrations of salts present in seawater result in an osmotic pressure that exceeds the capability of the RO membranes used here. A simple calculation demonstrates this effect where π (osmotic pressure) = molarity x R (gas constant) x T (temperature in K). The total molarity of the major components in seawater is ~ 1.14 moles L^{-1} (Berner and Berner, 1996) and the calculated osmotic pressure generated by this molarity is 410 psi, exceeding the 250 psi that the RO membranes used here can handle. Membranes designed specifically for saltwater desalination can be used, however, as the ions in the saltwater become concentrated during RO processing, their solubility will be exceeded resulting in their precipitation onto the RO membrane. Removal of cations by an H^+-saturated cation exchange resin limits the formation of carbonate salts and their precipitation onto the RO membrane but the high concentration of cations in seawater limits the effectiveness of this method. Salts precipitated on the RO membrane limit its efficiency and permeate flow slows or stops completely.

The use of ED to significantly lower the salt content of seawater prior to RO followed by a combination of RO and ED to further lower the salt content and simultaneously concentrate the DOM is developed and tested here. The method described here shows that 75 ± 12% of marine DOM can be recovered, far exceeding the

30% recovery by UF and probably including classes of compounds not isolated previously.

3.2 Methods

3.2.1 RO/ED System

A 100 membrane-pair electrodialysis stack (Type 100, Deukum) was used. The membranes had a cross-sectional area of 100 cm^2 and consisted of Neosepta CMX cation exchange and AMX anion exchange membranes. The 200 L sample was held in a 210 L cylindrical high-density polyethylene tank (HDPE) with a conical bottom and the concentrate was held in a 230 L HDPE tank. Electrode rinse was in a 20 L tank. Seal-less pumps were used for circulation through ethyl vinyl acetate tubing.

The RO used a FilmTec TW30-4021 membrane in a stainless steel pressure vessel. Tubing and fittings for the RO system were stainless steel.

Prior to processing a new sample, the entire RO/ED system was rinsed with 50 L of the sample, which was then drained from the system. ED was performed until the conductivity of the sample was lowered to < 15 mS/cm so that the RO could then be used to reduce the volume of water. After RO, ED was used again to further concentrate the DOM. In most experiments, ED and RO were simultaneously used.

On board ship, the electrode rinse solution for electrodialysis was prepared by dissolving sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) in RO permeate water to make a 0.23 M solution. This solution was used in all shipboard experiments.

To ensure better recovery of the DOM, the RO membrane was rinsed with an ~0.01 M NaOH solution at the end of the run.

As for the ED of freshwater described in Chapter 2, the system was run at 10 – 15% below the limiting current density to prevent excess loss of DOM, a decrease in the pH of the sample, and precipitation of $CaCO_3$ and $MgCO_3$.

3.2.2 Preliminary Experiments with ED

Preliminary laboratory experiments were carried out using saltmarsh water and artificial seawater to test the efficacy of the removal of sulphate and the recovery of TOC by ED. All experiments were performed using a 100 membrane-pair electrodialysis stack (Type 100, Deukum).

Water was collected in May 2004 from the saltmarsh at the Skidaway Institute of Oceanography near Savannah, GA. The water was passed through a sand filter into a 24 L HDPE carboy and transported back to the laboratory where it was filtered through a 0.45 μm filter. Artificial ocean water was made in the lab by adding DI or post-RO water to a commercial mixture of synthetic sea salt (Instant Ocean, Aquarium Systems) at a ratio of ~ 1.5 pounds of Instant Ocean dissolved in 20 L of water.

3.2.3 RO Optimization Experiments

Because the RO system will not operate efficiently at the high salt concentrations in seawater, the permeate flow from the RO system was determined for a range of conductivities to determine at what point the RO system could be turned on to begin reducing the volume of water, thereby reducing the time required for ED.

A second purpose of these RO optimization experiments was to test whether there is a difference between seawater that is diluted with DI water and seawater that has had

its conductivity reduced by ED (i.e., dilution by ED). The rationale is that the process of ED does not eliminate all ions from water at the same rate, with some ions such as Cl^- and Na^+ preferentially moving through the anion and cation exchange membranes of the ED stack and others such as SO_4^{2-} moving more slowly through the membranes. In contrast, a straightforward dilution with water maintains the same concentration ratios between all ions.

These experiments were conducted with artificial ocean water made by adding DI or post-RO water to a commercial mixture of synthetic sea salt (Instant Ocean, Aquarium Systems) at a ratio of ~ 1.5 pounds of Instant Ocean dissolved in 20 L of water.

3.2.4 Sampling

The RO/ED method was field-tested during two cruises on the Atlantic Ocean off the coast of Georgia. During the first cruise in July 2006, nine seawater samples and one estuarine water sample were processed; five seawater samples and one estuarine water sample were processed during the second cruise in October 2006. Samples were retrieved from different depths and locations (Table 3.1) using Niskin bottles that were lowered into the water to the desired depth at which point a spring-loaded mechanism closed both ends of the bottles that were then brought back to the surface. The water was drained from the Niskin bottles into 24-liter HDPE carboys and then transferred to 200-liter HDPE holding tanks. During the transfer of the water to the RO/ED system, the water was passed through a 0.45 μm polypropylene filter (Flotrex, Osmonics, Inc.). During RO/ED, the sample was held in a 210-liter cylindrical HDPE tank with a conical bottom.

Samples for analysis were collected at various stages during the RO/ED process. Specifically, samples for DOC analysis were collected in glass vials conditioned overnight in a muffle furnace at 450^{o}C to eliminate any carbon residues. The samples were refrigerated and analyzed within one week of collection.

Table 3.1: Location and depth of the seawater sampling sites. S706 are the samples collected in July 2006 and S1006 are the samples collected in October 2006.

Sample ID	Latitude	Longitude	Depth, m	Description
S706-1	31° 05.9'	77° 28.5'	20	Open Atlantic - East of Gulf Stream
S706-2	31° 06.1'	77° 28.9'	860	Open Atlantic - East of Gulf Stream
S706-3	31° 05.9'	77° 27.2'	20	Open Atlantic - East of Gulf Stream
S706-4	31° 28.2'	79° 21.4'	20	Gulf Stream
S706-5	31° 30.3'	79° 18.7'	322	Gulf Stream
S706-6	31° 29.5'	79° 19.2'	77	Gulf Stream - CDOM Maximum
S706-7	31° 30.1'	79° 19.4'	20	Gulf Stream
S706-8	31° 30.0'	79° 20.3'	20	Gulf Stream
S706-9	31° 29.4'	79° 20.2'	20	Gulf Stream
S706-10	31° 51.6'	81° 09.2'	2	Ogeechee River Mouth, High Tide
S1006-1	31° 30.3'	79° 19.4'	20	Gulf Stream, Station 4
S1006-2	31° 32.8'	79° 14.3'	20	Gulf Stream, Station 5
S1006-3	31° 32.6'	79° 15.7'	20	Gulf Stream, Station 6
S1006-4	31° 32.5'	79° 13.8'	84	Gulf Stream, Station 7
S1006-5	31° 32.5'	79° 13.8'	84	Gulf Stream, Station 7
S1006-6	31° 51.6'	81° 09.1'	2	Ogeechee River Mouth, High Tide

3.2.5 TOC Analysis

TOC concentrations were determined by high temperature catalytic oxidation (Shimadzu TOC-VCSN). A known problem with high-temperature combustion

instruments is the system blank (Sharp et al., 1993), which can be substantial in relation to the TOC concentrations present in seawater, thereby biasing the calculated percentage recoveries of TOC. By comparing the TOC concentrations of DI water on a Sievers 800 TOC analyzer (UV-persulfate oxidation with no system blank) and the Shimadzu, it was determined that the system blank for the particular instrument used here was 0.16 mg/L. This value is the average difference over the six month period immediately preceding the first research cruise in July 2006.

3.3 Results and Discussion

3.3.1 Preliminary Experiments with ED

Preliminary laboratory experiments were carried out using saltmarsh water and artificial ocean water to test the efficacy of the removal of conductivity and the recovery of TOC by ED (Table 3.2). For all samples, recovery of TOC was 90% or better while 83 to 90% of the conductivity was removed from the sample. For sample RO/ED 3, if ED was continued for 1.5 extra hours (designated as RO/ED 3+ in Table 3.2) to remove a total of 99% of the conductivity, a significant loss of TOC occurred. This loss of TOC occurs because of a lack of competing anions such as Cl^- to prevent excessive loss of DOM across the anion exchange membrane (see discussion in Chapter 2 on this topic).

In the case of the one saltmarsh sample where SO_4^{2-} was measured, 78% of the SO_4^{2-} was removed while TOC recovery was 95%, (Table 3.2). This experiment demonstrates that ED can significantly reduce the salt concentration in seawater with little loss of DOM.

These laboratory experiments demonstrate the ability of the ED apparatus to remove significant quantities of salt from highly saline waters while recovering 90+% of the TOC.

Table 3.2: Preliminary laboratory experiments to test the efficacy of ED to remove conductivity and recover TOC from saltmarsh and artificial seawater.

		RO/ED 1	RO/ED 2	RO/ED 3	RO/ED 3+
Water	Saltmarsh	Artificial seawater	Artificial seawater	Saltmarsh	Saltmarsh
Volume, L					
initial	22.8	20.7	45.9	98.5	98.5
final	22.7	18.6	41.3	94	84.7
ED time, min	90	83	200	150	240
Cond, mS/cm					
initial	35.6	46.0	46.7	31.4	31.4
final	6.23	6.84	7.1	3.36	0.4
% removed	83	87	86	90	99
TOC, mg					
initial	78.2	37	N/A	391	391
final	74.0	33	N/A	354	282
% recovered	95	90	N/A	90	72
SO_4^{2-}, mg					
initial	40918	N/A	N/A	N/A	N/A
final	8982	N/A	N/A	N/A	N/A
% removed	78	N/A	N/A	N/A	N/A
SO_4^{2-}/TOC*					
initial	94	N/A	N/A	N/A	N/A
final	15	N/A	N/A	N/A	N/A

* Molar ratio.

3.3.2 RO Optimization Experiments

Efficient use of the RO system requires that it be operated at 250 psi and that the percentage rejection of solutes by the RO membrane is ~98%, meaning that 98% of the solutes in the sample being processed are retained whereas pure water is being discarded. The RO/ED 1 and RO/ED 2 experiments shown in Table 3.2 were also used to determine

at which TDS the RO system becomes efficient. At certain times during the ED process, the sample was diverted to the RO so that permeate and retentate flow rates could be measured at various pressures. For these two experiments, the dilution process occurred by ED but the dilution of ions was not the same for all ions as the ED process preferentially moves certain ions across the ED membranes at a faster rate than others. The relative proportions of the ions in solution change during the ED process as a result of the specific conductances of the ions and their charge-to-size ratios. An experiment where artificial ocean water was diluted with post-RO water was also conducted. In this case, the relative proportions of the ions in solution remained constant.

At conductivities exceeding 18 mS/cm, permeate flow was not observed. Below 18 mS/cm, permeate flow increased as the conductivity decreased. The results presented in Figure 3.1 show that the type of dilution used does not significantly affect the permeate flow rates from the RO at the operating pressure of 250 psi.

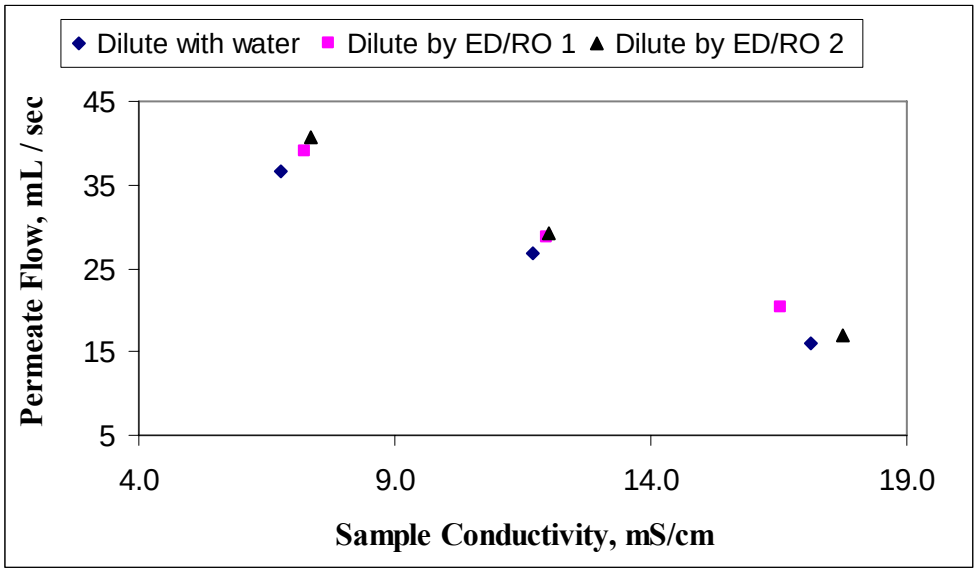

Figure 3.1: RO permeate flow rates at 250 psi. No permeate flow was observed at a conductivity exceeding 18 mS/cm. These experiments show that the dilution process does not significantly affect the permeate flow rate at the operating pressure of 250 psi.

3.3.3 Shipboard RO/ED

The above experiments were all conducted with highly saline waters; saltmarsh water and artificial ocean water. The purpose of the two cruises was to demonstrate that the RO/ED process is applicable to real ocean water and is able to isolate more than 50% of the DOM in sufficient quantities in a reasonable amount of time.

One of the experiments run shipboard was a blank. Permeate water from the RO was used as the sample; the ship's water supply was used to make the RO permeate water. Two hundred liters of RO permeate water was continually circulated through the ED stack for three hours with the power supply turned off. No ED could take place as the water had a very low conductivity. The RO system was then turned on and the sample volume was reduced to 6.2 L over two hours.

The initial measured TOC concentration of the RO permeate water was 0.225 mg L^{-1}, which is very close to the system blank described in section 3.2.5. The final TOC concentration was measured to be 2.55 mg L^{-1} and the percent TOC recovered was calculated to be 157%. The high percent recovery indicates either a potential source of TOC in the RO/ED system or a slight error in the initial TOC concentration of the RO permeate water. An analysis of the TOC concentrations of the samples as they were transferred from the storage tanks into the RO/ED tank, in the RO/ED tank as soon as it was full (time 0 minutes), and after nine minutes of circulating through the RO/ED system (with the ED and RO turned off) is presented in Table 3.3. These three measurements should all yield the same TOC concentration. The variation inherent in the measurement of TOC by the high-temperature TOC analyzer results in a mean standard deviation of 0.039 mg TOC L^{-1} for these three samples. Sample S706-10, an estuarine

sample, is not included in this analysis: it has a significantly larger initial TOC concentration and its standard deviation of 0.097 mg L^{-1} would bias the mean standard deviation to a higher value. An initial TOC concentration for the blank sample of 0.262 mg L^{-1}, which is within the standard deviation, would result in a revised percent recovery of TOC of 99.4%. An initial TOC concentration for the blank sample of 0.188 mg L^{-1}, also within the standard deviation, would result in a revised percent recovery of TOC of 384%.

Table 3.3: Sample (diluate) TOC concentrations while filling the RO/ED tank, once the tank is full (0 minutes), and after 9 minutes of circulating through the RO/ED system (with the ED and RO turned off) have a mean standard deviation of 0.039 mg L^{-1}. During the second cruise, only one sample was taken prior to RO/ED so that a similar analysis is not possible. TOC concentrations are blank-corrected.

	TOC, mg L^{-1}				
Sample*	Fill	Tank	Diluate	Mean TOC	sd
S706-1	1.035	0.960	-	0.998	0.053
S706-2	0.771	0.581	0.718	0.690	0.098
S706-3	1.029	1.005	0.988	1.007	0.021
S706-4	1.046	0.983	1.090	1.040	0.054
S706-5	0.664	0.613	0.616	0.631	0.029
S706-6	0.935	0.924	0.969	0.943	0.023
S706-7	1.057	1.022	1.010	1.030	0.024
S706-8	0.937	0.964	0.950	0.950	0.014
S706-9	0.857	0.884	0.933	0.891	0.039
				Mean sd =	0.039*

* Excludes samples S706-0 (blank) and S706-10 (estuary).

The time sequence of the concentrations for these three samples shows them to be random. There is no systematic increase in TOC concentration from fill to tank to diluate so that leaching of TOC from the RO/ED system is not likely to be the cause of the over-recovery of TOC seen for the blank sample. Variation in TOC concentrations accounts for the observed error. An analysis of the reproducibility of TOC concentrations was

carried out for the first five samples from the October cruise. Each sample was analyzed three separate times for TOC resulting in a mean standard deviation of 0.017 mg L^{-1}, a more accurate reflection of the reproducibility of the Shimadzu high-temperature TOC analyzer. The difference between the two sets of analyses is that for the second set each sample was analyzed three times whereas for the first set three supposedly identical samples were each analyzed one time.

During the two cruises, a total of fourteen ocean water and two estuarine samples were collected (Tables 3.1 and 3.4) and processed shipboard. Blank-corrected TOC concentrations in ocean water ranged from 0.631 to 1.040 mg L^{-1} (53 to 87 μM) with a median value of 0.943 mg L^{-1} (79 μM). These concentrations are similar to those found in other studies (e.g., Sharp 1993; 1995). There is no apparent relationship between TOC concentration and sample site (Gulf Stream versus open ocean) but there is an indication that the TOC concentration decreases with depth (Figure 3.2), confirming previous studies.

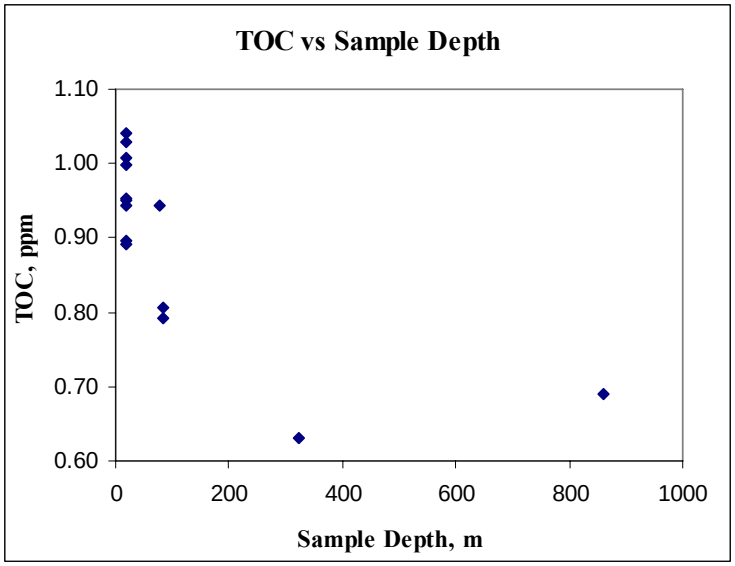

Figure 3.2: TOC concentrations tend to decrease with increasing depth.

Table 3.4: Initial conditions for all seawater samples processed by RO/ED.

	Initial		
Sample	TOC[a], mg/L	Volume, L	Cond. mS/cm
S706-1	0.998	198.38	47.1
S706-2	0.690	198.38	49.7
S706-3	1.007	198.38	44.2
S706-4	1.040	198.38	49.8
S706-5	0.631	198.38	51.2
S706-6	0.943	198.38	53.2
S706-7	1.030	198.38	52.6
S706-8	0.950	198.38	51.2
S706-9	0.891	394.06	52.6
S706-10	4.948	198.38	44.8
S1006-1	0.943	103.72	47.2
S1006-2	0.954	198.38	51.3
S1006-3	0.896	198.38	54.3
S1006-4	0.792	198.38	56.2
S1006-5	0.807	198.38	56.4
S1006-6	4.196	100.54	47.7
Average=	0.898[b]		
Median=	0.943		
Std. Dev.=	0.125		

[a]TOC concentrations are blank-corrected. [b]Open ocean samples only. The statistics exclude the estuarine samples S706-10 and S1006-6.

The samples were processed by RO/ED; percent recovery of TOC ranged from 61 to 95% with a median recovery of 72% while removing 99 to 100% of the conductivity from the water (Table 3.5; Figure 3.3). These recoveries far exceed the 30% recovery that has been the norm for recovery of marine DOM. There is no apparent correlation between percent TOC recovered and sample site or depth.

The percent recovery of TOC had a standard deviation of 12%. The process of RO/ED may have an impact on the percent recovery of TOC. As was discussed in Chapter 2, DOM can be lost by adsorption onto the ED membrane if there is an insufficient amount of competing Cl^- ions for the charged sites on the membrane. Some

of this DOM may be recovered by employing a wash of the ED stack. Other operational parameters, including whether the current is continuous or pulsed, and temperature of the sample during processing (higher temperature may result in increased loss of DOM) may impact the percentage of TOC recovered. Variations in TOC concentration measured by the high-temperature analyzer account for at most 1.6% of the variation in percent TOC recovery.

Table 3.5: % recovery of TOC, % removal of water and conductivity, and final conditions for all seawater samples processed by RO/ED.

	Recovery	Removal		Final		
Sample	TOC	Water	Cond	TOC*	Volume	Cond.
	%	%	%	mg/L	L	mS/cm
S706-1	75.7	96.8	99.6	23.58	6.28	6.25
S706-2	93.7	96.6	99.4	14.16	6.77	9.25
S706-3	77.2	96.6	99.3	21.31	6.84	8.98
S706-4	65.5	96.8	99.4	20.41	6.30	8.79
S706-5	95.4	95.9	99.3	14.50	8.14	8.75
S706-6	67.9	97.0	99.4	20.49	5.90	10.79
S706-7	61.2	96.5	99.4	17.03	7.00	9.45
S706-8	67.6	95.7	100.0	14.43	8.48	0.187
S706-9	73.3	97.6	99.5	25.87	9.57	10.89
S706-10	94.2	95.5	98.9	103.24	8.84	11.42
S1006-1	92.2	93.8	100.0	11.27	6.44	0.096
S1006-2	69.8	97.0	100.0	18.36	5.89	0.050
S1006-3	71.9	97.6	100.0	20.86	4.78	0.096
S1006-4	72.2	96.3	100.0	13.11	7.27	0.099
S1006-5	61.9	97.5	100.0	15.81	4.97	0.075
S1006-6	61.0	91.1	100.0	25.01	8.99	0.052
Average	75.0	96.1	99.6			
Median	72.0	96.6	99.5			
Std. Dev.	12.2	1.7	0.4			

* Blank-corrected.

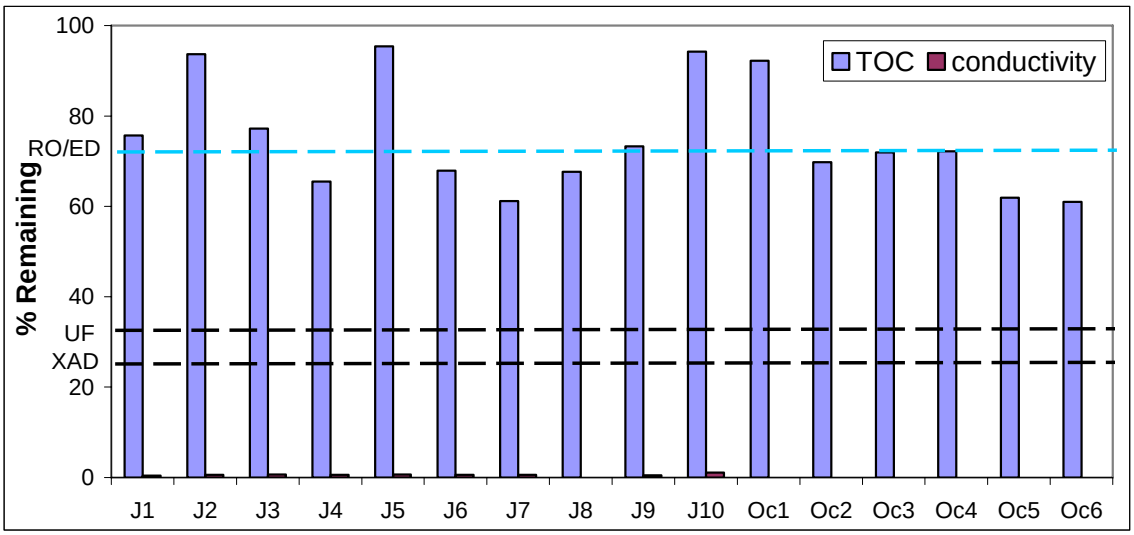

Figure 3.3: RO/ED of seawater resulted in a median recovery of 72% of the TOC and the removal of 99 to 100% of the conductivity. The recovery of TOC far exceeds what is possible by UF and XAD resins. J# are samples 1 – 9 from the July cruise and Oc# are samples 1 – 6 for the October cruise.

While the samples processed during these cruises have relatively high concentrations of DOM and >99% of the conductivity has been removed, they still contain significant amounts of salts. At infinite dilution, one mole of Na^+ ions has a conductivity of 50.1 mS/cm and one mole of Cl^- ions 76.4 mS/cm for a total of 126.5 mS/cm. Assuming that the remaining conductivity of the samples is due only to NaCl, it can be shown that the samples still have a molar NaCl/TOC ratio of 0.19 to 61, meaning that for every 1 C there are up to 61 Na^+ ions and an equivalent number of Cl^- ions (Table 3.6). Further processing of the samples with a combination of RO/ED and an H^+-saturated cation exchange resin is required to lower the salt to levels that will not affect chemical analyses such as determination of elemental composition, titratable carboxyl and phenol groups, and mass spectrometry.

Table 3.6: Calculated moles of NaCl and molar NaCl/TOC ratio remaining in the processed samples.

Sample	Remaining Moles NaCl	Molar NaCl/TOC
S706-1	0.049	25
S706-2	0.073	61
S706-3	0.071	40
S706-4	0.069	41
S706-5	0.069	57
S706-6	0.085	50
S706-7	0.075	52
S706-8	0.001	1
S706-9	0.086	40
S706-10	0.090	10
S1006-1	0.00076	0.80
S1006-2	0.00040	0.26
S1006-3	0.00076	0.43
S1006-4	0.00078	0.70
S1006-5	0.00059	0.44
S1006-6	0.00041	0.19

The assumption that the remaining conductivity is due only to NaCl is not entirely accurate. There are other salts (e.g., sulfates) still present in the sample but their equivalent conductivities are all sufficiently similar not to result in much bias in the above calculation. Furthermore, by virtue of the presence of unprotonated carboxyl groups, organic matter itself produces conductance. Conductance measurements for the titration of soil NOM with NaOH (Schnitzer and Skinner, 1963) revealed a minimum conductivity of ~20 μS/cm for 30 mg TOC/L. Assuming that soil NOM is sufficiently representative of marine NOM, and knowing that the final TOC concentrations for the cruise samples varied from ~ 11 to 26 mg/L (mean = 18 mg/L), then 18 mg/L should produce a conductivity of 12 μS/cm. Subtracting this value from the conductivities does

not result in any significant changes in the final molar NaCl/TOC ratios for the S706 samples presented in Table 3.6. For the S1006 samples the final recalculated molar NaCl/TOC ratios are 0.15 to 0.70, somewhat lower than those shown in Table 3.6.

3.4 Conclusions

This part of the research was intended to demonstrate a new and more efficient method of isolating and concentrating marine DOM in quantities sufficient for chemical and physical analysis. The effort successfully recovered a median of 72% of the TOC from 200 L samples within six to nine hours of processing through a combination of ED and RO, greatly exceeding the current norm of 30%. Recovering 72% of the DOM implies that classes of DOM previously missing are included in these samples and should yield new insight into the chemistry of marine DOM.

The samples processed here require further ED to remove the remaining salts. Experiments are under way to determine the best method to achieve near-complete salt removal while recovering most of the DOM. Experiments are also under way to determine why the percent recovery of TOC varies by as much as 12% from similar sampling sites.

3.5 Future Research

The protocol for isolating marine NOM by RO/ED requires further development so that losses of TOC can be minimized as the sample is further isolated from inorganic constituents. ED processing of the samples is needed to lower the sulphate and silica levels. For sample S706-8, the SO_4^{2-}/TOC ratio for is 0.27 and the H_4SiO_4/TOC ratio is

0.086, both of which exceed the required levels. The protocol described in Chapter 2, whereby Cl^- ions are added and the pH is maintained at ~ 9.0 to 9.5, will have to be applied and improved upon to successfully remove sulphate, silica, and other anions from the samples.

3.6 Acknowledgments

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CHAPTER 4

BIOAVAILABILITY OF FRESHWATER DISSOLVED NATURAL ORGANIC MATTER

4.1 Introduction

4.1.1 What is Bioavailability?

The nutrient and energy source available to bacteria and other micro-organisms in surface waters consists of a complex mixture of molecules of dissolved organic matter (DOM) (Volk et al., 1997) of which only a fraction is actually used by the micro-organisms (Sun et al., 1997). Some molecules of DOM will be easily utilized by the micro-organisms present in the water, thus having short lifetimes, whereas others will be harder to break down and yet others will be recalcitrant. Those molecules that are utilized in an arbitrarily defined time-period are deemed bioavailable or labile.

A survey of several studies by Sondergaard and Middelboe (1995) found that 3 to 35% (median = 25%) of the DOM in river waters is labile, which they defined as the DOM that can be decomposed by bacteria within two weeks. Volk et al. (1997) noted that biodegradability (their term) of DOM is probably influenced by the type of bacteria used in the experiment. They speculate that bacteria associated with a particular sample is probably better suited to utilize the DOM present than bacteria from another source that is unacclimated to the DOM. This idea was previously put forth by Vannote et al. (1980) as part of the 'river continuum concept'; this concept will be discussed in a later section.

Because DOM is an energy source for aquatic micro-organisms, it is an essential component of the microbial food web (Sun et al., 1997; Vallino et al., 1996). Land-use

change (Hopkinson et al., 1998) and climate change may alter the amount and chemistry of DOM available in surface waters. It is therefore important to have the necessary methods to properly evaluate the impacts these potential changes may have on the dynamics of stream ecosystems.

4.1.2 Biological Oxygen Demand

Biological oxygen demand (BOD) is a measure of the amount of O_2 consumed by bacteria while transforming DOM into CO_2, intermediate compounds, and/or new biomass under aerobic conditions in a closed system for a specified time-period. O_2 is also consumed by the oxidation of nitrites, ammonia, and inorganic ions such as Fe^{2+}, sulfide, and sulfite (Sawyer et al., 1994; Standard Methods, 1989).

Bioavailability as defined in the present study is equivalent to BOD. BOD has traditionally been based on a 5-day incubation period (though there are variations) (Standard Methods, 1989) whereas the incubation time used in bioavailability and bioassay studies varies from 3 days (e.g., Sun et al., 1997) to 16 days (e.g., Leff and Meyer, 1991) to as long as 98 days (e.g., Moran and Hodson, 1990). Bioavailability is a relative term: if any of the above studies had lasted for a longer period of time, the amount of DOM consumed would have been greater. Relative bioavailability, however, would probably remain the same. The bioavailable fraction itself is made up of molecules that range from easily biodegraded (i.e., labile) to relatively refractory (Kaplan and Newbold, 1995).

4.1.3 Bioavailability and Elemental Composition

Previous work by Sun et al. (1997) has indicated that bioavailability is positively correlated to the bulk atomic H/C and N/C ratios of river DOM and negatively correlated to the bulk O/C ratio ($R^2 = 0.93$; n = 20). The results of this work suggest that the more aliphatic the DOM the more bioavailable it is. A reanalysis of the data by Hunt et al. (2000) questions this conclusion; they claim that Sun et al.'s data support the importance of the N/C ratio as being the primary indicator of the bioavailability of DOM and that the H/C ratio is insignificant. They also try to downplay the importance of the O/C ratio by unjustifiably ignoring data for the fresh leachate samples.

It is at first unexpected that the N/C ratio of bulk DOM is correlated with bioavailability. N is only a small part (0.5 – 5.0 %) of DOM (Perdue and Ritchie, 2003) and only 25% of DOM is typically bioavailable, further masking the effect of N. The N in DOM is mostly in peptides (amides) which are believed to be part of the labile DOM fraction (Marschner and Kalbitz, 2003, and references therein).

All of the samples in the study by Sun et al. (1997) were from one river, the Ogeechee in Georgia, so that the samples are in some way all related to one another. It is conceivable that this may improve the correlation between bioavailability and the bulk elemental composition of the DOM. Based on evidence from the literature (e.g., Sabater et al., 1993 and references therein), the bioavailable components present in fresh DOM injected into the stream water near its source are quickly used up. As the water courses downstream, the fraction of bioavailable DOM decreases, assuming no other significant sources of fresh and labile DOM are present downstream. This is akin to the river continuum concept put forth by Vannote et al. (1980) which states that the diversity of

DOM molecules is greatest in the headwater and progressively decreases downstream. A further consequence of such activity would be that the age of the remaining DOM would increase downstream. This is indirectly supported by the work of Raymond and Bauer (2001) who, using ^{14}C, concluded that relatively younger riverine DOM is preferentially biodegraded, leaving older DOM to be exported to the oceans.

Hopkinson et al. (1998) used the elemental composition and aliphatic content of four coastal rivers to rank them according to anticipated bioavailability. Their ranking was supported by bacterial growth assays; samples that were relatively more bioavailable supported more bacterial growth. Moran et al. (1999) found large geographical and temporal variations in the biodegradability of DOM in estuaries of the southeastern USA. Leff and Meyer (1991) and Sun et al. (1997) both found that bioavailability decreases downriver.

4.1.4 Hypotheses

The logical extension of the work by Sun et al. (1997) is to investigate whether the same type of correlation between bioavailability and bulk elemental composition is seen for riverine DOM from geochemically diverse rivers, not only coastal rivers and estuaries as was the case for Hopkinson et al. (1998) and Moran et al. (1999). The purpose of the research presented here is to answer the following questions:

- Is bulk elemental composition related to the bioavailability of DOM from geochemically distinct rivers?
- Does the bioavailability of riverine DOM vary spatially in non-coastal areas?

- Can the BOD/TOC ratio be used to predict the bioavailability of geochemically diverse riverine DOM?

The following hypotheses were used for this research:

- The bioavailability of riverine DOM varies geographically in the southeastern US.
- Relatively higher values of bioavailability will be found in regions where allochthonous sources of DOM are relatively important to the total DOM make-up in the water. Further downstream, where the river is larger and allochthonous sources of DOM are not as predominant, it is expected that relatively lower values of bioavailability will prevail.
- The ratio of BOD/TOC can be used to predict the bioavailability of geochemically diverse DOM.
- Bulk elemental composition for a range of freshwater NOM will be related to bioavailability. The H/C and N/C ratios are expected to increase with increasing bioavailability whereas the O/C ratio is expected to decrease.

In summary, there are two uses for BOD for this research. One is to use it as a surrogate for the bioavailability of DOM and the second is to use it to identify potential sampling locations so that DOM representing a wide range of potential bioavailability is obtained for this research allowing for the above hypotheses to be tested.

4.2 Literature Review

4.2.1 Bioavailability, Biodegradability, Assimilable Organic Carbon

Marschner and Kalbitz (2003) define bioavailability as the uptake but not necessarily the breakdown of DOM by micro-organisms. Because micro-organisms also release exo-enzymes which promote extracellular degradation, bioavailability is seen as the potential for micro-organisms to interact with DOM. They define biodegradability as a measure of the actual utilization of DOM, encompassing uptake or breakdown and complete mineralization while recognizing that if only DOM consumption is being measured then the distinction between uptake and mineralisation is not possible. The terms bioavailable and biodegradable will be used interchangeably in this chapter, especially with reference to the literature, and are meant to imply one thing: the uptake and partial or complete breakdown of DOM.

The term assimilable organic carbon (AOC) is that part of the TOC that is easily assimilable by bacteria to increase biomass (Escobar and Randall, 2000). Jegatheesan et al. (2004) describe AOC as the readily utilizable form of biodegradable DOC. AOC, commonly referred to in the water treatment literature, is thought to be the main source of bacterial growth in drinking water (Chien et al., 2007). There is a distinction between AOC and biodegradable DOC and how they are measured. AOC specifically refers to that fraction of DOC which results in bacterial growth whereas biodegradable DOC refers to the DOC that is both mineralized and assimilated (Escobar and Randall, 2001).

Moran et al. (1999) found large geographical and temporal variations in the biodegradability of DOM in estuaries of the southeastern USA, indicating that perhaps the chemistry of the DOM at the various sites was quite different. Similarly, Hopkinson

et al. (1998) found a range of bioavailability for DOM from a set of geographically diverse coastal rivers feeding estuaries along the east and west coasts.

DOM is being continually affected by physical processes such as photolytic degradation (Kaiser and Sulzberger, 2004; Bano et al., 1998) and biological means (bacterial breakdown). Consequently, the bioavailability of freshwater DOM varies temporally (e.g., Volk et al., 1997) and geographically. The chemical composition of DOM may also be impacted by the nutrient status of the water from which the DOM is extracted. Ziegler and Brisco (2004) speculate that the higher levels of refractory DOM in nutrient-rich streams is due to increased *in situ* bacterial utilization of the available DOM. Miettinen et al. (1999) found that the addition of nutrients to humus-rich drinking waters increased the amount of carbon taken up by the bacteria present.

The most common method of measuring bioavailability is to use bioassays that measure changes in bacterial populations. There are numerous studies examining bacterial growth on either whole DOM or fractions of DOM (molecular weight fractions and humic and fulvic acids). It is implicit that the greater the bacterial growth on a sample, the more bioavailable the sample components are. Studies examining bacterial growth include those on molecular weight fractions of stream and river DOM (e.g., Amon and Benner, 1996; Sabater et al., 1993; Meyer et al., 1987), humic and non-humic components of lake and marsh water (e.g., Moran and Hodson, 1990), oligotrophic lake water (e.g., Tranvik, 1988), polluted lake water (e.g., Markosova, 1991), DOM from several sites along a river including leachates (e.g., Sun et al., 1997; Leff and Meyer, 1991), mountain bog pool water (e.g., Satoh and Abe, 1987), the humic fraction extracted

from stream water and peat (Hunt et al., 2000) and estuarine waters (e.g., Moran et al., 1999; Hopkinson et al., 1998).

Should bacterial growth be used as an indicator of the bioavailability of DOM, then the study by Amon and Benner (1996) shows a shortcoming of this method: they found the high molecular weight fraction for both marine and freshwater DOM to be relatively more degradable but the low molecular weight fraction better supported bacterial growth. In a study of the mineralisation of aquatic organic materials, Bitar and Bianchini (2002) found that the overall uptake of oxygen by bacteria was greatest for the labile fraction but that the refractory fraction was easier to process.

Given that measuring bacterial biovolume is a difficult and time-consuming task, other approaches to infer DOM utilization have been developed. Volk et al. (1997) used plug-flow biofilm reactors colonized by micro-organisms, defining the biodegradable fraction of DOM as the difference in DOC concentration between the influent and effluent water of the bioreactors. Lucena et al. (1990) used a similar approach. Moran et al. (1999) followed the consumption of O_2 by bacterial cultures exposed to the DOM from estuaries.

Some studies have attempted to model the bacterial utilization of organic matter. Vallino et al. (1996) developed a bioenergetic model examining the growth kinetics associated with bacterial utilization of DOM and reduced nitrogen species. They found that the oxidation state of the *labile* portion of the DOM determines bacterial growth rate and yield: the more oxidized the DOM the less bacterial growth it will support. Using complex wastewaters as their focus, Mao and Smith (1995) proposed a two-staged biodegradation process linked by a transformation process.

To the author's knowledge, no study exists whereby the bioavailability of *freshwater* DOM is measured by monitoring the amount of O_2 and DOM consumed and CO_2 produced over time. Several studies however, have done so for soil organic matter and marine DOM (see Marschner and Kalbitz, 2003 for a review). A comprehensive approach where O_2, DOC, and CO_2 are monitored is essential to gain a better understanding of the processes occurring during the degradation of natural DOM. In this manner, the research presented here is different from what is currently available in the literature.

A large number of the studies quoted above deal with fractions of DOM. Although this may be useful for certain applications, it fails to deal with the reality that surface water DOM is not made up only of low or high molecular weight molecules, nor is it composed only of humic or fulvic acids; rather it is a complex mixture of all of these. The current study uses whole DOM and in this way is different from most of the existing work on bioavailability.

4.2.2 Stoichiometry

The nature of the organic matter (e.g., complexity of the molecules, bonding energy, degree of oxygenation) and the conditions of incubation (mixing, ratio of bacteria to organic matter) determine the stoichiometry of the reaction (Peret and Bianchini, 2004; Dilly, 2001; Busch, 1966). Assuming complete mineralization, the stoichiometry of the breakdown of glucose is one mole of CO_2 produced per mole of dissolved organic carbon (DOC) consumed. Extensive tests have shown that for a 20-day BOD analysis using glucose as the substrate, at most 85% of the theoretically maximum amount of O_2 will be

consumed (Sawyer et al., 1994) (i.e., 0.85 mole of O_2 consumed per mole DOC) because some of the DOC is incorporated into biomass. Bitar and Bianchini (2002) have shown that for the breakdown of glucose over a 16-day period, only 20% of the DOC is mineralized while 60% becomes part of the biomass and the remaining 20% is humified.

There are other considerations affecting the stoichiometry of the oxidation of organic matter. The makeup of the starting material is the most obvious factor. During the incubation, physical and chemical processes in addition to biological breakdown may remove DOM. The remaining DOM may have been produced by the bacteria, i.e., transformation of the original DOM. Finally, the rate of O_2 uptake may be different for degradation and transformation reactions (Mao and Smith, 1995).

4.2.3 Correlations between COD, BOD, and TOC

Chemical oxygen demand (COD) determines the amount of oxygen required to oxidize the DOM in a sample using a strong oxidizing agent. COD does not differentiate between bioavailable and biologically inert DOM. With the exception of pyridine, aromatic hydrocarbons, and perhaps volatile organic compounds, all DOM is oxidized. As with BOD, reduced inorganic ions may also be oxidized during the COD analysis. Thus, COD is greater than BOD and will be much greater for samples containing significant amounts of non-labile DOM (Sawyer et al., 1994; Standard Methods, 1989).

Careful consideration reveals that correlations between COD and BOD for different samples are not possible because BOD is affected by the chemistry of the DOM whereas COD is not. Unless the chemistry of the DOM is similar between samples as far

as the bacteria using the DOM are concerned, then a correlation between BOD and COD is not possible.

For unpolluted waters, correlations between BOD and TOC should be possible. Sondergaard and Middelboe (1995) found that the greater the DOM concentration, the greater the concentration of labile DOM.

4.2.4 Average Oxidation State of Carbon

Assume that the average empirical formula for a DOM sample is $C_C H_H N_N O_O$. Given that the average oxidation states of H, N, and O are +1, -3, and –2, respectively, and that the DOM mixture is uncharged, then:

$$C(Zc) + H(+1) + N(-3) + O(-2) = 0 \quad (1)$$

where Z_C is the average oxidation state of carbon in the DOM. Dividing by C yields molar H/C, N/C, and O/C ratios and upon rearranging the above equation, Z_c can be expressed as:

$$Z_c = 3(N/C) + 2(O/C) – (H/C) \quad (2)$$

Alternatively, Z_c can be defined from COD and TOC (Stumm and Morgan, 1996):

$$Z_c = 4(TOC – COD)/TOC \quad (3)$$

which can be rearranged to:

$$COD/TOC = 1 – Z_c/4. \quad (4)$$

Using this equation, the TOC and COD values of a water sample can be used to calculate the average oxidation state of organic carbon in DOM.

The two extreme oxidation states for carbon are +4 (for CO_2) and –4 (for CH_4). Using theses extreme values of Z_c in equation (4), it is shown that 0 <= COD/TOC <=

2.0. However, by observing the makeup of organic matter as displayed on a Van Krevelen plot (e.g., Sun et al., 1997; Rice and MacCarthy, 1991) it seems that Z_c varies at most between 2 and –2 so that 0.5 <= COD/TOC <= 1.5.

The smallest possible value for the BOD/TOC ratio is 0, which occurs when none of the DOM is bioavailable. From the above discussion, we know that BOD should be less than COD. Given these constraints, the range of BOD values is restricted to 0 <= BOD/TOC <= 1.5. If BOD can be used as a surrogate for bioavailability, then perhaps the BOD/TOC ratio can be used as an indicator of the relative bioavailability of surface water DOM.

4.2.5 Degree of Reduction

The degree of reduction of an organic compound, Ψ, is defined as the number of electrons transferred to oxygen when it is oxidized to CO_2, H_2O, NH_3, and H_2SO_4 (Hopkinson et al., 1998). For a compound with elemental composition $C_cH_HO_ON_NS_S$ and charge a, the degree of reduction is calculated from the valence state of each element as

$$\Psi = 4C + H - 2O - 3N + 6S - a \quad (5)$$

or on a per mole C basis as

$$\Psi_c = 4 + (H - 2O - 3N + 6S - a) / C \quad (6)$$

According to Vallino et al. (1996), if DOM is being completely oxidized and there is no significant accumulation of biomass in the samples during the incubation period then Ψ_c can be calculated as:

$$\Psi_c = 4\ \Delta DO / \Delta DIC \quad (7)$$

where DO is the dissolved O_2 consumed and DIC is the dissolved inorganic carbon (i.e., CO_2) produced during the incubation period. For bioassays where there is an accumulation of bacterial biomass, the above equation must be expanded to include the DOM that is not completely oxidized:

$$\Psi_c = [4.2\ (\Delta DOC + \Delta DIC) + 4\ \Delta DO] / \Delta DOC \quad (8)$$

where 4.2 is the bacterial Ψ_c (Vallino et al., 1996). Using this equation, the average degree of reduction of the *labile* portion of a DOM sample can be calculated.

4.2.6 Chemical Composition and Bioavailability

Linking the chemical composition of DOM and its bioavailability has been attempted in some studies. Amon and Benner (1996) found that the low molecular weight fraction (< 1000 Da) of both marine and freshwater DOM supported greater growth efficiency for bacteria but that the high molecular weight fraction was more degradable. Their findings suggest compositional differences exist between the size fractions. Volk et al. (1997) found that a third of the biodegradable fraction of streamwater DOM was made up of carbohydrates and amino acids, which are low molecular weight molecules, but that three-quarters was made up of humic substances, which are thought to be of high molecular weight. They noted that half of the carbohydrates and amino acids were not utilized, their point being that general classifications of what compound classes may be bioavailable are not foolproof and as such care must be taken in making general classifications of what is bioavailable. Volk et al. (1997) also claimed that to determine which molecular classes contribute to bacterial

growth requires knowledge of the rates of utilization (kinetics) and growth yields. Interestingly, most of the carbohydrates and amino acids utilized were bound to humic substances.

The study by Hertkorn et al. (2002) elucidates some of the observations made by Volk et al. (1997). They found a complex mechanism at work with regards to the utilization of humic substances by autochthonous micro-organisms. It was found that the utilization of humic substances was aided by the presence of glucose through a process of co-metabolism and concluded that the act of biodegrading humic substances resulted in humic substances with decreased aliphatic and aromatic structures.

With the advent of electrospray ionization mass spectrometry (ESI-MS) the composition of the bioavailable fraction of DOM can further be elucidated. Using ESI-MS to achieve a molecular-level characterization of the bioavailable fraction of streamwater DOM, Seitzinger et al. (2005) found that low molecular weight molecules (< 600 amu) contribute significantly to bioavailability and that some compounds are not utilized at all. They also found that biological degradation of NOM may result in the formation of new compounds as seen by new masses appearing in the MS spectra. In a study of tropical and stream DOM metabolism, Kim et al. (2006) reported an increase in lower molecular weight molecules and that O-rich molecules were degraded. It would appear from their results that higher molecular weight molecules of relatively high O-content were selectively degraded to lower molecular weight molecules.

4.3 Materials and Methods

4.3.1 STORET Database

For more than 30 years various entities including the USGS, EPA, and state agencies have been collecting surface water samples at varying frequencies and analyzing them for numerous characteristics including COD, BOD, and TOC. EPA has gathered this data into a large database named STORET (STOrage RETrieval) which is available online at www.epa.gov/storet.

From this database was obtained water quality data of surface waters since the late 1960s for eight southeastern states: Virginia, North Carolina, South Carolina, Georgia, Tennessee, Alabama, Mississippi, and Florida. The original dataset consisted of more than 83,000 lines of data. Because our interest is the DOM in natural streams and rivers, the dataset was edited by eliminating all data for lakes, estuaries, reservoirs and any non-natural or polluted samples, such as the outflow from industry and sewage treatment plants. In addition, only streams and rivers containing data for BOD, COD, and TOC were kept. If any of these was at the detection limit, these data were also purged. Values for the average oxidation state of carbon (Z_c) were calculated for each sample and the dataset was further constrained by the elimination of data with Z_c values falling outside the range of –2 to +2 (see section 4.2.4). Furthermore, BOD/TOC ratios falling outside the range of 0 to 1.5 were also eliminated. After going through all of these stages of data quality control, a dataset with approximately 40,000 samples was left. There is no guarantee that the remainder of the dataset is without error, however, the quality control applied here produces a dataset that is internally consistent for this research.

4.3.2 Sample Collection

Once the STORET dataset was purged of all data that were deemed incorrect (see previous section), mean molar BOD/TOC ratios were calculated for each site for the summer season, defined as May to September. To minimize the effect of outliers, only those sites sampled a minimum of ten times were included in our analysis. The BOD/TOC values were plotted on a map and sorted into arbitrarily defined categories of low (0.0 – 0.49), medium (0.50 – 0.99), and high (1.00 – 1.50) values. The calculated ratios and maps were used as a guide in the selection of sampling sites covering a potentially wide range of DOM bioavailability.

Numerous sites were visited in July and August 2001. Some were not sampled because of potential pollution sources upstream (e.g., agricultural fields, factories, towns), disturbances, low water levels, stagnant water, site located on private land, and the inability to get close enough to the site with the sampling equipment. Six river samples were collected by using reverse osmosis (Figure 4.1; Table 4.1). A detailed description of the reverse osmosis method is given elsewhere (Sun et al., 1995; Serkiz and Perdue, 1990). Briefly, water was collected by pumping it through a 1 μm pre-filter, followed by a 0.4 μm filter, then through an H^+-saturated cation exchange resin to remove major cations before being pumped at high pressure (200 - 250 psi) through the RO membranes. At each site, 250 to 500 L of river water was concentrated to a final volume of 12 to 25 L, resulting in a 10 to 20- fold concentration of the DOM in the water. The recovery of DOM was 83 to 100 %, with an average of 92 % (Table 4.1). All samples were maintained on ice until our return to the laboratory, where they were immediately stored in the dark at 4^{o}C.

Table 4.1: Characteristics of the six river samples.

		Raw water	*Concentrate*	
	pH	DOC (mg C L^{-1})	DOC (mg C L^{-1})	% Recovery of DOC
Suwannee (GA)	3.89	75.5	743.0	91
Withlacoochee (GA)	7.48	10.7	104.7	93
Congaree (SC)	7.63	3.43	68.5	83
New (VA)	7.61	1.84	30.8	94
Ocmulgee (GA)	8.16	2.02	34.7	100
Broad (SC)	7.70	3.45	59.4	92

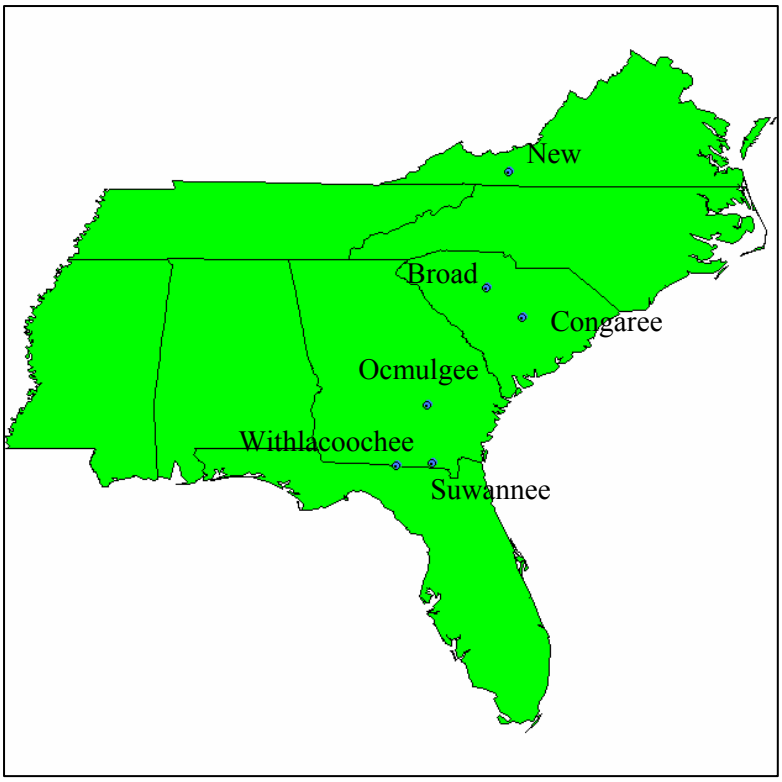

Figure 4.1: Location of the six sampling sites.

4.3.3 Incubations

All glassware, pipette tips, and filters were autoclaved and all work was carried out in a positive pressure hood to prevent contamination of samples by foreign bacteria.

Bioavailability measurements were done using bacteria isolated from the Chattahoochee River upstream of Atlanta. The DOM from this river was not included in the study of bioavailability. Using non-native bacteria allows for a cross-system evaluation of bioavailability. Native bacteria are acclimated to the DOM, preferentially degrading it and thereby potentially biasing the results. One goal of this research is to rank DOM by bioavailability in a non-biased manner, and using non-native, non-acclimated bacteria is a possible way to do so.

The bacteria from the Chattahoochee River were isolated by filtering 2 L of river water through a 0.7 μm pre-filter to remove protozoa and a 0.22 μm filter on which the bacteria were trapped. The filters were then suspended in 150 mL of autoclaved nano-pure water in an autoclaved glass bottle and shaken for 48 to 72 hours to remove the bacteria from the filters, suspending them in the water.

In preparation for the incubations, the concentrated water samples were sterilized by filtering them through 0.22 μm filters into autoclaved flasks. The concentrates were then diluted to a DOC concentration of ~ 0.38 mM using autoclaved nano-pure water. Samples were then poured into 1L autoclaved glass bottles to which 10 mL of inorganic nutrients (Leff and Meyer, 1991) was added to avoid the possibility that nutrients would be a limiting factor in the experiments. Samples were then adjusted to the pH of the river water from which the bacteria were isolated. Blanks consisted of autoclaved nano-pure water and the nutrient media. Controls contained a glucose solution prepared in autoclaved nano-pure water and the nutrient media. Blanks and controls were treated in the same way as samples.

Once all blanks, controls and samples were prepared, 15 mL of the bacterial inoculum was added to each 1L sample bottle. The contents of the bottles were shaken to properly disperse the bacteria and then poured into triplicate 60 mL glass incubation bottles conditioned by three hours in a muffle furnace at 540^{o}C to eliminate any carbon residues. The incubation bottles were quickly closed using stoppers having a tapered ground glass end so that all air within the bottle was displaced. The bottles were sealed by pouring water onto the flared mouth of the bottle thus preventing the possibility of the loss of gases (water vapor and carbon dioxide) from the incubation bottle. The incubation bottles were placed in a temperature controlled incubation bath and kept in the dark at 25^{o}C. Bottles were sacrificed immediately and after 3 and 10 days for dissolved oxygen and complete carbon analyses.

4.3.4 Analyses

Dissolved O_2 was measured in triplicate at the time of incubation, after 3 days, and after 10 days using the Winkler method (Carpenter, 1965). TOC, total inorganic carbon (TIC), and total carbon (TC) analyses were done at the time of incubation and after 10 days on a Sievers TOC 800 analyzer, which directly measures TC and TIC and calculates TOC by difference. The incubation bottles represent a closed system so that a TC balance must be maintained.

Elemental analyses for C, H, N, and S were carried out by Huffman Laboratories (Golden, CO) using methods described by Huffman and Stuber (1985). The samples were vacuum-dried to a constant weight prior to analysis. Two sets of freeze-dried samples were submitted for analysis. The first set consisted of all six NOM samples.

Upon analysis, it was found that five of the samples had very high ash contents; only the Suwannee had an ash content <8% by weight. The five high-ash samples were subjected to electrodialysis (see Chapter 2 for details) to reduce the ash content and three were resubmitted for elemental analysis. The Congaree NOM was lost during the final stage of preparation and there was an insufficient amount of the New NOM for re-analysis of elemental composition.

Samples were also analyzed by capillary zone electrophoresis (Chapter 5), ^{1}H- and ^{13}C-NMR (Chapter 6), and electrospray ionization mass spectrometry (Chapter 7). The results from these analyses will be discussed in their respective chapters.

4.4 Results and Discussion

4.4.1 STORET Database

The molar ratio of BOD/TOC calculated for each site examined in the STORET database exhibit geographical trends (Figure 4.2 and 4.3). Note that each point on these maps represents the average of many samples. The highest bioavailabilities (>= 1.00 mol O_2 / mol TOC) are found mostly in Virginia whereas very few sites having relatively high bioavailability are found in the coastal plain regions of South Carolina, Georgia, Florida, Alabama, and Mississippi. For Georgia, the sites identified as having DOM of high bioavailability are in the Atlanta area. Despite our efforts to eliminate urban-related data, there may still be other such sites within the data set. The presence of sites having high BOD/TOC ratios in Virginia and the relative absence of such sites in other states may be due to a sampling artifact. The data set contains many more samples from Virginia than

any other state so that statistically there will be more samples identified as having high BOD/TOC ratios in Virginia.

Assuming that there is no sampling artifact, the presence of relatively higher bioavailabilities in restricted regions is consistent with the idea of the river continuum concept (Vannote et al., 1980) where the sources of fresh (and thus labile) DOM are relatively larger for headwaters and lower order streams.

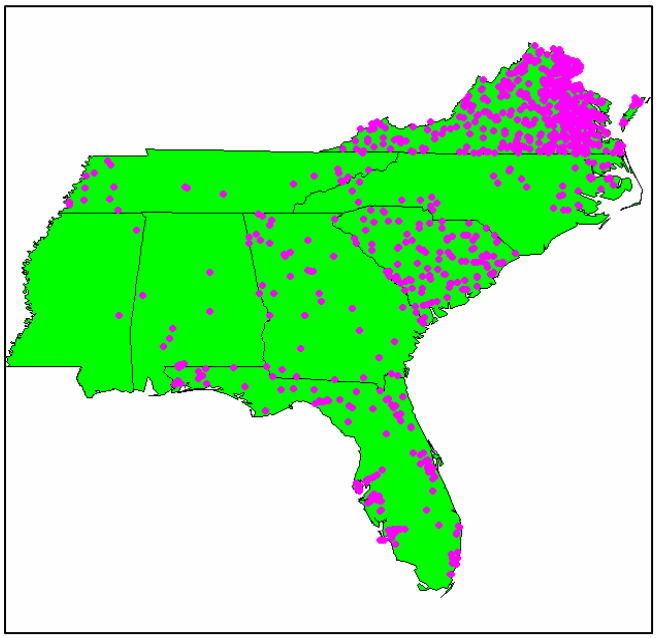

Figure 4.2: Of all the sites examined in the STORET data set, 967 of them had a mean molar BOD/TOC ratio in the range 0.001 – 0.055 mol O_2 /mol TOC. Note that the sites are scattered over the entire southeast.

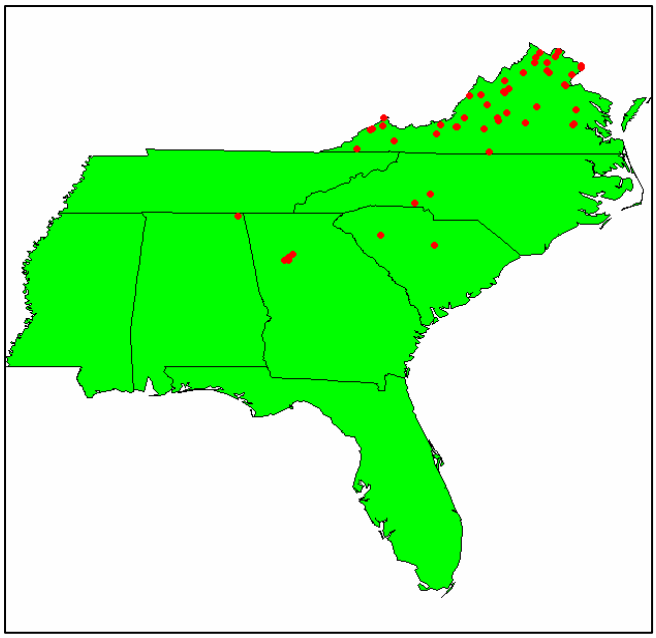

Figure 4.3: Of all the sites examined in the STORET data set, 52 of them had a mean molar BOD/TOC ratio in the range 1.00 – 1.50 mol O_2 /mol TOC. Note that most of the sites are found mainly in Virginia, presumably nearer the headwaters and relatively large sources of fresh DOM.

4.4.2 Carbon Mass Balance

TOC, total inorganic carbon (TIC), and total carbon (TC) determinations were made at the time of incubation and after 10 days. The incubation bottles represent a closed system so that a TC balance must be maintained. The standard deviations for triplicate analysis were 5.17 x 10^{-3} mM for TOC, 5.42 x 10^{-3} mM for TC, and 4.50 x 10^{-3} mM for IC. The mean TC lost for 10-day incubations was 5.17 x 10^{-3} mM or 0.84 % of the initial TC concentration, indicating a near-perfect mass balance of carbon in the closed system of the incubation bottles. It seems unlikely that the missing TC was adsorbed to the sides of the incubation bottles, because putting the bottles in a sonic bath for 5 minutes immediately prior to analysis did not alter the measured TC.

For a 60 mL bottle, the 5.17 x 10^{-3} mM of TC lost represents 3.10 x 10^{-4} mmol C which translates into 1.36 x 10^{-2} mg CO_2. For some samples, it was noticed that tiny (< 0.1 mL) air bubbles had formed in the incubation bottles. Assuming that the air bubbles occupied a total of 0.1 mL, then the concentration of CO_2 in the air bubbles would have to be 136 mg L^{-1} to account for the average TC lost, which is ~ 191 times the current atmospheric concentration of the gas (~ 360 uL L^{-1} or 0.71 mg L^{-1}) but is ~ 15 times less than the density of CO_2 (1980 mg L^{-1}). The measured loss in TC is attributed to a loss of CO_2 due to the occasional formation of air bubbles which are lost as soon as the incubation bottle is opened. The air bubbles formed during the incubation period because the incubation temperature (25^{o}C) exceeded that of the solutions (20 - 22^{o}C) when they were first poured and sealed in the incubation bottles.

4.4.3 Dissolved O_2 Analyses

The average standard deviation for triplicate measurements was 0.22 mg O_2 L^{-1} and the coefficient of variation (CV) was 2.8%. For comparative purposes, Moran et al. (1999) reported a CV of 0.4% using an automated titration method and Standard Methods (1989) imposes a standard deviation of 0.04 mg O_2 L^{-1} which for a mean O_2 concentration of 7.0 mg O_2 L^{-1} equals a CV of 0.6 %.

The values for reproducibility reported here are greater than the expected norm. A possible reason is a differential loss of O_2 due to the formation of air bubbles during the incubation period (see previous section). At 25^{o}C, an air bubble of 0.1 mL would contain 0.027 mg O_2, the loss of which would impact the reproducibility of measured dissolved O_2 if bubble formation and outgassing were not uniform for all incubation

bottles. Other than human error in detecting the endpoint of the titration, there is no other possible reason for the larger than expected CV reported here. Nonetheless, as will be seen, the CV is not large enough to mask differences between samples.

4.4.4 Quality Control

The blank was identical to the samples except that it had no DOM other than the bacterial inoculum and traces present in the DI water and the nutrient broth. The uptake of O_2 by the blank during the incubation period was almost as large as for the samples (Table 4.2). It is believed that in the absence of DOM (the energy source for the bacteria), cannibalism was taking place within the BOD bottles of the blank. Because of this effect, the O_2 consumption of the blank was not subtracted from the samples. Standard Methods (1989) requires blanks without bacteria and other studies (e.g., Moran et al., 1999) have followed suit. By comparison, blanks containing only DI water and the nutrient media and incubated for four days under slightly different conditions had an O_2 consumption of only 0.0094 mM.

Glucose was run as a control to ensure that the incubation set-up was operating to its fullest potential. It has been shown that for BOD analyses lasting 20 days, the oxidation of glucose will result in at most 85% of the theoretically maximum amount of O_2 to be consumed (Sawyer et al., 1994) (i.e., 0.85 mole of O_2 consumed per mole TOC). In the present setup, using glucose as the substrate for a 5 day incubation resulted in 0.76 mole of O_2 uptake per mole of glucose (Table 4.2), which is slightly less than the maximum theoretically possible of 0.85 because of the shorter time for incubation.

Table 4.2: TOC uptake, TIC gain, TC lost, and O_2 uptake during 10-day incubations of the freshwater NOM samples. The respiratory quotient, CO_2 produced/O_2 consumed, is 1.0 for the glucose control, as expected. The samples were not processed by electrodialysis prior to analysis. All concentrations are in mM.

	TOC_i (initial)	ΔTOC (used)	ΔTIC (gained)	ΔTC (lost)	ΔO_2 (used)	ΔO_2 / TOC_i	ΔO_2 / ΔTOC	ΔTIC /ΔO_2	ΔTIC / ΔTOC
blank	0.042	0.013	0.0071	0.0059	0.022	0.527	1.69	0.32	0.54
glucose	0.18	0.14	0.1069	0.0271	0.105	0.591	0.76	1.02	0.78
Suwannee	0.40	0.0095	0.0096	0.0000	0.029	0.074	3.09	0.33	1.01
Wchee	0.37	0.026	0.026	0.0000	0.047	0.126	1.78	0.56	1.00
Congaree	0.37	0.022	0.015	0.0058	0.056	0.152	2.58	0.27	0.70
New	0.39	0.061	0.048	0.0132	0.056	0.145	0.93	0.85	0.79
Ocmulgee	0.40	0.033	0.033	0.0024	0.064	0.158	1.94	0.52	1.00
Broad	0.38	0.036	0.025	0.0096	0.068	0.177	1.90	0.37	0.70
Mean*	**0.38**			**0.0052**					

*For NOM samples only.

Another measure of the adequacy of the incubation setup is the respiratory quotient (RQ) which is defined as the molar ratio of CO_2 formation to O_2 uptake (Dilly, 2001). Aliphatic compounds, refractory compounds, and mixtures low in O and having refractory compounds will have an RQ < 1. Mixtures with higher O content may have an RQ greater than 1. The latter is also true of environments where there are electron acceptors other than oxygen that are participating in the degradation of DOM, as would occur under anoxic conditions. The complete oxidation of glucose to CO_2 and H_2O

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O \qquad (9)$$

yields an RQ of 1. The oxidation of humic acids will have an RQ < 1 (Dilly, 2001). For the system described here, glucose had an RQ = 1.0, as expected (Table 4.2). With the exception of the New NOM which had an RQ of 0.85, all NOM samples had RQs < 0.60 indicating that the compounds being mineralized in these samples had relatively low O contents perhaps because of high aliphatic or high aromatic content. Furthermore, low

RQ's indicate that mineralization is incomplete so that not all of the oxygen consumed produces CO_2.

All samples had initial TOC concentrations that were approximately equal, ranging from 0.37 to 0.40 mM (Table 4.2). The molar ratio of TIC (i.e., CO_2) produced to TOC consumed for 10-day incubations varied from 0.70 to 1.01 reflecting different stoichiometry because of the differing NOM chemistry between samples. As for the RQ data, these data indicate that during the oxidation process not all the O_2 consumed results in production of CO_2; some intermediate compounds or new biomass may also be formed. In addition, O_2 may also be consumed in the oxidation of inorganic ions.

4.4.5 Bioavailability

Bioavailability was calculated as the molar ratio of O_2 consumed to the initial TOC concentration in the sample (i.e., $\Delta O_2 / TOC_i$) and is plotted for the six rivers in Figure 4.4. Carbon-normalized O_2 consumption increases over time for all samples and there are distinct differences in the bioavailabilities of the various DOM samples. Suwannee River DOM has the lowest bioavailability with an O_2 consumption of 0.074 mol O_2/mol TOC_i. This value is within the range found by Moran et al. (1999) for blackwater estuaries in the southeast (0.046 to 0.11 mol O_2/mol TOC_i) . The Suwannee River was sampled a few miles downstream of the Okefenokee Swamp. It is a blackwater river with a relatively high concentration of DOM (see Table 4.1). For surface waters in the US, 50 % of the DOM consists of humic and fulvic acids (Malcolm, 1985) but their content may be higher in waters draining swamps, as is the case for the Suwannee River where humic and fulvic acids represent 75% of the DOM (Malcolm et

al., 1995). Humic acids tend to be more recalcitrant and thus less bioavailable than non-humic components (e.g., Moran and Hodson, 1990) and therefore the bioavailability of DOM in surface water draining a swamp, as the Suwannee River does, is expected to be relatively low.

The Withlacoochee River is also a blackwater River in south Georgia; its DOM had the second lowest bioavailability, but because the river does not directly drain a swamp, its DOM probably has a relatively smaller proportion of humic and fulvic acids than the Suwannee River DOM. As such, the bioavailability of the Withlacoochee River is greater than that for the Suwannee River DOM but still less than that for the other DOM samples studied.

The Broad River (SC) DOM had the highest bioavailability (0.177 mol O_2 consumed/ mol TOC_i) of all the rivers studied. The relatively high bioavailability indicates that the chemistry of the DOM is different from that found in the blackwater rivers (Suwannee and Withlacoochee). Elemental analysis of the freeze-dried DOM samples indicates differences in the bulk elemental make-up of the samples (see section 4.4.6).

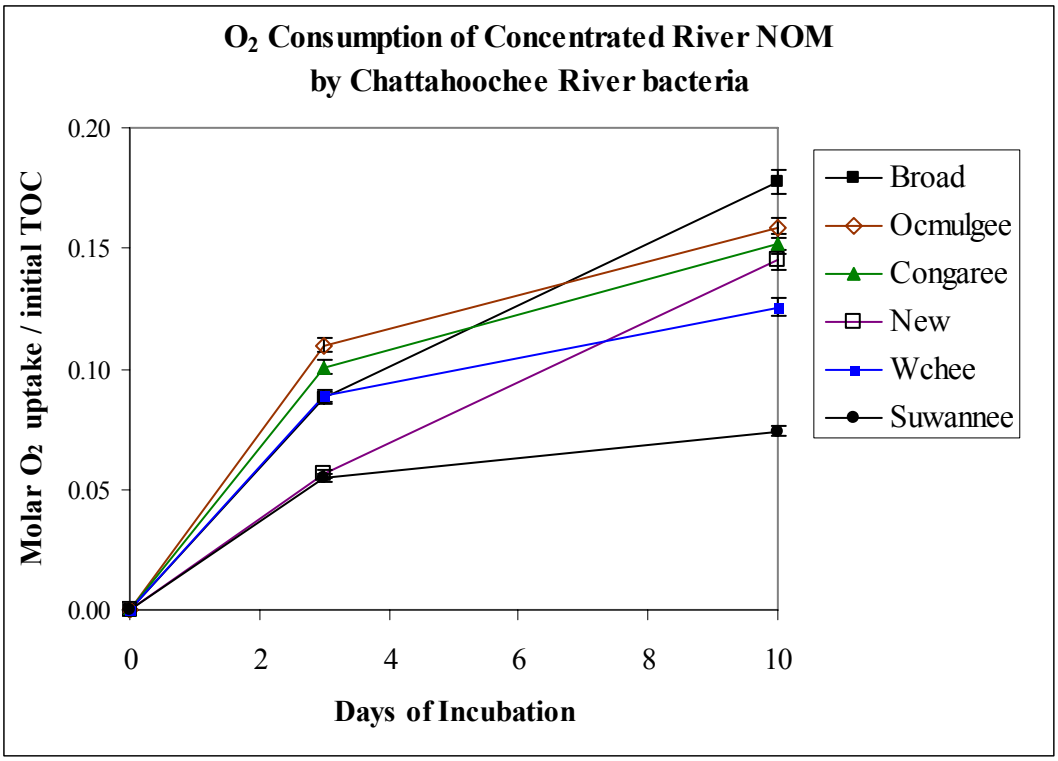

Figure 4.4: Measured bioavailability (10-day incubations using non-native bacteria) for the six river DOM samples. The Suwannee River DOM has the lowest bioavailability and the Broad River DOM the highest. Error bars represent a 2.8% coefficient of variation.

Figure 4.5 is a plot of the measured bioavailability data (ΔO_2 /TOC_i) as a function of the molar BOD/TOC ratio calculated from the STORET dataset. Interestingly, the sampling sites are distributed by geographical area. All three south Georgia rivers, which are on the coastal plain, plot above the 1:1 line indicating that the measured bioavailabilities of their DOM are greater than the calculated bioavailability values (BOD / TOC). Conversely, the measured bioavailabilities for the DOM of the two South Carolina rivers, both located on the Piedmont, are much less than the calculated bioavailabilities. The New River DOM plots just below the 1:1 line so its bioavailability is also less than the calculated bioavailability.

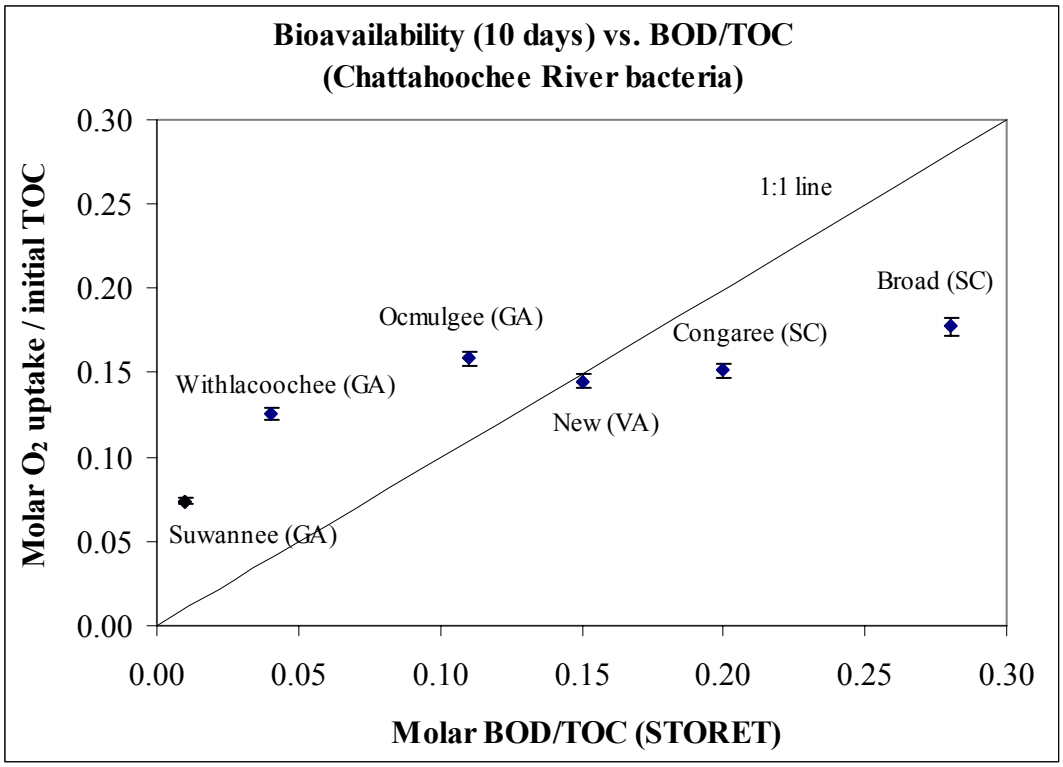

Figure 4.5: Measured bioavailability (after 10 days) compared to the BOD/TOC ratio calculated from the STORET dataset. The measured bioavailability is expected to be greater than the calculated bioavailability (see text) but is not for three of the samples. Error bars represent a 2.8 % coefficient of variation.

The BOD data in the STORET data set are for 5-day incubations whereas our incubation time was 10 days. A longer incubation should result in greater O_2 consumption since there is more time for the bacteria to consume the DOM. Thus, it is expected that our measured bioavailabilities should be greater than that calculated from the STORET data set (BOD/TOC), as they are for the three Georgia Rivers.

There are several possible reasons that could account for the Congaree, Broad, and New Rivers falling below the 1:1 line. First, ammonia, nitrite and other reduced species (e.g., Fe^{2+}) present in the samples may be oxidized, using up O_2 in the process and biasing the BOD results. The protocol for BOD (Standard Methods, 1989) calls for

the use of a nitrite inhibitor if these levels are expected to interfere with the BOD analysis. There is no indication in the STORET data set that the samples were treated in this manner. Assuming they were not, then samples having a high nitrogenous oxygen demand would result in erroneously high BOD values. During the collection of our samples, most cationic species, including NH_4^+ (pK_a = 9.3 at 25°C), are removed by the H^+-saturated cation exchange resin. Hence, given a sufficiently high ammonia concentration in the water, it is possible for the STORET BOD/TOC values to exceed our measured bioavailabilities.

Secondly, the TOC concentrations in the Congaree, Broad, and New Rivers are low (Table 4.1) so that even a relatively low concentration of inorganic nitrogen ions and other reduced ions will have a large impact on measured BOD, yielding erroneously high results.

A third possibility has to do with the measurement of TOC. An automated wet-chemical method (Sievers TOC 800 analyzer) was used in the present study. In the past, TOC has been measured by a variety of methods some of which may not completely oxidize the DOM present in freshwater samples (Koprivnjak et al., 1995). That being the case, it may be that some of the BOD/TOC ratios calculated from the STORET data set may be erroneously high.

It is possible to remove the contribution of reduced forms of N from the calculated BOD values in the STORET data set. Ammonia is oxidized to nitrate as (Stumm and Morgan, 1996):

$$NH_3 + 2O_2 \rightarrow NO_3^- + H^+ + H_2O \qquad (10)$$

There are 2 moles of O_2 consumed for each mole of ammonia oxidized. The BOD values can be adjusted by subtracting 2 moles of O_2 from the BOD value for each mole of ammonia present. Figure 4.6 is the same as Figure 4.5 except that the calculated BOD / TOC ratios do not include the contribution of ammonia nitrogen to BOD. Five of the DOM samples now lie above the 1:1 line whereas the Broad River DOM falls right on the line. Thus, 10-day incubations yield greater O_2 consumption values than the 5-day BOD incubations, as expected. This observation does not negate the possibility that the calculated values are still too high because of the impact of low inorganic nitrogen ions on low TOC concentrations and the possibility of incorrectly low TOC concentrations.

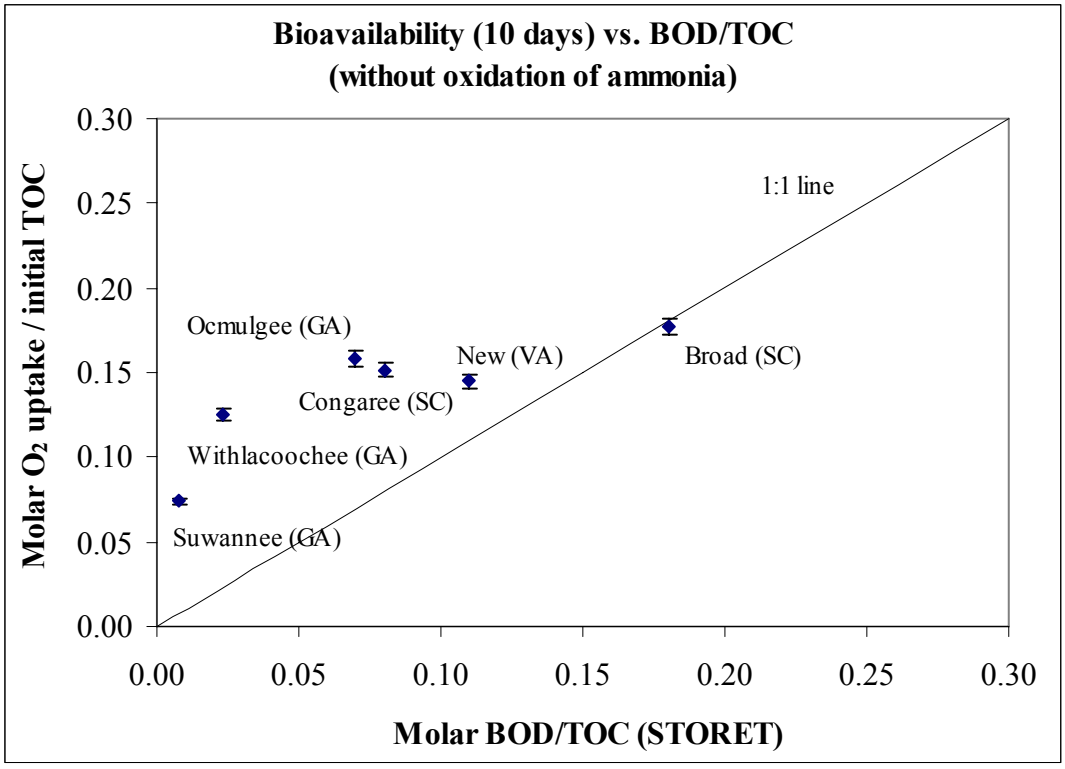

Figure 4.6: This is the same plot as Figure 4.5 except that the contribution of the oxidation of ammonia has been removed. For all samples, the measured bioavailability is now greater than the calculated bioavailability, as expected. Error bars represent a 2.8 % coefficient of variation.

For each sample, the percent bioavailable TOC can be calculated as the fraction of the TOC used during the 10-day incubations. Except for the New NOM, bioavailability ($\Delta O_2/TOC_i$) increases with % bioavailable TOC (Table 4.6). In the case of the New NOM, there is a larger percentage of TOC used during the incubation period but O_2 consumption per mole of TOC consumed is less than for any other sample (Table 4.2).

4.4.6 Elemental Analyses

Elemental analyses were first performed on high-ash freeze-dried samples. Subsequently, these samples were processed by electrodialysis (see Chapter 2 for details). The presence of ash will bias the elemental analysis results because ash consists of the inorganic components in freshwater NOM that were not removed prior to analysis. For the samples analyzed here (freeze-dried freshwater NOM collected by reverse osmosis), ash consists mainly of silicic acid (H_4SiO_4) and sulfate (SO_4^{2-}) (Sun *et al.*, 1995; Serkiz and Perdue, 1990). Most cations were removed by the H^+-saturated cation exchange resin in conjunction with reverse osmosis during sample collection. The resulting decrease in pH also causes inorganic carbon to be largely removed as CO_2(g). Other anions that produce relatively volatile acids (Cl^- and NO_3^-) are largely eliminated during the freeze-drying process. Results for elemental analysis are presented in Table 4.3. The presence of ash should have little effect on the N content. As for C, certain cations can combine with CO_2 to form carbonates (e.g., $CaCO_3$, $MgCO_3$) (see section 1.2.4 in Chapter 1) thereby reducing the organic C content and raising the ash content. For the Suwannee NOM, the already low ash content means that the elemental composition

presented in Table 4.3 is reliable; it is also comparable to that found for Suwannee NOM in the past (e.g., Perdue, 1998).

Table 4.3: Results of elemental analysis for high-ash and electrodialysed freshwater DOM samples. All results are reported on a dried, ash-free basis as wt %. After the samples have undergone electrodialysis (ED), the remaining ash content is primarily due to the presence of NaCl.

Sample	% C	% H	% O*	% N	% S	% sum	% ash
High-ash							
Suwannee	52.04	4.15	42.07	1.13	0.61	100.0	7.78
Withlacoochee	48.37	5.12	32.15	2.26	12.10	100.0	49.58
Congaree	40.60	5.41	0.62	4.24	49.14	100.0	77.35
New	32.25	5.46	21.55	2.98	37.76	100.0	72.14
Ocmulgee	35.29	7.13	-14.66	4.46	67.78	100.0	83.18
Broad	34.44	5.35	15.87	3.91	40.45	100.0	76.71
After ED							
Withlacoochee	52.87	5.28	40.03	1.82	N/A	100.0	66.50
Ocmulgee	46.40	5.40	46.50	1.71	N/A	100.0	52.36
Broad	46.49	5.58	45.84	2.08	N/A	100.0	39.07

* Calculated by difference; N/A: not analyzed.

Prior to electrodialysis, ash contents were extremely high for all samples (designated high-ash) except for the Suwannee NOM (Table 4.3). The electrodialysis process removed significant quantities of sulfate and silica so that the final molar sulfate/TOC ratio was < 0.012 and the silica/TOC ratio was < 0.079 (Table 4.4). Subsequent to electrodialysis, the ash content for these samples was still high (Table 4.3) due to an inadequate desalting process, on an H^+-saturated cation exchange resin, which left behind significant quantities of Na^+ (Table 4.4).

Table 4.4: % recovery of TOC and molar ratios (normalized to TOC) of Na, sulfate, and silicic acid remaining in the DOM samples after electrodialysis (ED). The remaining ash content is primarily due to the presence of NaCl.

Sample	% Rec. TOC	Na^+/TOC	SO_4^{2-}/TOC	H_4SiO_4 /TOC
After ED				
Withlacoochee	92	3.4	0.00096	0.013
Congaree	63*	0.019	0.00027	0.027
New	71*	0.081	0.0021	N/A
Ocmulgee	84	3.1	0.0012	0.0032
Broad	79	0.095	0.011	0.078

* Excludes rinse of ED membrane stack to recover adsorbed NOM.
N/A: Not available.

For the samples processed by electrodialysis, the H/C, O/C, and N/C values increase with increasing bioavailability of the NOM (Table 4.5). Mass spectrometry (MS) analysis of these samples (see Chapter 7) also indicates increasing H/C with increasing bioavailability. The MS results for the O/C and N/C are inconsistent with the results presented here probably because of the low sensitivity of the MS instrument at the time of analysis, limiting the number of peaks for analysis.

The average oxidation state of carbon (Z_c) for each sample was calculated from the elemental composition data. Z_c values fall within the range 0.04 – 0.32 (Tables 4.5 and 4.6); these values are similar to those calculated from the data in Hopkinson et al. (1998) for estuaries. Z_c for NOM, calculated using median values for H/C, O/C, N/C, and S/C (Perdue and Ritchie, 2003; Table 7.2) is 0.21, comparable to the values found here.

Table 4.5: Atomic ratios of elemental composition, molar ΔO_2 /TOC_i (bioavailability) and molar BOD/TOC ratios, and the calculated average oxidation state of carbon in the mixture, Z_c. The H/C, O/C, and N/C ratios shaded in gray are not reliable due to the presence of high contents of ash in these samples (see Table 4.3). For the samples processed by ED, the O/C ratio is questionable (see text).

Sample	H/C	O/C	N/C	molar ΔO_2 /TOC_i	STORET* BOD/TOC	Z_c**
High-ash						
Suwannee	0.95	0.61	0.02	0.074	0.008	0.32
Withlacoochee	1.26	0.50	0.04	0.126	0.023	-
Congaree	1.58	0.01	0.09	0.152	0.080	-
New	2.01	0.50	0.08	0.145	0.110	-
Ocmulgee	2.40	-	0.11	0.158	0.070	-
Broad	1.85	0.35	0.10	0.177	0.180	-
After ED						
Withlacoochee	1.19	0.57	0.030	N/A	0.023	0.037
Ocmulgee	1.38	0.75	0.032	N/A	0.070	0.22
Broad	1.43	0.74	0.038	N/A	0.180	0.17

* Adjusted for oxidation of ammonia.
** Calculated from elemental composition.

4.4.7 Degree of Reduction

According to Vallino et al. (1996) the average degree of reduction of the *labile* portion of a DOM sample can be calculated using equation 8 (see section 4.2.5)

$$\Psi_c = [4.2\ (\Delta DOC + \Delta DIC) + 4\ \Delta DO] / \Delta DOC \qquad (8)$$

Recall equation (2) for the average oxidation state of carbon:

$$Zc = 3(N/C) + 2(O/C) - (H/C) \qquad (2)$$

and equation (6) for the degree of reduction of the labile portion of DOM:

$$\Psi_c = 4 + (H - 2O - 3N + 6S - a) / C \qquad (6)$$

By combining these two equations and assuming no charge on the DOM sample, it can be shown that the average oxidation state of carbon in a DOM sample, Z_c, is related to the average degree of reduction of the organic compounds in that sample, Ψ_c, where:

$$Z_c = 4 - \Psi_c \quad (11)$$

The terms in equation (8) have all been determined during the bioavailability measurements done for the current study. Equation (8) however, cannot be applied to the current study for two reasons outlined in Vallino et al.'s (1996) paper: (i) the presence of NO_3^-, added here as a nutrient supplement, alters the equation but they did not specify how, and (ii) this equation is not applicable for a system where the microbial food web is not intact, which is probably the case for the current study. To demonstrate this point, a value of Z_c for glucose was calculated from the experimental data using equation (8) to calculate Ψ_c and equation (11) to calculate Z_c. The value of Z_c for glucose was 0.34 which is incorrect; using equation (2), glucose has a Z_c of 0, the correct value. Consequently, the average degree of reduction of the six riverine DOM samples cannot be calculated using equation (8).

Given elemental compositional data and using equation (11), it is possible to determine Ψ_c and Z_c for the riverine DOM samples (Table 4.6). The calculated Ψ_c are similar to those found by Hokinson et al. (1998) for coastal rivers. For each site, the long-term average for Z_c was calculated from the STORET dataset. With the exception of the Withlacoochee NOM, the long-term average Z_c values are all less than Z_c at the time of sampling. Thus, for three of the four sites at the time of sampling, the organic carbon was more oxidized than it normally is. This is part of the normal variation in the DOM chemistry at these sites. There is no correlation between bioavailability and either the Z_c values specific to the samples collected for this study or the long-term Z_c values.

Table 4.6: Average degree of reduction of labile DOM (Ψ_c) before electrodialysis and average oxidation state of carbon (Z_c) as determined from elemental composition and calculated from the STORET dataset. Experimental data for glucose, run as a control, are included for comparison. The samples are listed in order of increasing bioavailability. Ψ_c values are calculated directly from Z_c using equation (11).

Sample	Ψ_c $(4-Z_c)$	Z_c^*	Z_c^{**}	$\Delta O_2/TOC_i$	% bioavailable TOC
Glucose***	3.66	0.34	N/A	0.53	77.4
Suwannee	3.68	0.32	0.24	0.074	2.4
Withlacoochee	3.96	0.04	0.10	0.126	7.0
Congaree	N/A	N/A	0.53	0.152	5.9
New	N/A	N/A	0.059	0.145	15.6
Ocmulgee	3.78	0.22	-0.32	0.158	8.2
Broad	3.83	0.17	-0.033	0.177	9.3

* Calculated from elemental composition using equation (2), except for glucose.
** Long-term average calculated from the STORET dataset.
*** Experimental data.

4.4.8 Correlation of BOD/TOC with O_2 /TOC

There is a moderate correlation between the calculated BOD/TOC ratios (adjusted for oxidation of ammonia) from the STORET dataset and measured bioavailability (ΔO_2 /TOC_i) ($R^2 = 0.68$; n=6). A stronger correlation is not possible because calculated BOD/TOC values are means for each site whereas the measured bioavailability is for one sample at each site. The conditions immediately prior to and during sampling may affect the concentrations and quality of the DOM and consequently the measured bioavailability.

4.5 Conclusions

BOD measures the amount of O_2 consumed in a closed system, as the organic matter is transformed into CO_2, some intermediate compounds, and/or new bacterial

biomass. BOD measurements, over a course of 10 days, were used as indicators of the relative bioavailability of DOM from different rivers in the southeast. It is possible to distinguish between the bioavailability of different river DOM samples by observing that the carbon-normalized dissolved oxygen consumption is least for samples containing relatively more non-labile carbon such as humic and fulvic acids. Analysis of the results indicates that the DOM is not completely mineralized to CO_2, implying the production of biomass.

Using an independent dataset (STORET), calculated BOD/TOC ratios appear to exhibit a geographical distribution with a significant portion of the high values restricted to regions of Virginia and low values being found in the entire southeast. Calculated BOD/TOC values are moderately correlated with measured bioavailabilities and thus can be used as a surrogate for bioavailability of geochemically diverse riverine DOM.

A strong correlation exists between the bulk elemental composition and the bioavailability of the DOM. The H/C and N/C molar ratios are positively and strongly correlated with bioavailability, as hypothesized.

4.6 Future Research

Future work should examine the question of whether the bioavailability and thus the chemistry of the DOM is affected by any one of the processes to which it is subjected to, namely reverse osmosis to concentrate the DOM, electrodialysis to remove ash, and freeze-drying.

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CHAPTER 5

CAPILLARY ZONE ELECTROPHORESIS (CZE)

5.1 Introduction

Capillary zone electrophoresis (CZE) is the separation of ionized molecules (deprotonated NOM molecules in this case) in an electric field. As the separation proceeds, molecules with the same charge-to-surface area ratio move together as a group, forming zones and moving at the same speed through the capillary column. Zones will form only if the velocities of the different charge-to-surface area groups are sufficiently different (Duxbury, 1989). The separation of cations and anions in a mixture is a function of their charge density (their effective charge-to-size ratio) which is governed by the pH of the buffer solution used (Schmitt-Kopplin and Kettrup, 2005).

The terminology used in the literature to describe the separation of molecules includes charge-to-mass and charge-to-size. In fact, molecules are separated based on their charge-to-*surface area* ratio in the form described by Offord (1966): $\mu = k\ Z / M^{2/3}$ where μ is the mobility, k is a viscosity-dependent constant, Z is charge, and M is the molecular mass. The term $M^{2/3}$ in Offord's equation is more accurately surface area, and Offord (1966) stated this point. On a molar basis, density (g/mL) is given by molecular weight (g/mole) over volume (mL/mole) as $\rho = M / V$. Rearranging, $M = \rho V$. If V is for a sphere, for example, then $V = 4/3\ \pi\ r^3$ so that

$$M = \rho(\frac{4\pi r^3}{3}) \quad \text{and} \quad M^{2/3} = (\frac{4\pi\rho}{3})^{2/3} r^2$$

Assuming molecules of the same density, the last equation shows that $M^{2/3}$ α r^2 which is surface area. The mobility of anions is formally defined as being inversely proportional to the Stokes radius of the analyte (Schmitt-Kopplin et al., 1999; Atkins 1998):

$$\mu_{eff} = \frac{q}{6\pi\eta R}$$

where q is the net charge, η is the viscosity of the buffer, and R is the Stokes radius. The Stokes radius is a hyrodynamic radius which takes into account the hydration sphere of an ion (Atkins, 1998). Surface area, r^2, is proportional to the Stokes radius, R: the greater the radius of an ion and its hydration sphere, the greater its surface area. From here on, the terminology used when describing mobility will be charge-to-surface area, keeping in mind that surface area is related to size and mass.

The core of a CZE system is a flexible fused silica capillary tube of 20 to 100 cm in length and an internal diameter of 20 to 100 μm. The silica capillary tube is enclosed in a temperature-controlled unit and each end is immersed in a buffer solution, allowing buffer to flow through the capillary. The introduction of the buffer into the silica column causes the inner surface silanol groups (pK_a 3 to 5) to ionize. The degree of ionization is dependent on the buffer pH; the higher the pH, the more silanol groups will be ionized. The presence of negatively-charged silanol groups attracts cations from the buffer solution, resulting in the formation of a double layer made up of an inner (fixed) layer and an outer (diffuse) layer. The thickness of the double layer is dependent on the ionic strength of the buffer solution, varying inversely with it (Baker 1995; Schmitt-Kopplin and Kettrup, 2005).

At a pre-programmed moment, the capillary inlet and anode are immersed into the sample vial. The sample vial is pressurized forcing a small volume (< 3 – 4% of total

column volume) of sample into the capillary. A voltage (5 – 30 kV) is then applied across the capillary (Schmitt-Kopplin and Kettrup, 2005), resulting in the buffer cations of the mobile layer to move toward the cathode. Because these cations are solvated, they drag along with them the bulk of the buffer solution in a process known as the electro-osmotic flow (EOF) (Baker, 1995). The negatively charged NOM ions attempt to flow in the opposite direction to the EOF, towards the anode, but eventually they also end up at the cathode as their velocity toward the anode is less than the EOF. Although it may seem counter-intuitive that negative ions flow to the cathode (-) under the influence of the EOF, this process can be envisioned as trying to swim upstream against a current but being dragged downstream by the stronger current. The greater the charge-to-surface area of the NOM molecule, the more resistance it will have and the longer it will take for it to migrate to the cathode. These molecules will have the highest mobilities (EOF + mobility in the opposite direction to the EOF). Molecules with low charge-to-surface area ratios will migrate through the capillary first followed by those with higher charge-to-surface area ratios. These latter molecules are the ones resisting the EOF most strongly and as such exhibit the greatest mobility (Baker 1995; Schmitt-Kopplin and Kettrup, 2005).

The type of buffer solution used and its pH are determined prior to analysis and depend on the goal of the research. Because the pH of the buffer solution determines the degree of ionization of the sample, any one sample will be run at a number of different pHs, so as to be able to detect changes in the peaks and the appearance and disappearance of peaks as a function of pH. Typically, buffers are either acetate or carbonate, and, in

some cases, borate, though the latter is not favored due to known interactions between borate and NOM molecules (Schmitt-Kopplin et al., 1998a).

Detection of the sample as it nears or exits the capillary outlet is by UV-visible spectroscopy, laser-induced fluorescence, electrochemistry, or mass spectrometry. For example, as the sample nears the cathode, absorbance at 254 nm can be measured using an optical window in the column (made by burning away a small section of the polyamide coating off the column), yielding an indication of the concentration of NOM with time (Baker 1995; Schmitt-Kopplin and Kettrup, 2005). The electropherogram (absorbance versus mobility) output can be thought of as a frequency histogram of molecules of differing charge-to-surface area ratios. Any given charge-to-surface area ratio will represent all molecules having the same charge-to-surface area but not necessarily the same charge nor the same mass (Ritchie, 2005).

Due to the complexity of an NOM mixture, a UV detector tuned to 254 nm may not be sufficient to distinguish NOM from various sources (Parlanti et al., 2002) and, as discussed in Chapter 1, not all NOM molecules absorb at this wavelength. In general, however, it has been observed that UV absorbance and dissolved organic carbon are well correlated.

5.2 Literature Review

There have been over 100 articles examining the separation of humic substances (HS) by capillary electrophoresis (CE) (Schmitt-Kopplin and Kettrup, 2005). In all cases, a similar mobility pattern is found, described as a Gaussian-like distribution (termed a “hump”) of charge density of the molecules in the sample, corresponding to

their charge-to-surface area ratios. The "hump" can be characterized by an average electrophoretic mobility which is dependent on the make-up of the sample, experimental conditions, and buffer pH (Schmitt-Kopplin and Kettrup, 2005). In some cases, there are also single peaks corresponding to single molecules that are polar and charged. Humic acids tend to give one or more "humps" whereas fulvic acids exhibit a set of sharp peaks, thought to be phenolic acids, on top of the "hump" (Schmitt-Kopplin and Kettrup, 2005). These sharp peaks must have either aromatic or other UV absorbing chromophores (Garrison et al., 1995). For a typical CZE plot of HS, the hump consists of an unknown, but very large number, of molecules which cannot be resolved. The information content of CZE lies in the fact that the hump represents a charge density distribution, governed by the distribution of molecular sizes and acidities (Schmitt-Kopplin et al., 1999). The hump has an average electrophoretic mobility which changes with changing pH, as discussed below.

The determination of molecular mass of a complex mixture requires statistical methods, because there is a continuous distribution of molecular mass (Perdue and Ritchie, 2003). Similarly, the average mobility of an electrophoretic distribution can be described by weight-average mobility (μ_w) calculated as (Schmitt-Kopplin and Kettrup, 2005):

$$\mu_w = \frac{\int_0^\infty A(\mu) \cdot \mu \cdot d\mu}{\int_0^\infty A(\mu) \cdot d\mu}$$

where $A(\mu)$ is the absorbance at 254 nm for a given mobility. The peak-maximum mobility, μ_p, is the mobility at the maximum peak height. For a symmetrical distribution, $\mu_p = \mu_w$; when $\mu_p / \mu_w > 1$, the mixture consists of a greater proportion of less mobile

molecules. When $\mu_p / \mu_w < 1$, the mixture has a greater proportion of more mobile molecules. As such, the ratio of μ_p / μ_w describes the heterogeneity of the sample. A value close to 1 indicates that there is a symmetrical distribution of the charge-to-surface area makeup of the sample whereas a value much greater or much less than 1 indicates a highly skewed mixture (Schmitt-Kopplin and Kettrup, 2005).

Garrison et al. (1995) carried out a systematic study examining the various parameters that may affect the outcome of capillary electrophoresis (CE) for the characterization of humic substances (HS). CE is useful for the study of HS because of their acidic functional groups, consisting mostly of carboxylic and phenolic groups. HS will have some charged molecules at any pH above about 3; it has been determined that the pK_a of carboxyl groups for simple organic acids lies in the range of 3 to 5 (Perdue, 1985) and that the pK_a for carboxyl groups in HS lies in the range of ~1.8 to 5 (Leenheer et al., 1995). Garrison et al. (1995) found that migration times will generally decrease as pH increases, because the EOF is greater and a greater proportion of the molecules in the sample are charged at higher pH. The separation of molecules by charge-to-surface area is affected by buffer pH. They suggested that detection is best at low pH when EOF is relatively low, but high pH may be preferred to get a better separation of the sample. At higher pH, most of the carboxyl and some of the phenolic groups are ionized, and the molecules are more mobile, resulting in a better separation of the sample. Increasing the applied voltage decreased migration times. The sharpest and most pronounced peaks were found when measuring the output at 254 nm.

The efficiency of CZE is determined by buffer solution ionic strength and pH, applied voltage, volume of sample injected, and capillary length. These parameters are

determined by the requirements of the research. The reader is referred to Garrison et al. (1995) and Schmitt-Kopplin's work for details on how each of these affects the separations.

Schmitt-Kopplin et al. (1998; 1999) have shown that the mobility of NOM molecules in a buffer will increase with increasing pH. As discussed above, this is an expected result, given that NOM are charged anions consisting of carboxyl and hydroxyl functional groups with a continuous pK_a distribution from ~1.8 to 11.

Parlanti et al. (2002) separated freshwater NOM into several fractions using high-pressure liquid chromatography (HPLC), analyzing each fraction separately by CZE. They found that despite the separation of the NOM into fractions, the CZE electropherograms resembled each other, so that the complexity of NOM persisted within each fraction. The same observation was made by Ritchie (2005) who separated NOM into fractions by size exclusion chromatography using Superdex 30. The complexity of an NOM sample is a known fact, but it should be noted that Parlanti et al. (2002) used a borate buffer, which has been shown, well before their study, to potentially interact with HS (Garrison et al., 1995; Schmitt-Kopplin et al., 1998a, 1998b). Furthermore, Parlanti et al.(2002) used electropherograms with a time scale instead of the mobility scale (i.e., 1 / time) that takes into account changes in the EOF from one measurement to the other (Schmitt-Kopplin and Junkers, 2003) and allows direct comparison of electropherograms generated under slightly differing conditions (e.g., pH, ionic strength, capillary length, applied voltage). Parlanti et al.'s work may have benefited from such considerations.

The interpretation of electropherograms is challenging. The mobility scale can be viewed as a frequency distribution of molecules having similar charge-to-surface area

ratios. Peaks appearing at either the high or at the low end of the mobility scale could be system peaks which form due to different concentration zones within the capillary. The sample contributes to the ionic strength within the capillary and could influence the ion mobility if the buffer ionic strength is too low. This results in field distortions and formation of system peaks due to the accumulation or depletion of ions within regions inside the capillary. Under normal conditions there is a continuous distribution of ion mobility (Schmitt-Kopplin and Junkers, 2003).

Peaks appearing at the low mobility region are not necessarily of high molecular mass, i.e., they do not necessarily have a low charge-to-surface area (Schmitt-Kopplin and Junkers, 2003) but may represent molecules of relatively low molecular mass (or surface area) and capacity for only a small charge density, such as lignin or lignin-derived compounds (Schmitt-Kopplin et al., 1998a). Molecules having the same charge-to-surface area ratio and capable of holding multiple charges will change their charge-to-surface area ratio with changing pH. This change may not affect all molecules in the same way if the pK_as of the functional groups differ, with the result that at a different pH they will be separated into different groups according to their new charge-to-surface area ratios.

The work by Schmitt-Kopplin et al. (1998a), where electropherograms representing different size classes of HA molecules were compared, showed that the low molecular weight fraction had the highest average mobility suggesting that the charge-to-surface area ratio was the highest for this fraction and implying that these low molecular weight molecules are, on average, more charged. When interpreting electropherograms for this research, fingerprint peaks appearing in the high mobility region of an

electropherogram will be considered to be relatively smaller molecules with high charge-to-surface area ratios.

5.3 Hypotheses

CZE was used to analyze six diverse freshwater NOM samples (see Chapter 4 for more information on the nature of the samples) with the following hypotheses in mind:

- The difference in weight-average mobility (μ_w) between the most and least bioavailable NOM is expected to be significant at each pH, reflecting the difference in elemental composition between the samples.
- The relative difference in the weight-average mobility between any two NOM samples will be different at different pHs. For example, for two samples, the difference in mobility at a low pH will be different than at a higher pH.
- The more bioavailable NOM will tend to exhibit distinctive peaks in CZE, not present in NOM of lesser bioavailability.
- The presence of a high concentration of inorganic ions (ash content) in a concentrated (by RO) NOM sample interferes with CZE analysis, probably through the prevention of charge formation and resulting in a lower weight-average mobility of the sample.
- The heterogeneity of the NOM, determined as the ratio of the peak-maximum mobility to weight-average mobility (μ_p / μ_w), is expected to be close to unity for all samples.

5.4 Methods

Freeze dried freshwater NOM samples that were processed by electrodialysis (ED) were dissolved in 0.250 mL of 0.1 M NaOH. High-ash freeze dried samples were dissolved in either 0.500 mL of 0.1 M NaOH or 1.200 mL of 1.0 M NaOH (Table 5.1). There is no significance to the difference in procedure between the high-ash samples and those processed by ED. The difference in the concentrations of TOC is due to differences in ash contents as determined by elemental analysis (see Chapter 4). The samples were well mixed, but an undissolved residue, presumably consisting of silica and some NOM, was generally present and was allowed to settle. The supernatant solution was used for analysis. All CZE analyses were carried out at three pHs with a different buffer at each pH. The high pH buffers (~ 9, ~ 11) were prepared with $NaHCO_3$ and Na_2CO_3 whereas the low pH buffer (~ 5) was prepared with CH_3COONa and CH_3COOH. Buffers were freshly prepared and their pH measured before analysis. Prior to analysis, the capillary was rinsed for ten minutes with 0.1 M NaOH.

The TOC concentrations of the samples (Table 5.1) are high (11 - 661 mM TOC). Initial tests using lower concentrations yielded unsatisfactory electropherograms with too small peaks. Concentrations that were too high produced peaks that exceeded the maximum peak absorbance of the UV detector. The concentrations used here were found to be suitable, with no visible complications. As a general rule, the buffer concentration should exceed the sample concentration by at least 100 times (Baker 1995), otherwise there may be differences in conductivity between the solute (NOM) and the surrounding buffer, causing a distortion in the electric field and leading to broad, skewed peaks. Even though this rule was violated during these experiments (buffer concentrations ranged

from 16.7 - 33.0 mM), there was no noticeable effect on the quality of the peaks nor was there any instability in the current during any of the runs. Garrison et al. (1995) used 42 mM TOC and buffers in the range of 50 to 100 mM and Schmitt-Kopplin et al. (1999) used 83 mM TOC with 25 to 50 mM buffers. In both studies the TOC concentrations used are comparable to the concentrations used here for the high-ash samples.

Table 5.1: Freshwater NOM sample concentrations used for CZE. High-ash samples were processed by electrodialysis (ED) to lower the sulfate and silica content of the NOM sample (see Chapter 2).

Sample	**mg dry sample**	**mM TOC***
After ED		
Suwannee**	4.13 in 0.250 mL 0.1 M NaOH	661
Withlacoochee	5.10 in 0.250 mL 0.1 M NaOH	301
Congaree	Not Analyzed	-
New	3.06 in 0.250 mL 0.1 M NaOH	226***
Ocmulgee	Not Analyzed	-
Broad	4.10 in 0.250 mL 0.1 M NaOH	303
High-ash		
Suwannee**	1.39 in 0.500 mL 0.1 M NaOH	111
Withlacoochee	2.10 in 0.500 mL 0.1 M NaOH	85
Congaree	3.28 in 1.200 mL 1.0 M NaOH	21
New	4.07 in 1.200 mL 1.0 M NaOH	25
Ocmulgee	2.63 in 1.200 mL 1.0 M NaOH	11
Broad	3.25 in 1.200 mL 1.0 M NaOH	18

* Taking ash content into account (determined by elemental analysis).
** Suwannee was the same (low-ash) sample in both cases. It was used as a reference to compare the CZE process over time.
*** Estimated.

Two sets of riverine NOM samples, obtained by reverse osmosis, were analyzed by CZE – samples with high ash content and samples whose sulfate and silica content was considerably lowered by the ED process (see Chapter 2). A Beckman P/ACE 5510 CZE instrument with the specifications shown in Table 5.2 was used.

Table 5.2: Specifications of the capillary electrophoresis instrument, a Beckman Coulter P/ACE 5510.

Parameter	*Specification*
Capillary type	uncoated fused-silica
Capillary dimensions:	
total length	57 cm
total length to window	50 cm
inside diameter	75 um
volume	2518 nL
volume to optical window	2209 nL
injection plug length	13.31 mm
plug % of length to window	2.66 %
Operating conditions:	
temperature	30^{o}C
injection pressure	0.5 bar
injection time	10-15 sec
buffer viscosity (relative to water)	1
electric potential field strength	25-30 kV 439-526 V / cm
Hydrodynamic Injection volume	58.8 nL
Diode Array detector	254 nm

The output (electropherogram) from the CZE instrument is a plot of absorbance (254 nm) versus time. CZE data are better interpreted when the time axis (minutes) is transformed into mobility ($cm^2\ V^{-1}min^{-1}$) because mobility takes into account changes in EOF that can occur from one measurement to the next due to slight differences in buffer chemistry (Schmitt-Kopplin and Junkers, 2003). The process of transforming the baseline from time to mobility sets zero mobility as the starting point, so that all electropherograms show only the negatively charged species present in the sample.

The transformation of the time axis to a mobility axis was done using the GelTreat software developed by I. Perminova and A.V. Kudryavtsev at the Lomonsov Moscow State University chemistry department in Russia (Schmitt-Kopplin and Kettrup, 2003), but all baseline corrections were made by the user and not by the software. The baseline correction is done using a spline function. The baseline can be positioned so that it hugs the bottom of all peaks, thereby creating peaks that all return to the baseline. Alternatively, the baseline can be placed so that peaks do not return to the baseline, but are floating above the baseline. How the baseline is positioned affects not only the visual aspect of the electropherogram but also, more importantly, the calculated weight-average mobility. The positioning of the baseline is subjective and must be consistent to ensure that relative mobilities are meaningful. To avoid inconsistency, all baseline manipulations were done at the same time by the author. As a result, any differences in mobility between samples are due to actual differences in mobility, reflecting charge-to-surface area differences, random error in the CZE process (if any), and human error in positioning the baseline. In the hands of an experienced technician, the error in positioning the baseline is minimized. Repeated efforts by the author to draw the baselines resulted in relative standard deviations (RSD) in weight-average mobility of 0.4 to 1.9% with an average of 0.9%.

Samples were re-run only when there was an obvious problem with an electropherogram such as weak peak formation. It was not part of the protocol to run duplicates of the samples. Samples that were run more than once because of perceived problems with the electropherograms indicate that the repeatability of the CZE process,

as determined by the weight-average mobility, is 4.9% (n=3; data not shown) which includes the 0.9% due to the baseline correction discussed above.

Schmitt-Kopplin (2002) using a mixture of benzoic, p-hydroxybenzoic, and vanillic acids determined the repeatability of the CZE process to have a relative standard deviation (RSD) for the mobility of < 0.25%. Given that the samples used in the present study were far more complex than a mixture of three acids, the figures presented in this chapter show a 2.0% error, consistent with the findings of the author.

The buffers were analyzed as a sample for each pH at which the CZE was run (Figure 5.1). This was to ensure that the various buffers did not contain any NOM-like characteristics due to contamination and did not produce peaks that would interfere with sample interpretation. No peaks of any significance other than the internal standard were detected in the electropherograms of the buffers. Internal standards were used when analyzing the high-ash samples only. They were p-hydroxy benzoic acid (pH 5.30 and 11.28) and mesityl oxide (pH 9.17).

The main interest in this study was to look for differences in weight-average mobility. The CZE system was not optimized during analysis to remove artefacts causing sharp peaks.

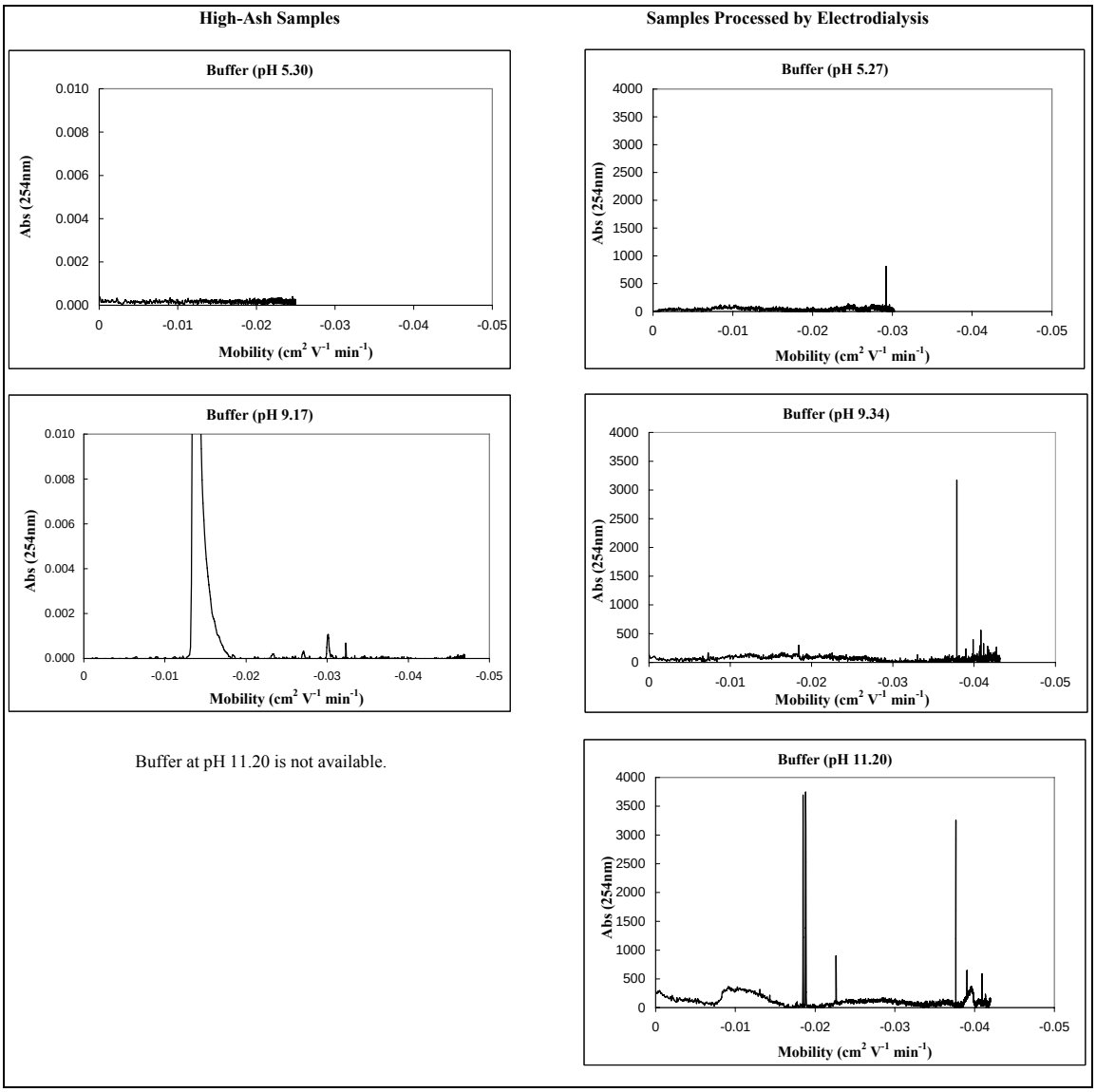


Figure 5.1: CZE plots for the buffers at all pHs used. The few peaks that appear have small absorbances relative to the samples and are well defined. The absorbance scales are different for the two runs because two instruments were used with different settings. For the pH 9.17 buffer, the peak at a mobility of ~ -0.015 is for the internal standard. No internal standard was used when analyzing the samples processed by electrodialysis.

5.5 Results and Discussion

5.5.1 Correcting for Differences Between Sample Runs

The calculated weight-average mobility for the Suwannee NOM was greater for each pH when measured for the samples processed by electrodialysis than when measured for the high-ash samples (Figure 5.2). The Suwannee NOM, however, was the same low-ash sample for each run; it had not been processed in any way in the intervening time (three years). The NOM was in freeze-dried form which is not expected to change significantly over time. The observed difference in mobility for the Suwannee NOM between the two runs must be due to the use of a different CZE instrument. Other contributing, though minor factors include slight differences in buffer pH, capillary and sample preparation, and sample concentration. The difference in mobility was significant at each pH (Figure 5.2) with no correlation to the small differences in operating pH between the two sets of analyses. During both runs, the operators were the author and a well-trained laboratory technician with substantial experience in the proper operation of the CZE instrument.

The results for the Suwannee NOM were used to correct for any procedural differences between the two analysis runs. It is not believed that differences in the TOC concentration (111 mM and 661 mM; Table 5.1) could be the cause of the difference in mobility. If there is a concentration effect, then it is accounted for by the described correction, because all samples had a higher TOC concentration during the second run (Table 5.1). To compensate for any differences between the two sample runs, the difference in mobility observed for the Suwannee NOM was subtracted from all samples, including the Suwannee (thereby setting the difference in mobility to be equal to zero for

the Suwannee NOM). Such a correction may over- or under-compensate for any changes that may have occurred for other samples, had they been measured in the same way, but in the absence of such analysis, it is the best that can be done under the circumstances. This correction is applied only when directly comparing the two sets of samples. For analysis within each group, the correction is not required and not used.

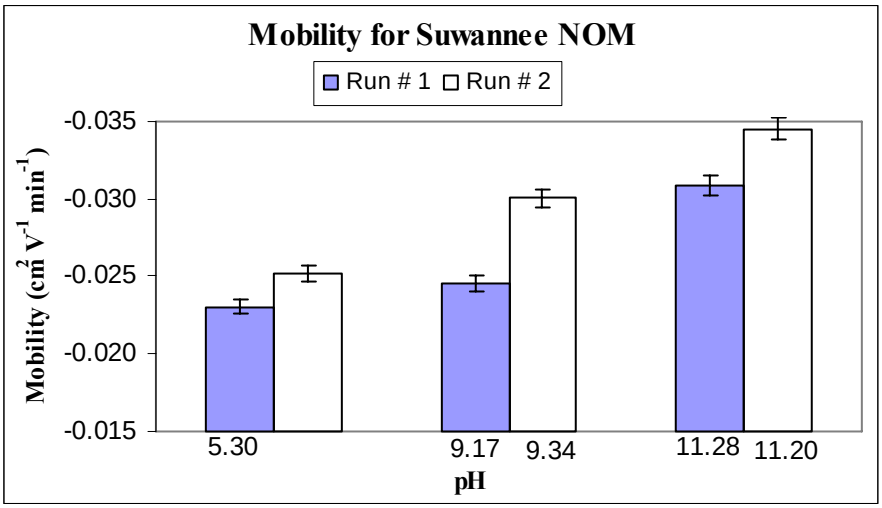


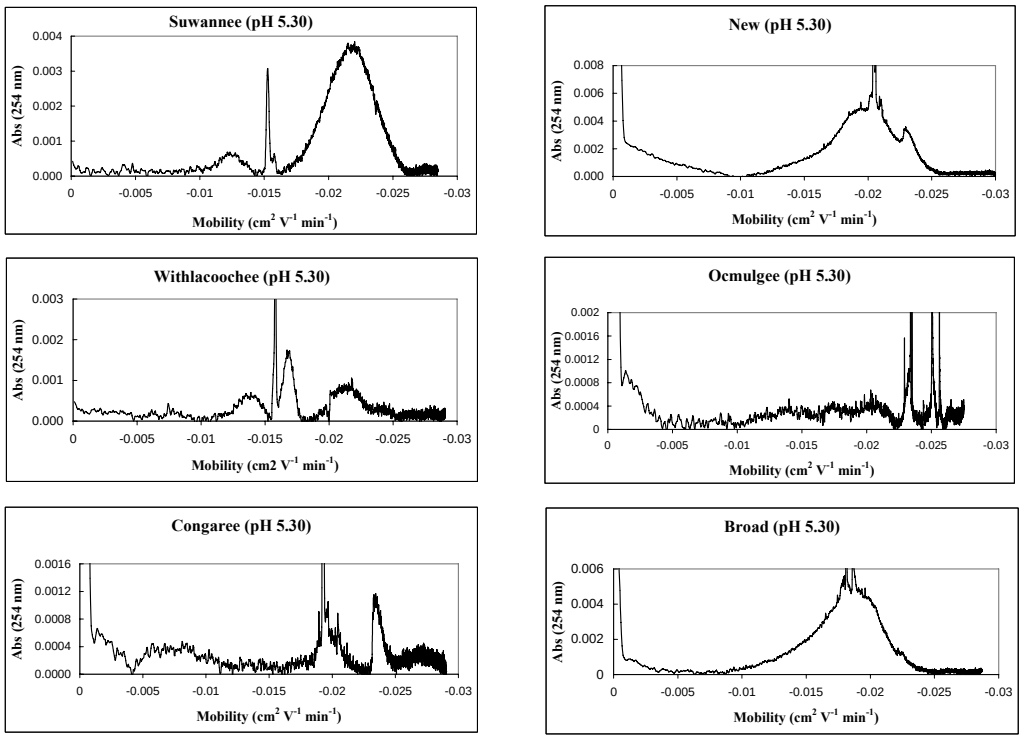
Figure 5.2: The mobility for the Suwannee NOM was significantly different for the two runs. Run #1 is associated with the high-ash samples and run #2 with the samples processed by electrodialysis. Error bars represent 2% of the mobility value.

5.5.2 Peaks

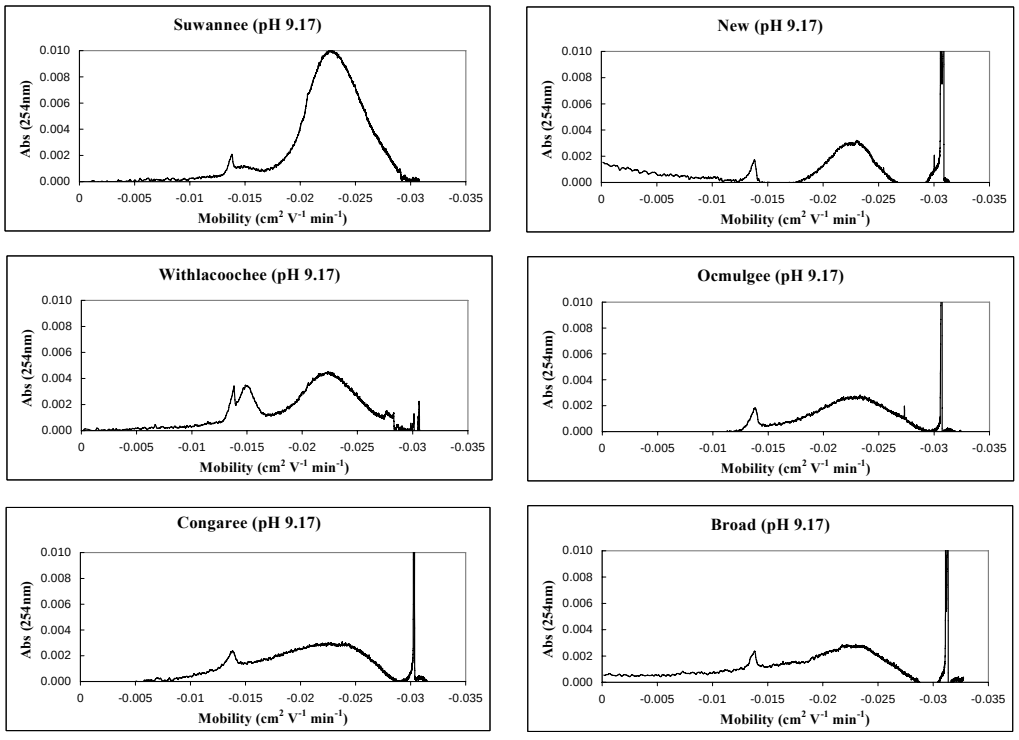
In theory, peak heights and areas are proportional to concentration. Peak heights and areas are not used here because of the complexity of a typical NOM mixture, the difficulty the author had obtaining reproducible electropherograms, and the fact that the CZE was not optimized to yield perfect electropherograms.

The first aspect to notice when looking at the electropherograms is the general peak shapes and any unique peaks appearing on top of the main, broad peak (or hump). For the high-ash samples (Figure 5.3) the following observations are made:

- At pH 5.30, the peak appearing in the mobility range of ~ -0.015 to -0.020 in the Suwannee and Withlacoochee electropherograms must contain aromatic or other UV-absorbing chromophores (Garrison et al., 1995). It may also represent molecules of high molecular weight or molecules having few ionized groups (e.g., high carbohydrate or non-oxidized lignin content (Schmitt-Kopplin et al., 1999)). The presence of aromatic compounds is consistent with the relatively low bioavailability of the two samples (see Chapter 4).
- At pH 9.17, the peak appearing at a mobility of ~ -0.030 in all electropherograms except for the Suwannee is a system peak possibly resulting from the difference in the ionic strength of the sample and the buffer resulting in a non-ideal interaction between the sample and buffer (Schmitt-Kopplin and Junkers, 2003). At the high end of the mobility scale, molecules with relatively high charge-to-surface area ratios appear, and by extension, higher ionic strength will be the result (ionic strength being a function of charge squared). The peak at ~ 0.013 to -0.015 in all electropherograms is the internal standard.



pH 5.30



pH 9.17

Figure 5.3: CZE electropherograms for the high-ash NOM samples at pHs 5.30, 9.17, and 11.28 (next page).

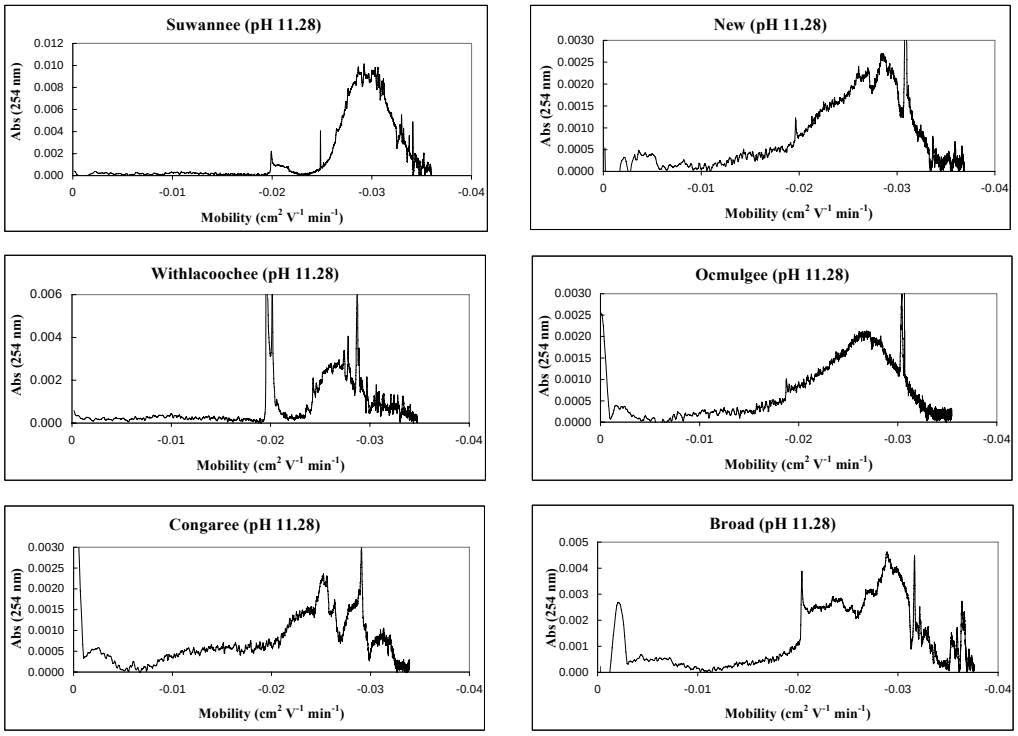


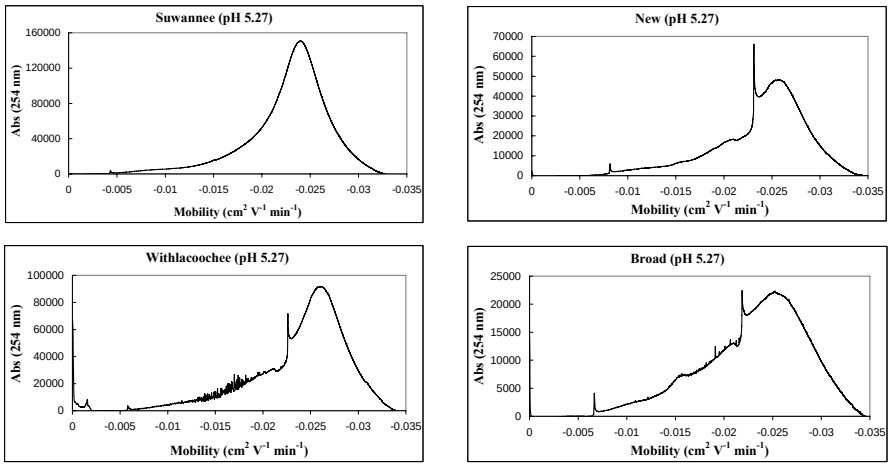
Figure 5.3 (continued): CZE electropherograms for the high-ash NOM samples at pHs 5.30, 9.17 (both on previous page), and 11.28.

- At pH 11.28, the peak appearing at a mobility of -0.028 to -0.031 in all samples except the Suwannee must contain UV-absorbing chromophores (Garrison et al., 1995). Being in the high mobility region of the electropherogram, this peak probably represents relatively smaller molecules with a relatively high charge-to-surface area ratio.
- There are no apparent distinct (fingerprint) peaks for any sample at any pH which would indicate that the samples are different.

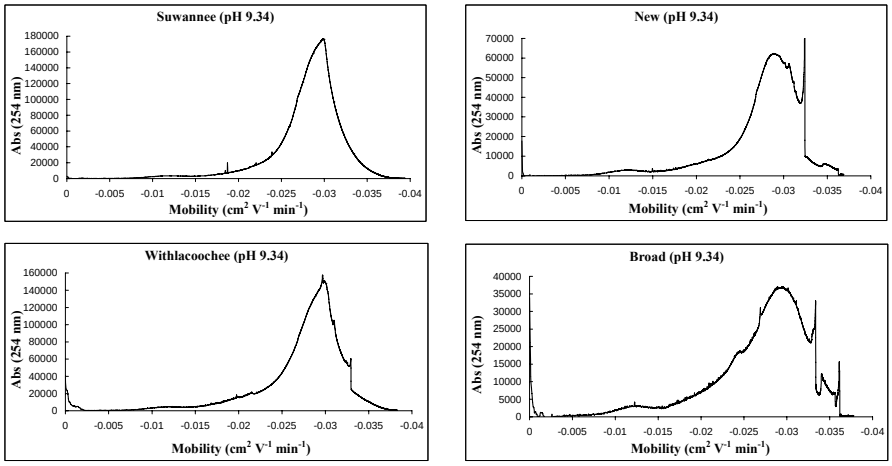
For the samples processed by electrodialysis (Figure 5.4) the following observations can be made:

- The electropherograms for the samples processed by ED were smoother, exhibiting distinct features not seen in the high-ash samples.
- At pH 5.27, the peak which appears at a mobility of ~ -0.023 for all samples except the Suwannee (where it is probably swamped out) is of relatively low mobility. This peak may represent molecules of high molecular weight or molecules having few ionized groups (e.g., high carbohydrate or non-oxidized lignin content (Schmitt-Kopplin et al., 1999) in either case resulting in molecules having relatively low charge-to-surface area ratios. For the Broad and Withlacoochee NOM, there are a number of small peaks on the leading edge of the main hump, in the low mobility region of the electropherogram. Broad NOM is also the most bioavailable of all the NOM samples whereas the Withlacoochee has the second lowest bioavailability. These peaks are not a distinctive characteristic which can be related to the differing bioavailabilities of these NOM samples.

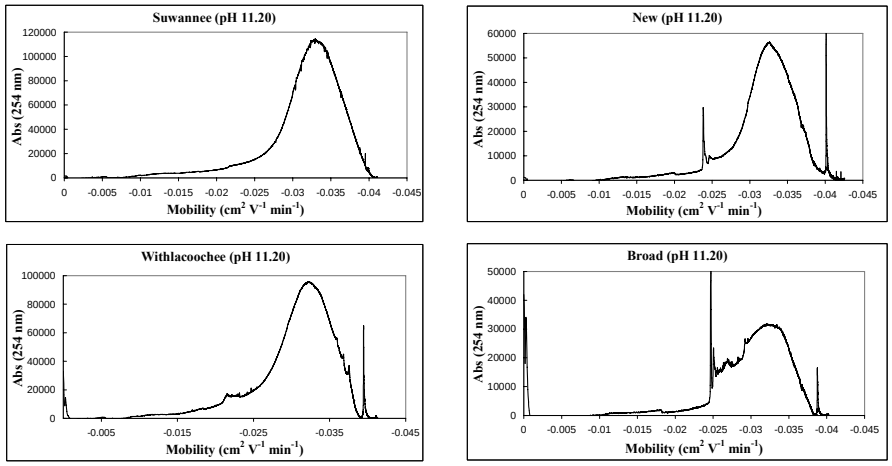
- At pH 9.34, the peak at a mobility of ~ -0.033 in the Withlacoochee, New, and Broad NOM (it is possibly swamped out in the Suwannee NOM sample) represents high-mobility molecules having high charge-to-surface area ratios. The most bioavailable NOM (Broad) exhibits a dip in absorbance at a mobility of ~ -0.033 probably due to the presence of non-UV-absorbing NOM with high charge-to-surface area.
- At pH 11.20, the peak at a mobility of ~ -0.023 to -0.024 in the Withlacoochee, New, and Broad NOM (it is possibly swamped out in the Suwannee NOM sample) represents low mobility molecules of relatively low charge-to-surface area ratios. This peak in the relatively low mobility region of the electropherogram may be indicative of the presence of phenolic acids, some of which are probably depronated at this high pH. The peak appearing at a mobility of ~ -0.040 is a system peak probably resulting from the difference in the ionic strength of the sample and the buffer.
- For all pHs, the maximum peak intensity of the main hump decreases with increasing bioavailability of the NOM (in the order of Suwannee, Withlacoochee, New, Broad). Initial sample concentrations were similar (Table 5.1), negating any concentration effect. A decrease in aromatic chromophores would result in decreasing absorbance. Such a decrease is supported by evidence that aromaticity may be inversely related to bioavailability (see Chapter 4).



pH 5.27



pH 9.34



pH 11.20

Figure 5.4: CZE electropherograms for the NOM samples processed by electrodialysis at pHs 5.27, 9.34, and 11.20.

In any of the electropherograms in Figures 5.3 and 5.4, all peaks, even those that appear sharp, more than likely represent many compounds, all with the same charge-to-surface area ratio. If a known phenolic compound is added to an NOM mixture and the mixture is subject to CZE, it would theoretically be possible to detect the presence of the added phenol as a sharp peak representing a particular charge-to-surface area ratio specific for the added phenol. If there are other molecules in the NOM which also have the same charge-to-surface area ratio, then the detected peak would be the sum of all these molecules. Given that a typical NOM sample consists of thousands of compounds with many of them certainly having the same charge-to-surface area ratio, it is highly unlikely that any peak in the electropherogram would represent only one compound; it is far more reasonable to assume that the peak would represent a class of compounds all having the same (or similar, depending on the resolution of the mobility scale) charge-to-surface area ratio.

The hypothesis that there would be fingerprint peaks distinguishing NOM of low bioavailability from high bioavailability is only seen for two cases. This is not surprising, given the complexity of an NOM sample. The main distinguishing factor related to the bioavailability is the decrease in the absorbance intensity of the main hump for the more bioavailable NOM. This decrease, only seen for the samples processed by electrodialysis, is evidence that CZE can be used as a tool to infer the presence or absence of certain groups of compounds, in this case the possible decrease in the concentration of aromatic compounds with increasing bioavailability. This finding may be enhanced by using other detectors which are not limited to detecting UV-absorbing

molecules. The absence of this observation for the high-ash samples further substantiates the need for low-ash samples for proper characterization.

It has been established that bulk compositional differences in, for example, elemental composition, do exist between NOM samples (e.g., Sun et al., 1997; Hopkinson et al., 1998). Because of the molecular-level complexity of a typical NOM sample, there are no charge-to-surface area differences between samples nor are there distinctive peaks.

5.5.3 Differences in Mobility

As discussed in section 5.4 (Methods), the calculated weight-average mobility has an error of ~ 2%. This error may be large enough to mask some of the subtle differences in mobility between samples. Nevertheless, conclusions may be drawn regarding some of the differences between the NOM samples.

It was hypothesized that each NOM sample would have a distinct weight-average mobility, perhaps reflecting the distinctive bulk elemental composition of each sample (see Chapter 4). For the high-ash samples there are fewer significant differences in mobility at pH 5.30 than at pHs 9.17 and 11.28 (Figure 5.5). This indicates that, at pH 5.30, the combination of mass and charge (determined by the deprotonated carboxylic groups) is similar between the samples. At the higher pHs, more carboxylic groups are deprotonated and phenolic groups also become deprotonated; differences in mobility at these pHs indicate different concentrations of carboxylic plus phenolic groups between the samples and that molecules capable of holding multiple charges differentially change their charge-to-surface area ratio with increasing pH.

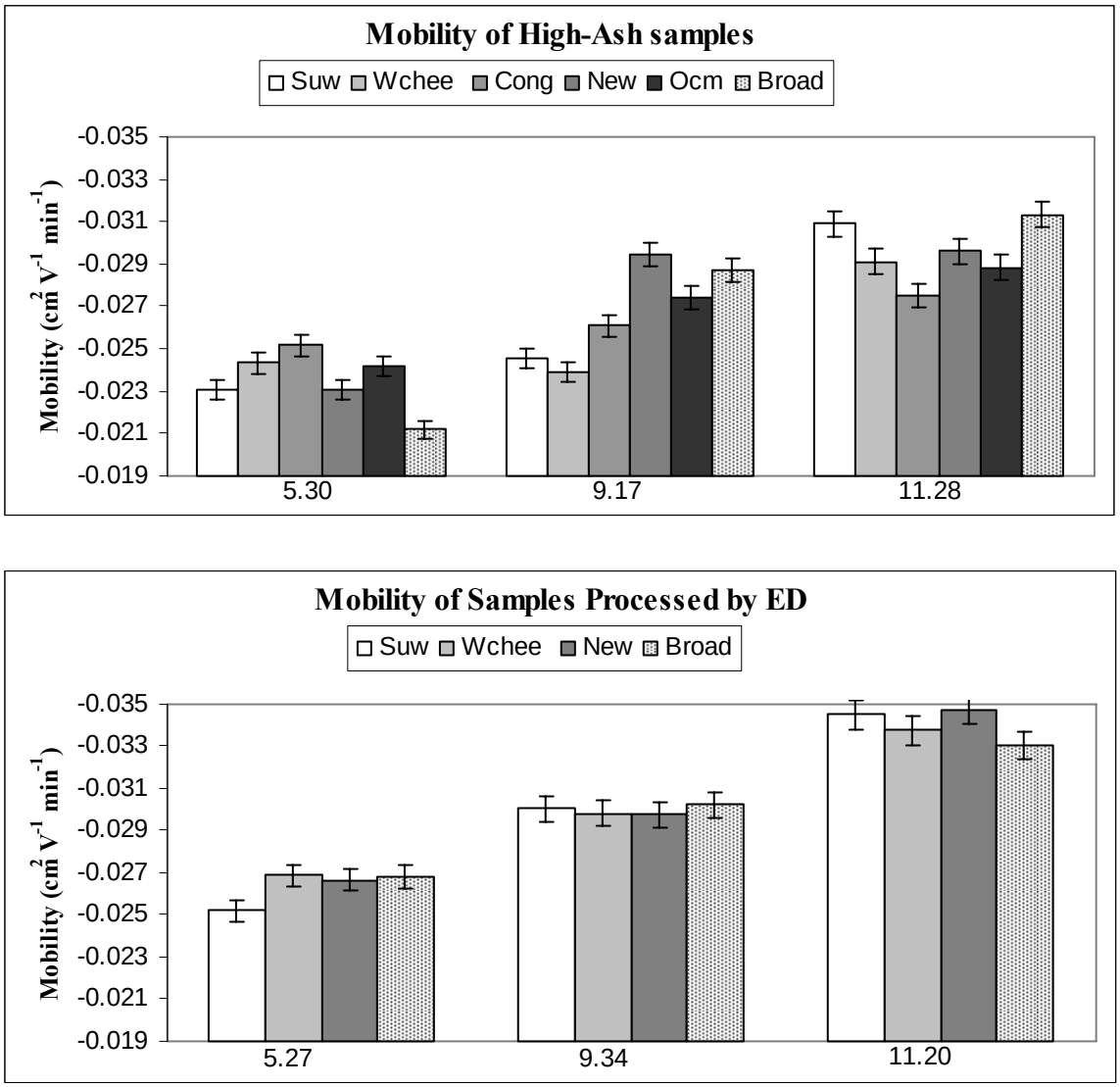


Figure 5.5: Weight-average mobility of the NOM samples at each pH. For the samples processed by electrodialysis there are fewer significant differences in weight-average mobility between samples than for the high-ash samples. The Suwannee NOM was the same sample in both runs (see text). These data are not corrected for the difference in the weight-average mobility for the Suwannee NOM between runs (see Figure 5.2 and text). Error bars represent 2% of the mobility value.

The above is best understood by looking at one pair of samples. For this purpose, the samples with the lowest bioavailability (Suwannee NOM) and highest bioavailability (Broad NOM) are chosen (see Chapter 4 for a discussion of bioavailability). Given the evidence that bioavailability is a reflection of differences in elemental composition, it was hypothesized that this may translate into significant differences in weight-average mobility. At pH 5.30, the Suwannee NOM has a significantly higher mobility than the Broad NOM with a high ash content, indicating that the combination of charge and mass of the molecules in the Suwannee NOM leads to a relatively greater charge-to-surface area ratio. There is also another, albeit unlikely, possibility. Assuming that there is no difference in the size distribution of molecules between the two samples, then a higher mobility for the Suwannee would indicate a greater concentration of carboxylic groups in that NOM. At pH 9.17, the Suwannee NOM has a significantly lower mobility than the Broad NOM but at pH 11.28 there is no significant difference in mobility between these two samples. These changes in relative mobility between the Suwannee and Broad NOM are indicative of the charge-to-mass ratio changing at different rates for each sample.

Other than the Suwannee-Broad NOM pair, there are no significant differences, at any pH, in weight-average mobility between any of the samples processed by electrodialysis. At pH 5.27, the Suwannee NOM has a significantly lower weight-average mobility than the Broad NOM. At pH 9.34 there is no difference in weight-average mobility. At pH 11.20, the Suwannee NOM has a significantly higher weight-average mobility than the Broad NOM. Note that these observations comparing the electrodialysed Broad NOM to the Suwannee NOM are all different than was the case for the Broad NOM containing a high ash content compared to the Suwannee NOM. In other

words, the differences in weight-average mobility between the Suwannee and the Broad NOM are not consistent between the high-ash samples and those samples processed by electrodialysis. This may be due to the ash significantly affecting charge formation and as a consequence the overall mobility of the Broad NOM of high ash content (see discussion in next section on effect of ash content).

Assuming that the samples processed by electrodialysis yield more reliable results, the observation that at pH 11.20 the Suwannee NOM has a significantly higher mobility than the Broad NOM means that the less bioavailable NOM has a greater average charge-to-surface area ratio at pH 11.20, perhaps indicative of the presence of smaller molecules which can carry a larger charge-to-surface area and thereby a larger concentration (mmol / g) of carboxylic plus phenolic groups. This is supported by the discussion on the relationship between bioavailability and bulk elemental composition in Chapter 4, where it was determined that bioavailability may be related to the oxidation state of the bulk NOM; the more oxidized the NOM, the less bioavailable it will be. Molecules having a greater concentration of carboxylic and phenolic groups are more oxidized, have higher O/C ratios, and more aromatic content. The NMR and MS data presented in Chapters 6 and 7 also show that the Suwannee NOM has a greater aromatic and carboxyl content and a greater O/C ratio that the Broad NOM.

It was also hypothesized that the relative difference in weight-average mobility for two NOM samples will be different at different pHs. As seen for the Suwannee and Broad NOM, there does not appear to be a consistent difference in mobility from pH to pH (Figure 5.5). This inconsistency probably results from nonlinear changes in the charge-to-surface area ratios of the NOM molecules

In general, using differences in weight-average mobility, it is possible to infer differences in overall charge-to-surface area ratio and perhaps phenolic content and carboxylic plus phenolic content; however, the plausibility of these differences is unclear in the absence of other analyses forthcoming in Chapters 6 and 7.

Each sample contains non-UV-active, negatively charged NOM molecules probably present in unequal proportions. (CZE output was monitored by a UV detector set at 254 nm). A relatively significant concentration of these molecules all having a particular charge-to-surface area would result in a dip in the absorbance intensity as they passed the optical window. As a consequence, the calculated mobility of the sample would be lowered because weight-average mobility is a function of absorbance.

Because of their small size, the charge-to-surface area of inorganic ions results in an effective mobility that will be higher than most organic molecules; recall from section 5.1 that the effective mobility μ_{eff} α net charge/surface area. Consequently, only the largest inorganic ions are expected to appear within the time frame of an electropherogram, while most inorganic ions never pass by the optical window because of their very high mobility. Inorganic ions do not absorb light at 254 nm so that their presence will also result in a drop in absorbance intensity as they pass by the optical window. Inorganic ions bound to the NOM would produce a different effect, as is discussed in the next section.

5.5.4 Effect of Ash Content

The mobility of each sample increased with increasing pH, irrespective of ash content (Figure 5.6). At pH 5, only carboxyl groups are thought to be ionized (Perdue

and Ritchie, 2003), resulting in mobility of the NOM molecules. At pH 11, both carboxyl and some phenolic groups are expected to be ionized and contribute to mobility. As a consequence, it is expected that mobility increases with increasing pH.

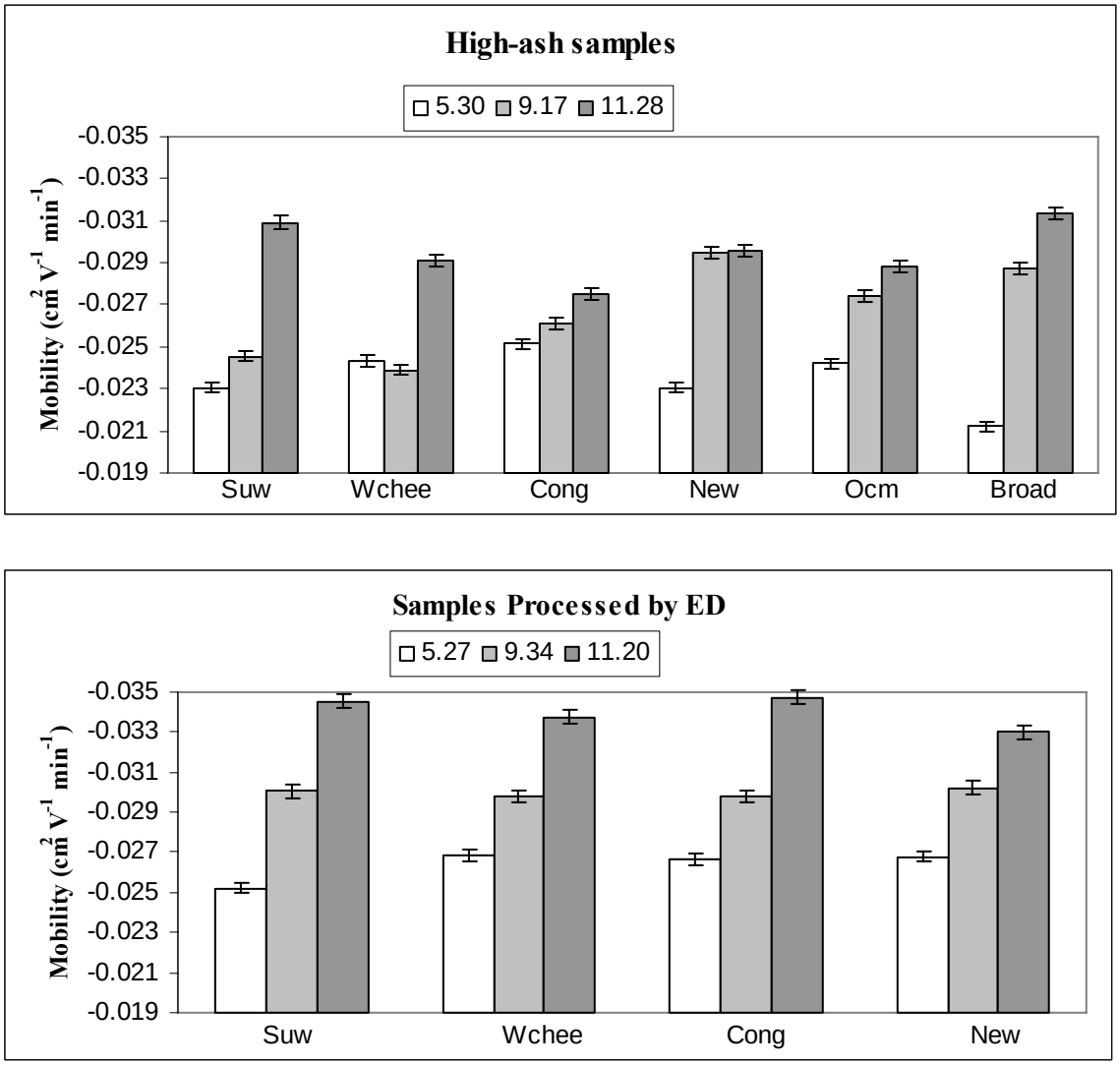


Figure 5.6: Increases in weight-average mobility as a function of pH for both high-ash samples and samples processed by electrodialysis. These data are not corrected for the difference in the weight-average mobility for the Suwannee NOM between runs (see Figure 5.4 and text). Error bars represent 2% of the mobility.

After correcting all sample mobilities based on the increased mobility for the Suwannee NOM from one sample run to another (recall Figure 5.2), it is found that the mobility for the samples processed by electrodialysis is inconsistently greater than that for the corresponding high-ash samples (Figure 5.7). It is unclear why this is so, but it may be related to the correction used. It is possible that the correction factor, based only on Suwannee NOM, is not representative of the differences in mobility that would be observed for the other NOM samples.

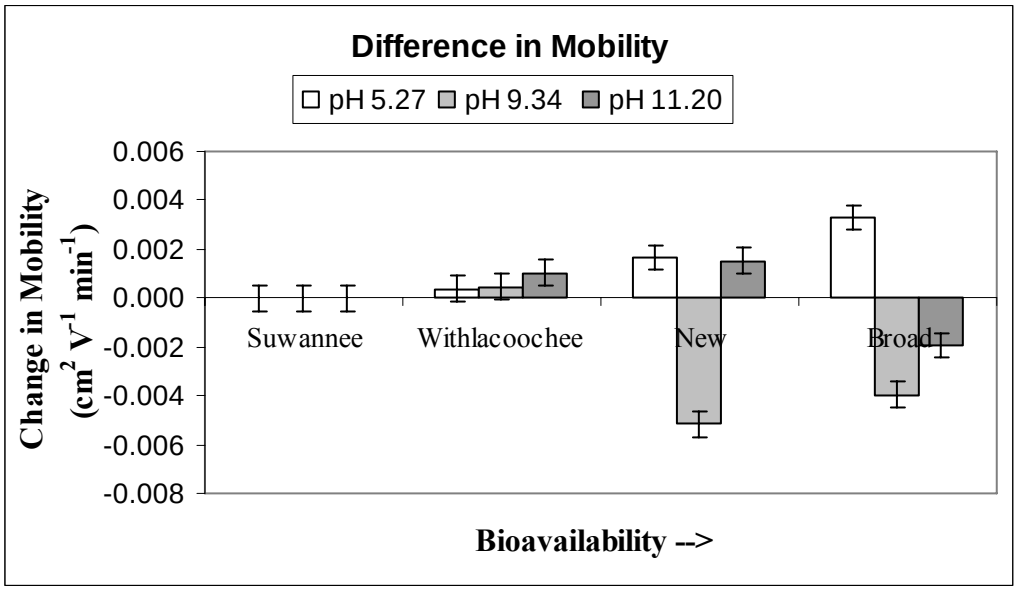


Figure 5.7: NOM samples processed by electrodialysis tend to have a higher weight-average mobility than high-ash NOM, especially at pH 5.27. There is no trend of increased mobility difference with increased bioavailability. Error bars represent 2% of the actual average mobilities the changes are based on.

It was expected that ash content would affect mobility through metal complexation with carboxyl groups at lower pH (Schmitt-Kopplin et al., 1999). The effect of ash content on mobility is best seen when comparing the mobility of Suwannee

NOM at various stages of processing (Figure 5.8). Three samples were available for study.

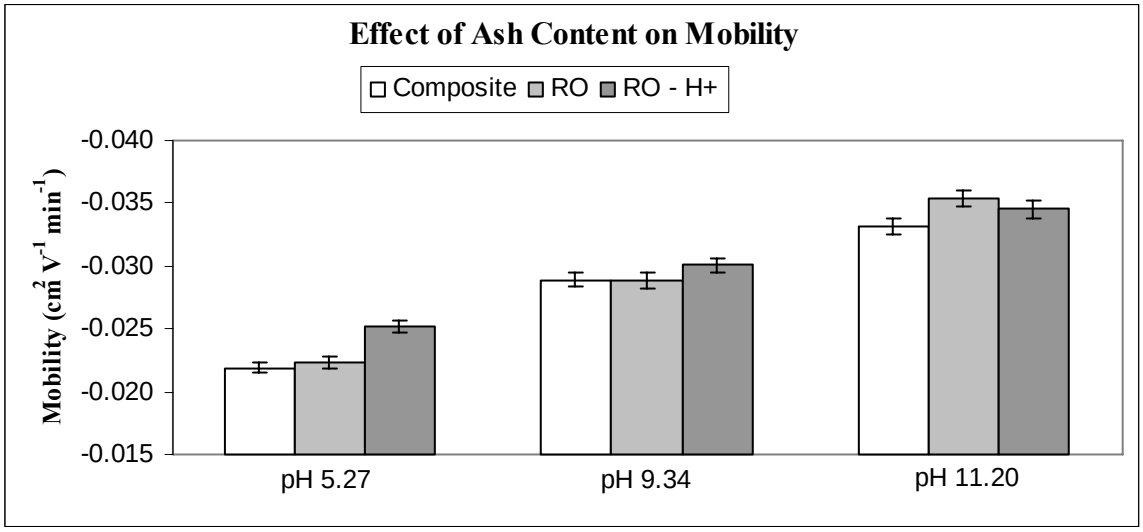


Figure 5.8: The effect of ash content on weight-average mobility is seen by comparing the mobility of Suwannee NOM at various stages of processing. The relative ash contents are composite > RO > RO – H^+. At each pH, there is a tendency for higher weight-average mobility as the ash content is lowered. Error bars represent 2% of the mobility value.

The composite sample is raw Suwannee river water, not having been processed in any manner and thus containing the most inorganic ash content per mole of NOM. A second sample is Suwannee NOM that has been processed by reverse osmosis without a cation exchange resin. The RO process results in the loss of some low molecular weight molecules. It turns out that 10 - 30% of the silica and 0 - 10% of the sulfate are lost through the RO membrane (E.M. Perdue, pers. comm.). Consequently, the sample processed by RO has slightly less inorganic ash than the composite sample. The third sample is Suwannee NOM that has been processed by RO using an in-line cation exchange resin (H^+ form) which removes major cations such as Ca^{2+} and Mg^{2+}. The

sample processed in this manner will contain even less inorganic ash. At pH 5.27 the observed weight-average mobilities are: composite < RO < RO – H^+, at pH 9.34 they are composite = RO < RO – H^+, and at pH 11.20 they are composite < RO ~ RO – H^+. Thus, other than at pH 11.20 where the difference between RO and RO – H^+ is not significant, the less the ash content the greater the weight-average mobility of the NOM and, by extension, the greater the average charge-to-surface area ratio of the NOM.

It is expected that complexation of divalent ions such as Ca^{2+} and Mg^{2+} will change the mobility of NOM. Consider a molecule of R-COO^- that has a charge of –1. If Ca^{2+} binds to this molecule, giving R-COO-Ca^+, then, its overall charge is now +1. This will result in a lower mobility of the ion, because it will have an opposite charge to the cathode and thus be attracted to it. The Ca^{2+} could also bind two molecules of R-COO^- forming R-COO-Ca-OOC-R, a neutral molecule which would be transported with the neutral components of the buffer and elute at the very beginning of the analysis. Now consider a complex mixture of 1000's of compounds having a wide range of charges, and one can see that sufficient concentrations of cations will lower the average charge-to-surface area ratio and as such the weight-average mobility of the solution.

5.5.5 Heterogeneity

For the high-ash samples, the heterogeneity ratio (peak-maximum mobility / weight-average mobility) is much > 1.00 for all samples at all pHs (Table 5.3). Samples processed by ED all have a heterogeneity ratio very close to 1.00. Assuming that the samples processed by ED yield more reliable results, the heterogeneity ratio close to one implies that none of the samples has an unbalanced mixture of molecules with regards to

the charge-to-surface area ratio. The molecular weights of NOM have been shown to exhibit polydispersity (e.g., Chin et al., 1994) which is defined as the ratio of the weight-average to number-average molecular weights. A survey of the literature shows that for NOM, the median weight-average molecular weight is 1590 Da and the median number-average molecular weight is 1100 Da (Perdue and Ritchie, 2003) resulting in a polydispersity of 1.44. As the polydispersity exhibited by molecular weight is not reflected in the charge-to-surface area ratio, this would indicate that charge formation is not balanced. In other words, molecular mass (size) and charge do not increase in step. This conclusion is supported by the work of Ritchie and Perdue (2003) who show that the total charge (normalized to carbon) on the standard Suwannee River fulvic acid does not increase linearly with pH. Ritchie (2005) shows that peak mobility for Suwannee NOM does not increase linearly with charge density. The results presented here also support the idea of an unbalanced charge formation where the charge-to-surface area ratio changes non-linearly with changes in pH.

Table 5.3: For the high-ash samples, the heterogeneity ratio (peak-maximum mobility / weight-average mobility) is much > 1.00 for all samples at all pHs. Samples processed by ED all have a heterogeneity ratio very close to 1.00.

	Heterogeneity Ratio		
High-ash	pH 5.30	pH 9.17	pH 11.28
Suwannee	1.24	1.25	1.16
Withlacoochee	1.19	1.29	1.19
Congaree	1.15	1.23	1.24
New	1.31	1.08	1.25
Ocmulgee	1.14	1.18	1.23
Broad	1.35	1.14	1.20
After ED	pH 5.27	pH 9.34	pH 11.20
Suwannee*	0.95	0.99	0.95
Withlacoochee	0.97	1.00	0.95
New	0.96	0.97	0.98
Broad	0.94	0.98	0.97

* Suwannee was same sample for both runs.

5.6 Conclusions

Samples processed by electrodialysis result in electropherograms that are smoother and exhibit distinct features not seen in samples containing relatively high ash contents. There are, however, no unique peaks to distinguish one sample from another and certainly not to infer about the bioavailability of the NOM. The main distinguishing factor related to bioavailability is a decrease in the absorbance intensity of the main hump as the relative bioavailability of the NOM increases. This observation may imply the relative absence of aromatic compounds in NOM of greater bioavailability. Only at pH 5.30 was there a peak present in the Suwannee and Withlacoochee NOM that may be indicative of aromaticity.

Other than the Suwannee-Broad NOM pair, there are no significant differences, at any pH, in mobility between any of the samples processed by electrodialysis. At pH 11.20, differences in mobility may reflect differences in phenolic content and carboxylic plus phenolic content, with the Suwannee NOM containing a higher content of these groups, further supporting the observation that the Suwannee NOM is less bioavailable partly because it is more oxidized and has a higher aromatic content than the Broad NOM.

Ash content affects mobility. The lower the ash content the greater the weight-average mobility of the NOM. Ash may interfere with charge development, thereby decreasing the average mobility of an NOM sample.

5.7 Acknowledgements

The author thanks Dr Philippe Schmitt-Kopplin at the Institute for Ecological Chemistry of the GSF (National Research Center for Environment and Health) in Neuherberg, Germany for advice and access to the CZE, and to Heidi Neumier and Agi Fekete for processing the samples. This research was supported by an NSF travel grant (# 3506B08) allowing the author to travel to the GSF in Germany to complete this work.

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CHAPTER 6

NUCLEAR MAGNETIC RESONANCE [NMR] SPECTROSCOPY

6.1 Introduction

Being a complex mixture of polydisperse molecules, NOM is not easily amenable to analysis available for simpler mixtures. Proton NMR is used to determine the functional and non-functional groups to which the various protons are associated. ^{13}C-NMR is used to study the carbon-containing functional groups and subunits. There are numerous possible types of NMR that are of potential interest in NOM studies (^{1}H, ^{13}C, ^{14}N, ^{15}N, ^{17}O, ^{31}P), but only ^{1}H and ^{13}C will be discussed here, because these were the methods used in this research.

Some of the earliest work with ^{1}H- and ^{13}C-NMR was to investigate seawater and terrestrial HS (Stuermer and Payne 1976). In the present work, only references to freshwater studies will be made, because only freshwater samples were analyzed.

6.2 Literature Review

6.2.1 ^{1}H-NMR

The hydrogen nucleus is a proton with two possible spin states. When placed in an external magnetic field, the proton's own tiny magnetic field assumes one of two possible orientations with respect to the external magnetic field: aligned with or against it. In proton (^{1}H) NMR, exposure of a compound containing H to a strong magnetic field while being irradiated by electromagnetic energy may result in the absorption of energy

by the H nucleus as it moves from a lower (aligned with the external magnetic field) to higher (aligned against the field) spin state in a process termed resonance. Resonance induces an electrical current in a coil surrounding the sample which is amplified and displayed as a series of peaks (the NMR spectra) (Solomons, 1980).

The magnetic field strength at which protons absorb energy depends on their surrounding environment of electrons and other protons. Protons in the vicinity of high electron density will be shielded and will require that a stronger external magnetic field be imposed before they are able to absorb energy (the electrons themselves produce a small magnetic field that also needs to be overcome by the external magnetic field) (Solomons, 1980).

The shielding (and de-shielding) effects cause the absorption energy of protons to be shifted from what it would be in the absence of any effects. Because these shifts in required energy result from electrons making up chemical bonds, they are called chemical shifts, denoted by δ. Chemical shifts are measured with reference to the absorption of protons in a reference compound, tetra-methyl-silane, whose protons absorb energy at a very high magnetic field strength (as its protons are all very highly shielded), a region where few other protons absorb energy.

^{1}H-NMR produces a spectrum of signals directly related to the types of hydrogen nuclei present in a mixture, all of which absorb energy at different magnetic strengths (Table 6.1). Though it is not reflected in Table 6.1, the change in chemical shift from one type of H to another is not sharp, but has an overlapping region (e.g., Solomons, 1980; Wilson, 1987) due, in part, to chemical shifts caused by interactions of the various protons. Thus, the limits for any one characteristic H are, to a certain extent, user-defined.

The ^{1}H-NMR spectrum is fairly complex with broad, overlapping peaks overlaid with sharp lines. This is a consequence of the complexity of the NOM sample and the narrow range of the spectral window (Cook, 2004).

Table 6.1: Characteristic regions used for ^{1}H-NMR analysis (after Lu et al., 2004).

Chemical Shift, ppm	Proton Characteristic
0.50 – 1.95	Aliphatic
2.00 – 3.10	Protons on carbon atoms next to aromatic, carboxylic, carbonyl, or other electron withdrawing groups
3.15 – 6.00	• Protons on carbon atoms next to O atoms (e.g., methoxy groups, aldehydes) • Alcohol, phenol, carboxyl groups • Amines
6.05 – 10.00	Aromatic

6.2.2 ^{13}C-NMR

The nuclei of ^{12}C have no net magnetic spin and thus do not give NMR signals, but ^{13}C nuclei (~ 1% natural abundance) do. ^{13}C-NMR allows for direct observation of the C skeleton (Solomons, 1980). An advantage of ^{13}C-NMR is that the chemical shift range of ~220 ppm is much larger than for ^{1}H-NMR with 10 ppm. The various types of carbon found in the different regions of the chemical shift spectrum are detailed in Table 6.2. A disadvantage is the potential interference of paramagnetic impurities which must be removed prior to analysis (Cook, 2004).

Characterizing NOM has been mostly done by ^{13}C-NMR using samples in the solid state. A discussion of the mechanism of solid-state NMR is beyond the scope of this work; however, a detailed review is provided by Cook (2004).

Table 6.2: Characteristic regions used for ^{13}C-NMR analysis (after Perdue and Ritchie, 2003).

Chemical Shift, ppm	Carbon Characteristic
0 – 60	Alkyl (-CH3, -CH2, -CH)
60 – 110	Alkoxy (O-alkyls: alcohols R-OH, hemiacetals R-CHOH-OR', ethers R-O-R')
110 – 160	Aromatic
160 – 190	Carboxyl (carboxylic acids R-COOH, esters R-COOR', amides $RCONH_2$)
190 - 220	Carbonyl (aldehydes RCOH, ketones RCOR')

6.2.3 Liquid-State NMR

Liquid-state NMR is used for ^{1}H- and ^{13}C-NMR. When studying NOM, the preferred solvent is water, but in ^{1}H-NMR, water produces a strong signal. The water signal can be suppressed in a number of ways including using solvents with limited or no exchangeable protons such as D_2O, NaOD, and DMSO (dimethylsulfoxide) (Cook 2004).

6.3 Hypothesis

NMR analyses were done with the following hypothesis in mind: the relative concentration of aliphatic- and aromatic-containing molecules will be different for NOM of differing bioavailabilities. Specifically, the more bioavailable NOM is expected to

exhibit a larger proportion of aliphatic protons and carbons and a smaller proportion of aromatic protons and carbons.

Inorganic ions (ash) are not expected to have an effect on ^{13}C-NMR results, unless they include paramagnetic impurities such as metal ions (Cook, 2004). In this research, metal ions are not expected to be present in significant quantities because of the use of an H^+-saturated cation exchange resin at the front end of the reverse osmosis unit when collecting the samples (see Chapter 4 for details of sample collection).

6.4 Methods

Samples were processed at the Institute for Ecological Chemistry of the GSF (National Research Center for Environment and Health) in Neuherberg, Germany. NMR analyses were performed with a Bruker DMX 500 at 303 K. For both types of NMR, the samples were dissolved in 0.1 N NaOD. No fractionation was observed in the samples. The reference for ^{1}H-NMR was $(H_3C)_3Si\text{-}CD_2\text{-}CD_2\text{-}COONa$ (-0.14 ppm) and for ^{13}C an external reference (CH_3OH in D_2O: 49.00 ppm) was used. All proton NMR spectra were acquired with a 5 mm z-gradient $^1H/^{13}C/^{15}N$ TXI cryogenic probe using 90^o excitation pulses (90^o(^{1}H) = 8.3 us; 90^o (^{13}C) = 19 us) (Hertkorn et al., 2006).

^{1}H-NMR spectra are limited to a chemical shift of 0 – 10 ppm. This scale was broken down into characteristic regions specific for different types of H. There are no definite boundaries; the classification of regions described by Lu et al. (2004) was applied here (Table 6.1). Each region was integrated yielding a relative percentage scale for each sample. Peaks in the chemical shift region of 4.55 – 4.75 were not integrated as

this region will exhibit the chemical shift for residual water in the sample (Lu et al., 2004).

The spectra for ^{13}C-NMR were overlain onto a grid and the intensity at each 5 ppm was recorded in the chemical shift range of 230 to 0 ppm. For each defined region of the ^{13}C-NMR spectrum (Table 6.2), the areas under the curves were integrated at 5 ppm intervals.

For the ^{1}H-NMR analyses, both high-ash samples and samples processed by electrodialysis were available. Due to time constraints and limited quantity of sample, only two samples were analyzed by ^{13}C-NMR, these being the least and most bioavailable NOM, the Suwannee and Broad, respectively.

6.4.1 Potential Effect of 0.1 N NaOD on NMR Interpretation

The protocol for dissolving NOM in 0.1N NaOD is commonly used in solution NMR work and it is beyond the scope of this thesis to dispute its validity. Exposure of NOM to very high pH always carries a risk of altering the NOM.

A likely consequence of using 0.1 N NaOD to dissolve NOM is ester hydrolysis resulting in the loss of an ester and the formation of a carboxylic acid and an alcohol:

$$R\text{-}COO\text{-}CH_3 + OH^- \rightarrow R\text{-}COO^- + H_3C\text{-}OH$$

As shown in Table 6.2, ester carbons and carboxyl carbons (shaded in the above equation) both occur in the same user-defined chemical shift region (160 to 190 ppm) in ^{13}C NMR. The carbons in R remain unchanged by the hydrolysis reaction. The hydrolysis of 1 mole of esters produces 1 mole of carboxylic acids, resulting in no change in the area under the curve in this chemical shift region. The C in CH_3 will experience a

slight change in chemical shift but this change will not be detected by the integration of the NMR spectrum across the wide range of chemical shift used here.

Similarly, dissolving the NOM in 0.1 N NaOD will produce an effect on the ^{1}H NMR spectrum but not its interpretation. The high pH will result in all of the carboxyl groups being de-ionized, so that in effect, carboxyl H will be absent and underestimated. The carboxyl H fall in the chemical shift region of 3.15 to 6.00 ppm, a region not used in the analysis presented here.

In conclusion, the alteration of the NOM that occurs by dissolving it in 0.1 N NaOD does not result in a different interpretation of the ^{1}H and ^{13}C NMR because of the integration ranges used and because the regions where there may be an effect are not used in the analyses.

6.5 Results and Discussion

6.5.1 Internal Consistency of the Data

As a check of the quality and consistency of the two methods, an internal check was done to ensure that the ^{1}H and ^{13}C data are mutually consistent. The literature survey by Perdue and Ritchie (2003) found that for freshwater NOM the median weight % C is 49.6% and for H it is 4.8%. Thus, a 100 g sample would be composed of 49.6g C and 4.8g H. It was also found that the median weight % aromatic C content is 24.7%. If a 100 g sample has 49.6 g of C then 24.7 % of it or 12.25 g (= 1.02 mole) is aromatic C.

Perdue and Ritchie (2003) did not provide a median weight % aromatic H content, but a maximal value can be calculated. In theory, an aromatic ring can have at most five H for every six carbons in the ring, for a maximum $(H/C)_{aromatic}$ ratio of 0.83 and a

minimum value of 0. Typically, $(H/C)_{aromatic}$ is closer to 0.33 (E.M. Perdue, pers. comm.). As $(H/C)_{aromatic}$ must be < 0.83 and aromatic C is 1.02 mole, then aromatic H must be < 0.85 mole. Thus, for a 100 g sample, there is at most 0.85 g of aromatic H or 17.7% (= 0.85 g/4.8 g) of the H in a freshwater NOM sample. For an $(H/C)_{aromatic}$ of 0.33, there must be 6.9% H. The data in Table 6.3 show that the % aromatic H is in the range of 5.1 – 9.4 %, well below the maximum allowable value and encompassing the typical value of 6.9%. A similar check can be made for the $(H/C)_{aliphatic}$ ratio. In this case, the possible range is 1.0 to 3.0 (Perdue, 1984). The calculated range of % aliphatic H is 23 – 69%. All values in Table 6.3 fall within this range.

Table 6.3: Percentage of the total area of the ^{1}H-NMR spectra within each characteristic chemical shift region (see Table 6.1 for types of protons in each chemical shift region). Note that the Suwannee is the same sample in both cases, a low-ash NOM, but is included with both high-ash samples and samples processed by electrodialysis (ED) for comparative purposes.

High Ash Samples: Increasing bioavailability →						
Chemical Shift	Suwannee	Withlacoochee	Congaree	New	Ocmulgee	Broad
6.0 - 10.0	8.41	7.79	5.12	6.04	6.15	5.21
3.1 – 6.0	31.98	26.99	22.91	27.87	14.40	28.08
1.95 – 3.1	30.01	23.75	25.18	22.01	23.26	23.69
0.5 – 1.95	29.60	41.47	46.78	44.09	56.19	43.02
Sum	100.00	100.00	100.00	100.00	100.00	100.00
Samples processed by ED: Increasing bioavailability →						
6.0 - 10.0	8.41	9.42	-	6.12	5.17	5.82
3.1 – 6.0	31.98	30.99	-	29.87	28.46	30.28
1.95 – 3.1	30.01	23.38	-	23.58	22.11	23.79
0.5 – 1.95	29.60	36.20	-	40.43	44.26	40.11
Sum	100.00	100.00	-	100.00	100.00	100.00

6.5.2 ^{1}H-NMR

Figures 6.1 and 6.2 show the spectra and Table 6.3 shows the integrated peaks by region. There are distinct differences in the aliphatic (0.5 – 1.95 ppm) and aromatic (6.0 – 10.0 ppm) contents between the least bioavailable NOM (Suwannee) and the most bioavailable NOM (Broad). The Suwannee NOM has a relatively higher aromatic content and a relatively lower aliphatic content than Broad NOM (Figure 6.3). These results imply that the Suwannee NOM will have a lower H/C ratio because aromatic rings have at most a 5:6 ratio of H/C whereas unsubstituted aliphatic chains have a ratio of at least 2:1 (for a theoretically infinitely long unsubstituted chain). This is in agreement with the mass spectrometry results (see Chapter 7) and with the work of Sun et al. (1997).

The use of electrodialysis appears to have altered the chemical makeup of the NOM: the relative proportions of the various hydrogens in a sample differ between the high-ash samples and samples processed by electrodialysis (Table 6.3). It is not clear whether the observed differences are within the limits of reproducibility of the NMR method or that the loss of NOM during the electrodialysis process was a non-uniform process resulting in slightly different proportions of NOM being lost. It is believed that loss of NOM during the electrodialysis process is partly responsible for the observed differences in the NMR results.

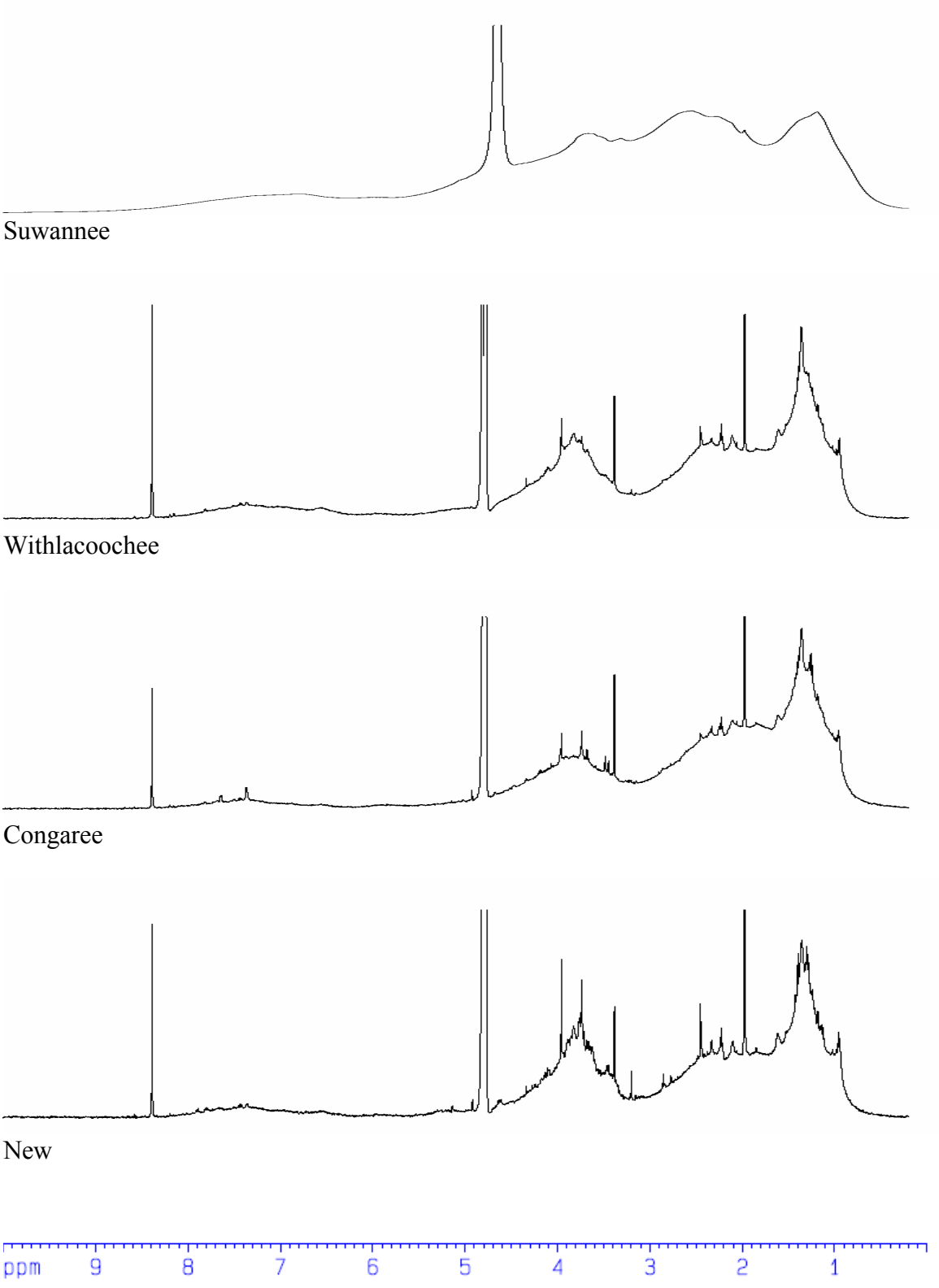


Figure 6.1: ^{1}H-NMR spectra for high-ash NOM samples. The peak at 4.7 ppm is due to the solvent used to dissolve the sample.

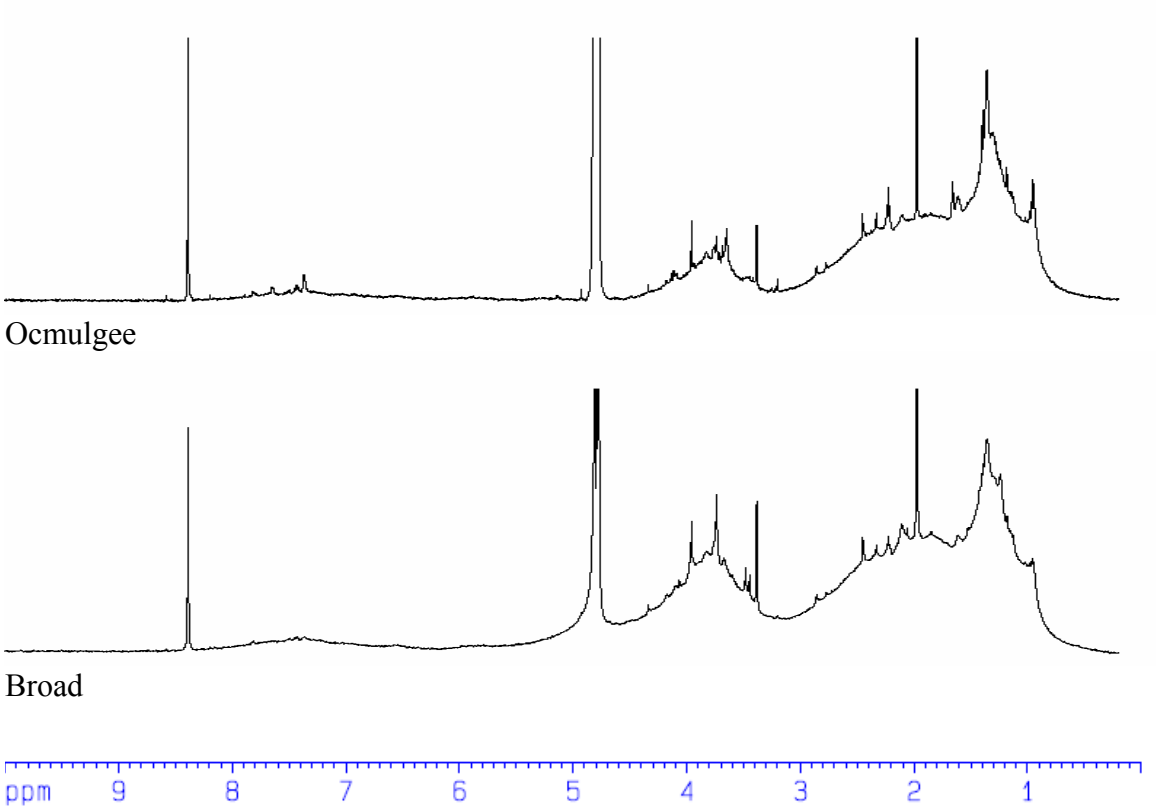


Figure 6.1 (continued): ^{1}H-NMR spectra for high-ash NOM samples.

6.5.3 ^{13}C-NMR

The relative proportions of the various carbons are shown in Table 6.4 and the spectra in Figure 6.4. As for the ^{1}H-NMR, there are distinct differences in the aliphatic and aromatic contents (Figure 6.3). The Suwannee NOM (least bioavailable) has relatively higher aromatic, carboxyl, and carbonyl contents and a relatively lower alkyl (aliphatic) content than Broad NOM (most bioavailable). These results imply that the Suwannee NOM will have a lower H/C ratio and will be relatively more oxidized whereas the Broad NOM will be relatively more aliphatic in nature and less oxidized. This is in agreement with the ^{1}H-NMR and the mass spectrometry results (see Chapter 6) and with the work of Sun et al. (1997).

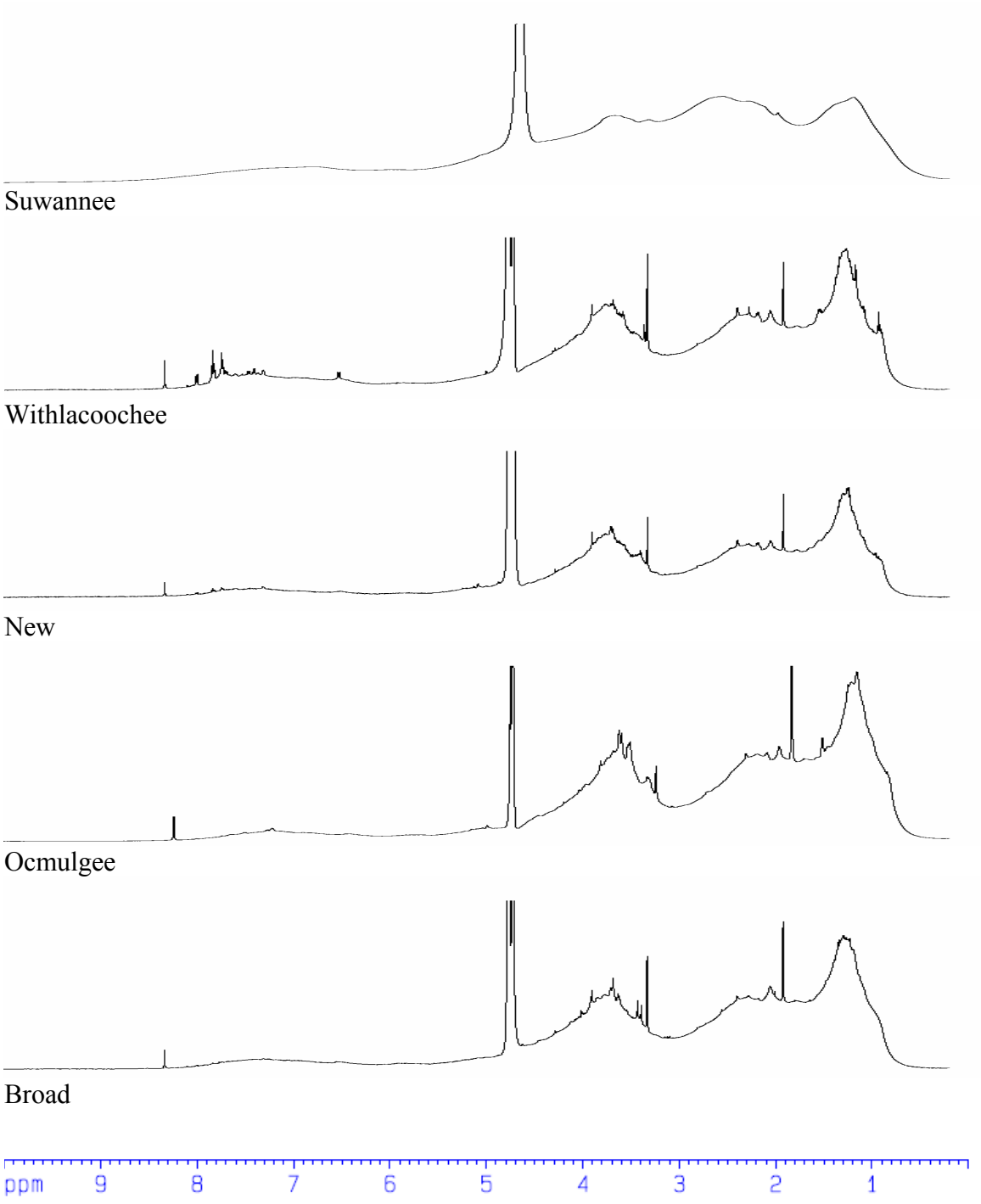


Figure 6.2: ^{1}H-NMR spectra for samples processed by electrodialysis. The peak at 4.7 ppm is due to the solvent used to dissolve the sample.

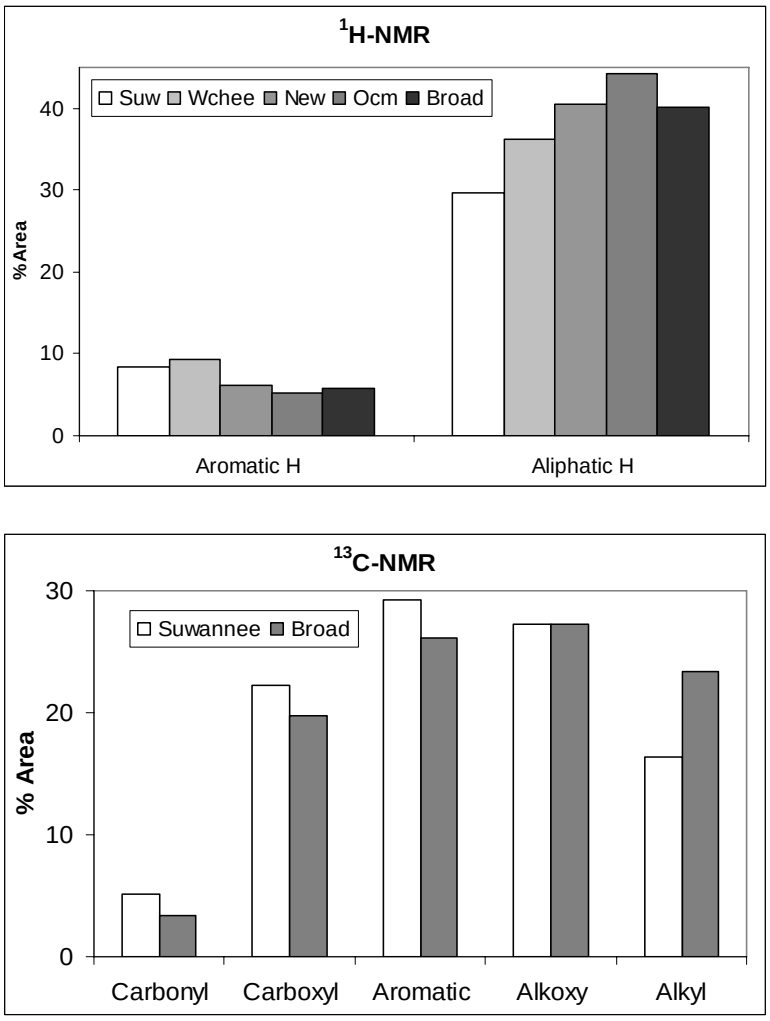
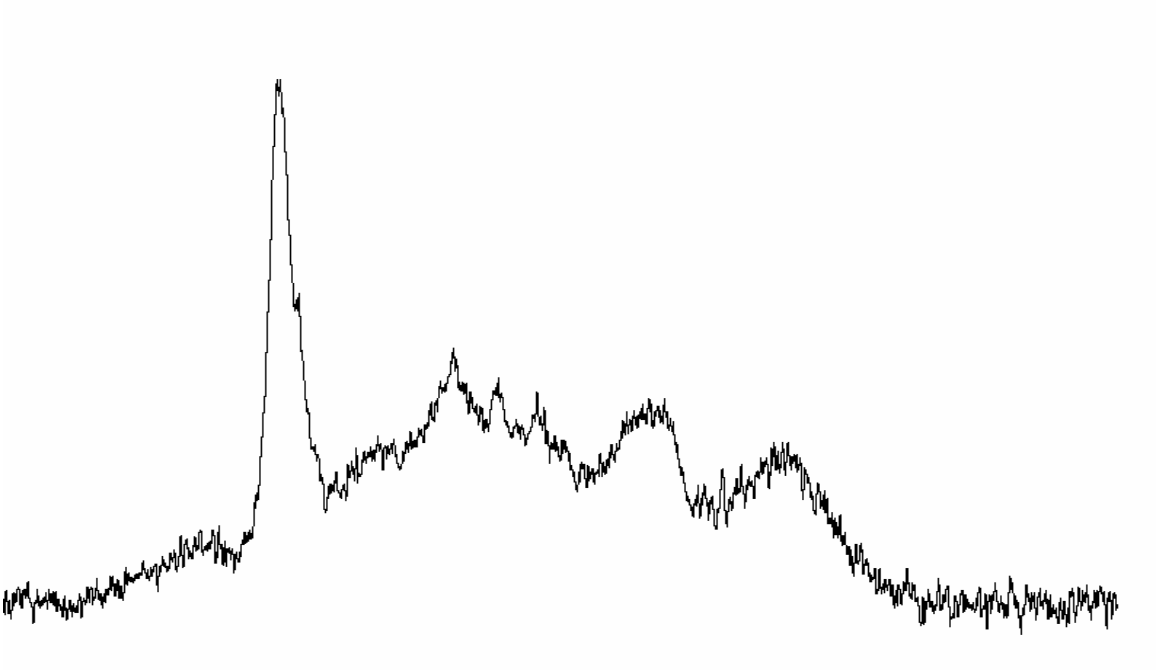
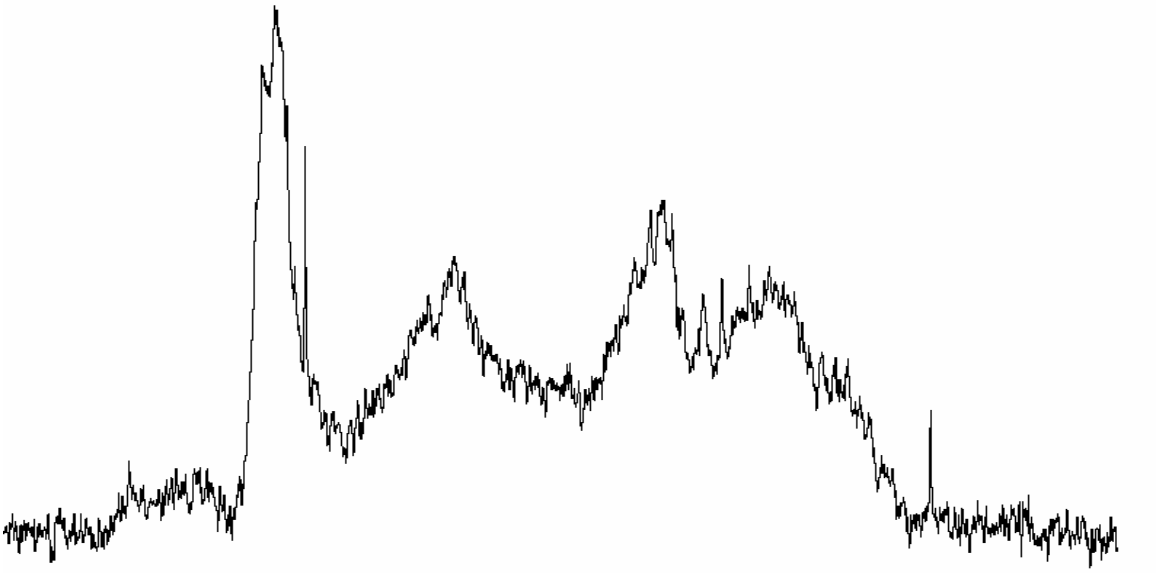


Figure 6.3: Percentage of the total area of the ^{1}H and ^{13}C NMR spectra within specific chemical shift regions for the samples processed by electrodialysis.



Suwannee



Broad

Figure 6.4: ^{13}C-NMR of Suwannee and electrodialysed Broad NOM.

Table 6.4: Percentage of the total area of the ^{13}C-NMR spectra within each characteristic chemical shift region. Also included are the median and range of values for freshwater NOM found in a literature survey by Perdue and Ritchie (2003).

				Literature*	
Chemical Shift	Suwannee	Broad	Type of C	median	range
190 - 220	5.1	3.4	carbonyl	4.7	1.5 - 12.6
160 – 190	22.2	19.8	carboxyl	19	13.7 - 23.0
110 – 160	29.2	26.1	aromatic	24.7	10.4 - 43.0
60 – 110	27.2	27.3	alkoxy	24.2	14.0 - 34.3
0 – 60	16.4	23.4	alkyl	26.7	12.0 - 39.4
sum	100	100		99.3	

6.6 Conclusion

The ^{1}H- and ^{13}C-NMR data are two more pieces of evidence linking bioavailabilty to the bulk chemistry of NOM. In particular, the ^{1}H NMR results show that the least bioavailable sample (Suwannee NOM) has relatively more aromatic and less aliphatic H than the most bioavailable sample (Broad NOM). ^{13}C NMR data show that the Suwannee NOM has relatively higher aromatic, carboxyl, and carbonyl contents and a relatively lower alkyl (aliphatic) content than Broad NOM. These results imply that the Suwannee NOM will have a lower H/C ratio and will be relatively more oxidized. The ^{1}H and ^{13}C NMR data support each other and the hypotheses that bulk elemental composition of NOM is related to the bioavailability of NOM.

6.7 Acknowledgements

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CHAPTER 7

ELECTROSPRAY IONIZATION MASS SPECTROMETRY (ESI-MS)

7.1 Introduction

In electrospray ionization mass spectrometry (ESI-MS), molecules are ionized in a way that singly charged particles are mostly produced. Combined with a powerful magnet, 12 T in this case, which allows mass detection to four decimal places (i.e., 0.0001 Da), the molecular composition for any peak can be assigned with reasonable accuracy. Each peak has a unique mass-to-charge (m/z) ratio. Because singly charged ions vastly outnumber multiply charged species, each peak then represents a unique molecular formula.

The purpose for analyzing the NOM samples by ESI-MS was to gain information regarding the molecular compositional makeup of the samples. Atomic ratios can be calculated for each peak: a weighted average can be calculated for the bulk sample and compared to the bulk elemental composition data from Chapter 4. The following hypotheses are proposed:

- The distribution of peaks will be different for all samples.
- The elemental make-up will be significantly different between samples. Based on previous research by Sun et al. (1997) (see Chapter 4) it is expected that the least bioavailable sample (Suwannee NOM) will have smaller H/C and N/C ratios and a larger O/C ratio than the Broad NOM, the most bioavailable NOM.

7.2 Literature Review

The earliest use of ESI-MS to analyze organic matter was by Fievre et al. (1997) and McIntyre et al. (1997). Subsequently, a number of studies of NOM using this method have appeared (e.g., Brown and Rice 2000; Kujawinski et al. 2002; Schmitt-Kopplin, 2002 (and references therein); Schmitt-Kopplin and Kettrup, 2003; Stenson et al., 2003; Kim et al., 2003a, 2003b, 2006; Kujawinski et al., 2004; McIntyre and McRae, 2005).

There are two parts to electrospray ionization mass spectrometry (ESI-MS), electrospray ionization (ESI) and mass spectrometry (MS). As it is a relatively new process, ESI will be reviewed more thoroughly, whereas the reader may chose to consult any of numerous textbooks for additional information on the topic of MS.

A typical mass spectrometer uses an electron beam to dislodge electrons from molecules, thereby creating molecular ions. The energy of the electron beam (about 70 electron volts) is high enough to also break covalent bonds, so molecular fragments are also created (Solomons, 1980). Mass spectrometry is the determination of the masses generated by fragmenting the molecules in a mixture. If a non-destructive method of ionization is used, however, then the mass spectrometer will detect molecular ions only and molecular fragments would not be produced. ESI is a method of "soft" ionization in which singly-charged molecules are mostly produced (see ensuing discussion). When applied to an NOM mixture, molecular ions of NOM are then detected on the mass spectrometer. Different types of mass spectrometers are available (ion trap, quadrupole time of flight, and Fourier transform ion cyclotron resonance [FT-ICR]) (Kujawinski

2002). The highest mass accuracy and resolution is with the FT-ICR (Kujawinski et al., 2004).

ESI is a soft ionization technique (fragmentation of molecules is minimal) that preferentially ionizes polar functional groups such as carboxylic acids and amines, producing negatively and positively charged ions, respectively, which are then introduced into an MS (Kujawinski 2002; Kujawinski et al., 2002). As polar compounds containing carboxylic and amine groups are preferentially ionized, non-polar compounds will not be present in the MS spectra. This is considered to be of little importance, however, as non-polar aliphatic compounds represent a very small component of NOM (Kujawinski et al., 2002; Kujawinski et al., 2004). Certain polar compounds may be under-represented if they do not easily form ions (McIntyre et al., 1997). A comparison of data generated from the determination of elemental composition and ESI-MS data suggests that ions generated by ESI are at least partially representative of the entire sample (Stenson et al., 2003).

Because ESI is a "soft" ionization method, it is believed that large molecules are not fragmented and remain singly charged (Brown and Rice 2000; Stenson et al., 2002 for HA and FA; Kujawinski et al., 2004). Stenson et al. (2003) and Kim et al. (2003) determined that all ions produced from Suwannee River humic and fulvic acid are singly charged by noting the presence of peaks at intervals of 1.0034/z higher in mass than the ^{12}C ion. The value of 1.0034 Da is exactly the difference in mass between ^{12}C and ^{13}C.

The main components of NOM are acidic in nature, composed of carboxyl and phenolic groups. Leenheer et al (1995) have shown that the pK_a for carboxyl groups in humic substances lies in the range of ~1.8 to 5. The pK_a of carboxyl groups for simple

organic acids lies in the range of 3 to 5 (Perdue, 1985). Thus, humic substances will have some charged molecules at any pH above about 2, well below the pH at which ESI experiments are carried out. Furthermore, models predict that fulvic acids have more than one carboxylic group per "molecule" (Brown and Rice, 2000). Despite the make-up of the sample, only singly charged ions are formed because of the mechanism of the electrospray process which consists of: (i) the production of charged droplets at the electrospray tip; (ii) shrinkage of the droplets as the solvent evaporates; (iii) disintegration of the droplets due to charge on the individual particles; and (iv) the formation of gas-phase ions (Gaskell, 1997; Schmitt-Kopplin and Frommberger, 2003).

The ionization process is dependent upon the experimental conditions used. These include sample preparation (solvents, salts), types of molecules in the sample, voltage, and flow rate Kujawinski (2002). The formation of a stable spray is difficult in pure water, necessitating the need for an alcohol to lower the surface tension. Other polar organic solvents may also be used. Non-volatile salts such as NaCl interfere with spray formation and suppress ionization by forming salt-solvent adducts and may also lead to aggregation of solutes (Brown and Rice, 2000). Interference due to the presence of salts in a sample reinforces the need for desalted samples, one of the major research themes of this thesis (see Chapters 2 and 3).

There are caveats associated with the ESI process, however, as pointed out by several authors, including Kujawinski (2002). A complex mixture such as NOM contains functional groups with differing ionization efficiencies, which may result in biased spectra. This implies that relative peak intensities are non-quantitative. The presence of

metal adducts further complicates interpretation. The presence of multiply charged species, inherently present in NOM, also poses problems.

Another potential problem with ESI-MS has to do with the use of alcohol-based solvents used to store and dissolve humic substances. Methanol and other high-polarity solvents offer the advantage of easily solubilizing humic substances. It has been shown by McIntyre and McRae (2005), however, that self-esterification of a strong acid in methanol is possible, within a few hours. This reaction is of the general form:

$$R\text{-}COO\text{-}H + R^{'}\text{-}OH \rightarrow R\text{-}COO\text{-}R^{'} + H_2O$$

where R and $R^{'}$ represent any organic chain. If esterification occurred, neutral compounds (esters) would be formed, compromising the ESI-MS spectra but only if a sufficient number of esters formed. Considering that esterification would create one molecule from two existing ones (i.e., a molecule of higher molecular weight would be formed) it would be expected that a shift in the spectra to higher m/z should result. Brown and Rice (2000) showed that changing the spray solution mixture from 30% methanol to 70% methanol resulted in a shift towards higher m/z. Though they did not discuss the reason for the observed shift, esterification of molecules leading to larger molecules could produce such a shift. A literature survey by McIntyre and McRae (2005) found that 17 of 26 studies used some form of alcohol-based solvent in their work and though they did not suggest it, these studies may have compromised MS spectra, especially if the samples were exposed to the solvent for several hours or more. McIntyre and McRae (2005) also showed that exposure to an alcohol-based solvent produces negligible changes in the amount of carboxyl groups in the first two hours.

Though the potential formation of adducts appears to be negligible, a brief description of their possible formation in the ESI process is warranted. Because the ESI process is gentle, it is believed that it may allow for non-covalently bound complexes (organic-organic; metal-organic) to also be ionized and transferred to the gas-phase. Ion clusters, where neutral species are attached to a charged species, are also possible (Schmitt-Kopplin and Frommberger, 2003). Schmitt-Kopplin and Kettrup (2003) were able to demonstrate that charge multiplicity, adduct formation, and thermolysis may occur under certain conditions of instrumental setup. They also showed that the charge developed on small molecules may not be kept when they are transferred into the gas phase.

Viewing MS data for complex mixtures made of potentially thousands of different molecules results in MS spectra with peaks for every possible mass-to-charge (m/z) ratio. When Suwannee river fulvic acid is subject to ESI, approx 4000 peaks are found within the mass-to-charge (m/z) range of 300 to 1000 (Kujawinski et al., 2004) and several compounds probably make up each peak. In the low mass range ($m/z < 500$), it is possible to determine the molecular formulae since there are only a limited number of possible formulae at that low range that can exactly match the masses which, if using an ultra-high resolution FT-ICR MS, are available to 4 decimal places (0.0001 Da). Using an FT-ICR MS with a 9.4T magnet produced 4626 individual formulae in 226 distinct homologous series for Suwannee river fulvic acid (Stenson et al., 2003).

A seemingly valuable step forward in the characterization of NOM is the combination ESI-MS with separation methods prior to ESI-MS, such as CE and free-flow electrophoresis (FFE) (Schmitt-Kopplin and Kettrup, 2003) and high-pressure liquid

chromatography (HPLC) (Fievre et al., 1997). This allows for a better resolution of the spectra. Another conceivable method is size-exclusion chromatography (SEC) followed by ESI-MS.

7.3 Methods

Samples were processed at the Institute for Ecological Chemistry of the GSF (National Research Center for Environment and Health) in Neuherberg, Germany. The ESI-MS was a Bruker Daltonics Apex Qe equipped with a 12T magnet. Data acquisition was made using Bruker ApexControl (v1.1). The MS was internally calibrated using arginine and then externally calibrated again with arginine. Only negative spectra were used, representing molecules having a negative one charge. Any positive ions formed, such as from amine groups, represent only a small fraction of the functional groups of NOM which are dominated by carboxylic and phenolic groups, both of which result in negative ions when ionized. The spectra were limited to the mass-to-charge (m/z) range of 200 to 1000.

Freeze-dried samples (~ 1 mg mL^{-1}) were dissolved in a 80:20 to 90:10 water:methanol mixture immediately prior to analysis. Fractionation of the samples was not observed. The injection volume was 2 ul/min if measured with microspray or a few hundred nanoliters/min (exact rate not determined) if measured with Nanomate.

Based on what is found in the literature the following assumptions were made:

- most NOM molecules with functional groups were ionized (i.e., the ESI process is nearly 100% efficient)
- only singly charged molecules are generated

- NOM molecules were not fragmented during the ionization process
- esterification due to use of methanol to dissolve the NOM does not occur to any significant extent because the samples were only dissolved in the solvent immediately prior to analysis
- metal adducts are not present or insignificant.

Peaks were validated only if the respective ^{13}C peak was detected: the mass of a molecule containing one ^{13}C is exactly 1.0034 Da more than if the molecule has only ^{12}C. For each validated peak, a possible molecular formula was generated, limited by C, H, O, N, S, while obeying the nitrogen rule (an odd nominal molecular mass indicates an odd number of nitrogen atoms in an organic molecule). Other than the nitrogen rule, the algorithm used does not constrain the molecular formulae according to well-established rules of chemistry. The list of possible molecular formulae was further constrained by the author by applying four rules: (i) C and H > 0; (ii) O >= 0; (iii) H <= 2C + 2 + N; and (iv) O <= C + 2. No constraints were imposed on N and S.

Determination of average H/C, O/C, and N/C was achieved by applying a Gaussian distribution model to frequency histograms for each ratio and using the Solver function in Excel to determine the best fit using a minimization function. Calculated in this manner, the mean atomic ratios are in effect weighted by the number of observations falling within the designated categories.

7.4 Results and Discussion

7.4.1 Atomic Ratios

The number of peaks generated was much less than expected. Only approximately 1/10 of the number of peaks detected by other studies listed above were detected here. This is due to the low sensitivity of the instrument at the time of acquisition. Furthermore, a mass balance between total NOM and the MS data was not attempted, so it is not known how much of the NOM the MS actually detects (Seitzinger et al., 2005).

Data generated for each sample were constrained to molecules containing C, H, O, N, and S. It is safely assumed that the presence of any other element is of minor significance and would not impact the results. For each sample, the output includes all possible molecular formulae, constrained as discussed in the methods section, and the intensity of each peak. Because of differing ionization efficiencies of the functional groups, peak intensity is not a quantitative parameter in this form of MS and as such it cannot be used as a weighting factor.

The results (Table 7.1) have relatively large standard deviations so that the differences in atomic ratios between the various NOM samples are not statistically significant. Nonetheless, trends can be inferred. The samples in Table 7.1 are listed in order of increasing bioavailability. As expected, the H/C ratio increases and the O/C ratio decreases with increasing bioavailability. This is in agreement with the work of Sun et al. (1997) and the results presented in Chapter 4. The N/C ratio, however, does not follow the expected trend of increasing with increasing bioavailability. The atomic ratios calculated from the MS data do not match the results obtained from elemental analysis

(see Chapter 4) implying that the low sensitivity of the ESI-MS instrument has resulted in inadequate and skewed peak formation. The calculated ratios, however, do fall within the range found for freshwater NOM (Table 7.2).

Table 7.1: Mean atomic ratios (standard deviation) for freshwater NOM. The number of peaks is limited due to low sensitivity of the mass spectrometer during analysis. Samples are listed in increasing order of bioavailability.

Sample	Total Peaks	**H/C**	# of Peaks	**O/C**	# of Peaks	**N/C**	# pf Peaks
Suwannee	283	1.23 (0.17)	283	0.55 (0.18)	283	0.17 (0.05)	66
Wchee	202	1.28 (0.17)	202	0.46 (0.11)	202	0.14 (0.08)	51
New	97	1.24 (0.12)	97	0.48 (0.09)	96	0.14 (0.03)	13
Broad	149	1.30 (0.13)	149	0.50 (0.09)	149	0.07 (0.03)	27

Table 7.2: Atomic ratios found in freshwater NOM based on a review of the literature by Perdue and Ritchie (2003).

Ratio	Observations	Lit. Range	Lit. Median
H/C	57	0.82 – 1.89	1.17
O/C	57	0.47 - 0.93	0.64
N/C	57	0.006 – 0.10	0.03
S/C	8	0.004 – 0.04	0.01

7.4.2 Anomalies

The H/C ratios exhibit a bi-modal distribution (Figure 7.1). The H/C ratios forming the smaller peak are very high, with a mean of ~ 2.3 in both cases. An analysis of the molecular formulae determined for these peaks by the algorithm presents some interesting anomalies. The high H/C ratios are associated with compounds that also have N and S contents (Figure 7.2) . Of the 283 molecular formulae generated for the

Suwannee NOM sample, 56 of them have H/C ratios >= 2.0, 66 of them have S, and 66 of them have N. Of the formulae containing S, 52 of the 66 are associated with H/C > = 2.0 and for N, 53 out of 66 are associated with H/C > = 2.0. Of the 56 formulae with H/C > 2.0, 52 of them have S and N in a ratio of 3:2.

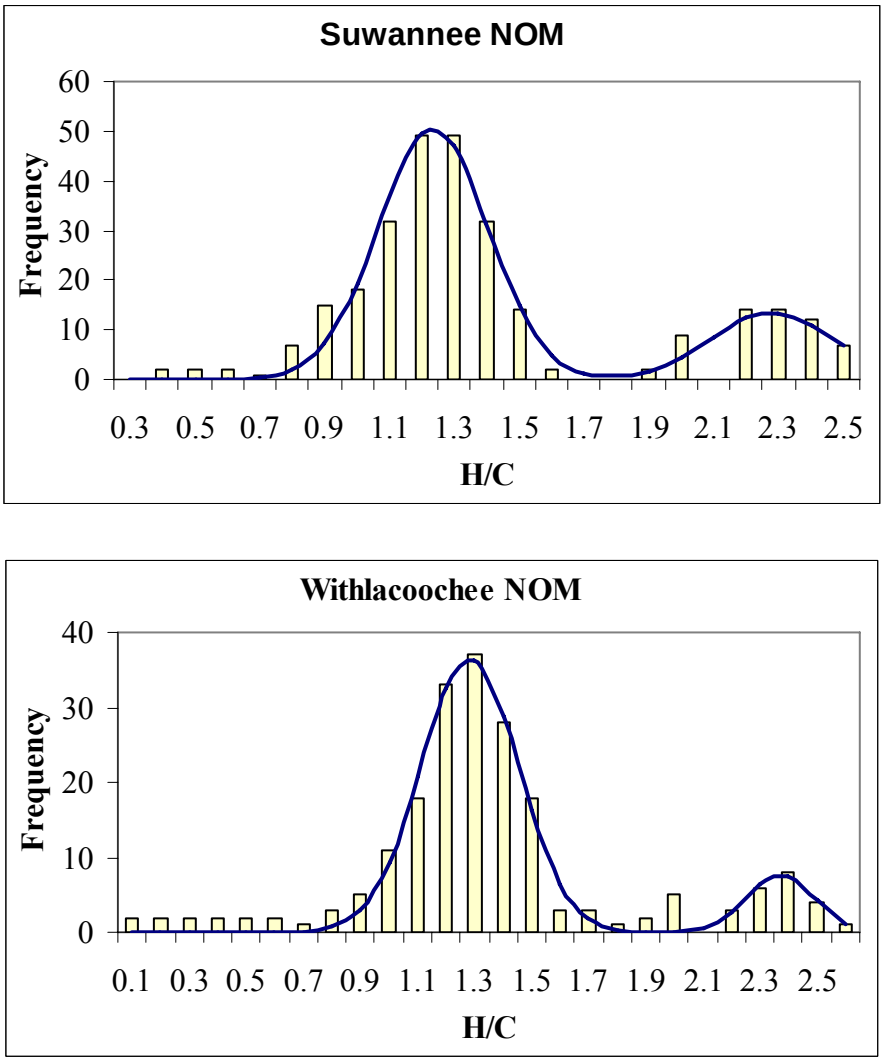

Figure 7.1: The H/C ratios for the Suwannee and Withlacoochee NOM exhibit a definite bi-modal distribution.

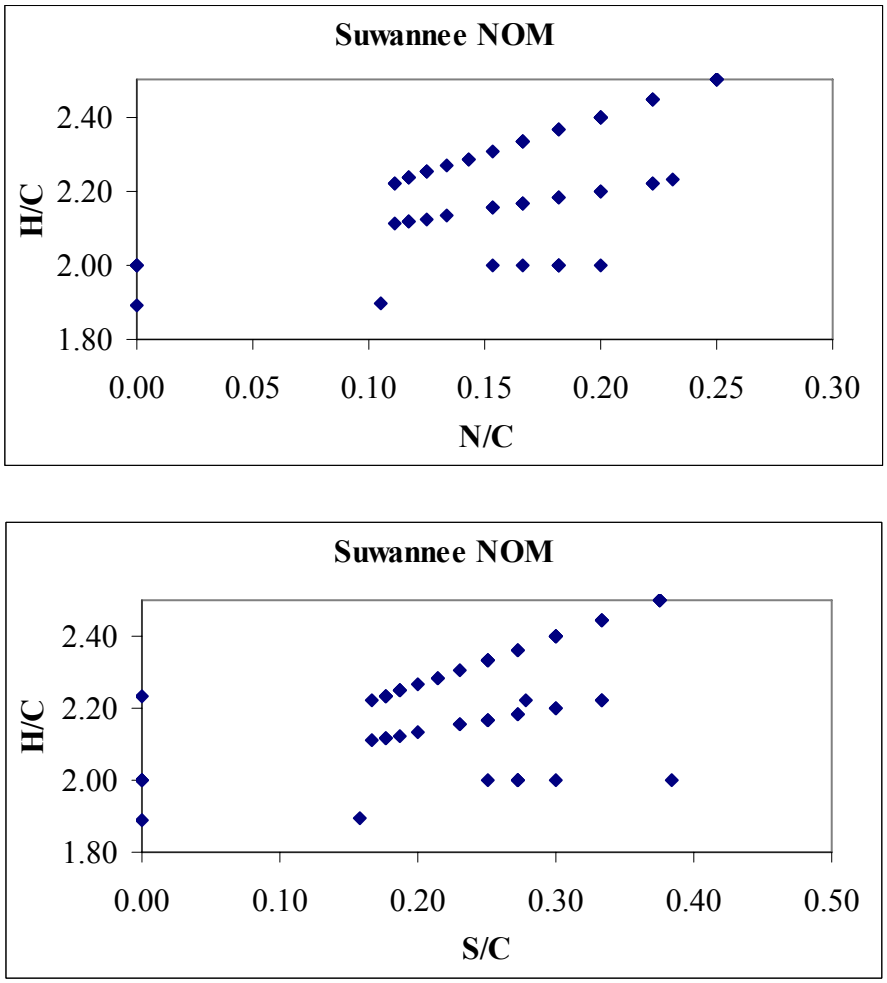

Figure 7.2: For the Suwannee NOM, high H/C ratios are associated with N and S.

In Figure 7.2, the plot of H/C versus N/C shows three straight lines that all converge at an H/C of 2.0. The slopes of the lines are 0, 1, and 2. Each line represents a homologous series of molecules. It can be shown that these lines represent the addition of N and S, always in a ratio of 2:3, and H.

The algorithm generating the molecular formulae is not constraining the possibilities according to established rules of chemistry. Instead it is performing a purely

mathematical operation, determining which combination of C, H, O, N, and S add up to the observed peak mass to within 0.0001 Da. The fact that most of the molecular formulae representing H/C ratios > 2.0 have S and N present in a 3:2 ratio, forming compounds that do not appear to be realistic, implies that these formulae are mathematical constructs and not true representations of what is actually present in the sample. An unsuccessful attempt was made to recalculate the molecular formulae using only combinations of C, H, and O.

7.5 Conclusion

The low sensitivity of the ESI-MS at the time of analysis resulted in a relatively small number of peaks, limiting further analysis of the data. The presence of a large number of compounds with S and N in a 3:2 ratio appears to be a mathematical anomaly and not a true representation of the molecular structures present in the NOM samples. If S and N are to be included in determining the molecular level make-up of NOM, then the method used to generate formulae requires further refinement.

7.6 Future Research

Since this research was completed, Dr. Frommberger, at the GSF, has improved the sensitivity of the ESI-MS instrument, which should allow more peaks to be detected. It is believed that this would accentuate the underlying differences in the molecular makeup of the NOM samples. The samples are being re-run on the ESI-MS instrument.

7.7 Acknowledgements

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CHAPTER 8

CONCLUSIONS AND FUTURE RESEARCH

8.1 Conclusions

The underlying theme of this thesis is the importance of electrodialysis (ED) for the isolation of natural organic matter (NOM) from freshwater and seawater. The application of ED for the isolation of NOM from freshwater enables the removal of sulfate and silica, previously difficult to remove, without either compromising or losing significant quantities of NOM in the process. It has been demonstrated through the use of real freshwater samples that sulfate and silica can be eliminated to pre-determined levels in relation to total organic carbon (TOC) while recovering >88% of the NOM and reducing the molar ratios of SO_4^{2-}/TOC and H_4SiO_4/TOC to a mean value of 0.0046 and 0.032, respectively, thereby surpassing the goal of 0.008 for removal of SO_4^{2-} and nearly achieving the goal of 0.021 for removal of H_4SiO_4. As a result, the samples cannot be compromised by sulphuric acid during the freeze-drying process and are more easily amenable to certain chemical and physical analyses where ash content interferes with the analysis: elemental composition, determination of carboxyl and phenol groups, and mass spectrometry. The mildness of the electrodialysis process minimizes the likelihood that NOM is altered by either chemical or physical conditions.

The application of this method to a large range of samples and with different research goals will lead to a better understanding of the chemical and physical characteristics of NOM and its interaction with the environment.

The coupled reverse osmosis (RO)/ED process that has been described in this study offers a fast, simple, chemically mild (relative to other methods), and reproducible method for isolating large quantities of relatively unfractionated, low-ash NOM from freshwaters.

The logical extension of the ED work on freshwaters was to apply RO and ED to the problem of the isolation and concentration of marine dissolved organic matter (DOM) in quantities sufficient for chemical and physical analysis. To date, marine DOM has at best been recovered with an efficiency of ~30%. The unrecovered 70% of the marine DOM may not be represented in the fraction being collected, thereby omitting entire classes of DOM in the analysis. The high inorganic content of seawater coupled with the low concentration of DOM represents a challenge that requires a different approach than for freshwater. The isolation of marine DOM requires that ED be used first to remove most inorganic solutes, followed by RO to concentrate the DOM, followed by another cycle of ED and RO or RO and ED in tandem. During two research cruises in the Atlantic Ocean, the effort successfully recovered a median of 72% (for 16 samples) of the TOC for 100 – 400 L samples within six to nine hours of processing using a combination of ED and RO, greatly exceeding the current norm of 30%. Due to the superior recovery, classes of DOM previously missing are included in these samples which should yield new insight into the chemistry of marine DOM.

While the samples processed during these cruises have relatively high concentrations of DOM and >99% of the conductivity has been removed, they still contain significant amounts of salts. Further processing of the samples using a combination of RO/ED and an H^+-saturated cation exchange resin is required to lower

salt content to a concentration that will not affect chemical analyses such as determination of elemental composition, titratable carboxyl and phenol groups, and mass spectrometry. The protocol used for freshwater where Cl^- ions are added (as NaCl) to the sample and ED is continued until SO_4^{2-} and H_4SiO_4 are reduced to the desired levels will have to be applied to the marine samples.

Experiments are under way to determine why the recovery of TOC varies by as much as 12% from similar sampling sites. This variation may be due to DOM being lost through adsorption onto the ED membrane if there is an insufficient amount of competing Cl^- ions for the charged sites on the membrane. A more thorough understanding of the parameters affecting percent recovery will enhance the usability and success of the RO/ED method and establish it as the new standard.

Even though the concentration of DOM in the oceans is relatively small (~40 to 80 μM), the size of the ocean reservoir means that the total DOM present in the oceans is comparable to that found on land. Given that oceanic DOM is part of the global carbon cycle, a better understanding of its chemistry and reactivity is necessary.

RO/ED was applied to six river NOM samples. These samples were collected by RO and analyzed for bioavailability. While bioavailability of NOM is not affected by the ash content of the sample, elemental composition and mass spectrometry are. These two analyses were required to link the bioavailability to the bulk elemental chemistry of the NOM. It was possible to distinguish between the bioavailabilities of the six different river NOM samples. A strong correlation has been found between the bulk elemental composition and the bioavailability of the NOM for samples processed by ED. The H/C

and N/C molar ratios are positively and strongly correlated with bioavailability, as hypothesized.

The use of STORET, a dataset of water quality parameters, showed that calculated BOD/TOC ratios appear to exhibit a geographical distribution with high values restricted to Virginia and low values in the entire southeastern US. Calculated BOD/TOC values are moderately correlated with measured bioavailabilities; as such, they can be used as a surrogate for bioavailability of geochemically diverse riverine DOM.

Six freshwater samples were analyzed by capillary zone electrophoresis (CZE) with the intention of discovering significantly differing weight-average mobilities and distinctive peaks in the respective electropherograms. It was hypothesized that differing bioavailability would be reflected in different charge-to-surface area ratios. The main distinguishing factor found to be related to bioavailability was a decrease in the absorbance intensity of the main peak as the relative bioavailability of the DOM increased. This observation, supported by the NMR data, implies that there is a relative absence of aromatic and other chromophore-bearing compounds in DOM of greater bioavailability. Samples analyzed prior to being processed by ED failed to produce this observation. The importance of samples having low sulfate and silica was further demonstrated and the usefulness of ED in removing sulfate and silica is noted.

Other than the Suwannee-Broad NOM pair representing the lowest and highest bioavailabilities, respectively, there were no significant differences, at any pH, in mobility between any of the samples processed by ED. Differences in mobility reflect differences in phenolic content and carboxylic plus phenolic content; increased mobility signifies higher contents of these groups. Increased carboxylic and phenolic group

content implies that the NOM is more highly oxidized and has a higher aromatic content. Suwannee NOM exhibited a higher mobility and higher aromatic content than Broad NOM.

Electro-spray ionization mass spectrometry (ESI-MS) data supported the notion that bioavailability is related to the bulk atomic H/C ratio of DOM. ESI-MS data also indicated that bioavailability was inversely related to the bulk N/C ratio which is counter to elemental analysis data and other work in the literature. The low sensitivity of the ESI-MS at the time of analysis resulted in a relatively small number of peaks and some anomalous mathematical artefacts, limiting analysis of the data. Analysis by ESI-MS would not have been possible on high-ash samples, providing further evidence for the importance of electrodialysis.

Analysis by ^{1}H-NMR revealed distinct differences in the aliphatic and aromatic contents between the least bioavailable NOM (Suwannee) and the most bioavailable NOM (Broad). The Suwannee NOM has a relatively higher aromatic content and a relatively lower aliphatic content than Broad NOM. These results, which agree with the mass spectrometry results, imply that the Suwannee NOM has a lower H/C ratio. ^{13}C-NMR also revealed distinct differences in the aliphatic and aromatic contents between the least bioavailable NOM (Suwannee) and the most bioavailable NOM (Broad) as well as differences in the carboxyl and carbonyl contents. The Suwannee NOM has relatively higher aromatic, carboxyl, and carbonyl contents and a relatively lower alkyl (aliphatic) content than Broad NOM. These results suggest that the Suwannee NOM has a lower H/C ratio and is relatively more oxidized, whereas the Broad NOM is relatively more aliphatic in nature and less oxidized. This is in agreement with the ^{1}H-NMR and the mass

spectrometry results and with the work of Sun et al. (1997). The ^{1}H- and ^{13}C-NMR data are two more pieces of evidence linking bioavailability to the bulk chemistry of NOM.

The impact of this research extends into the field of DOM chemistry, its characterization, and its compositional variability as related to environmental factors. The isolation of freshwater and marine DOM by the chemically "soft" method of electrodialysis and its subsequent characterization by methods requiring low-ash NOM may yield further insight into the properties of DOM and its impact on the global carbon cycle.

8.2 Future Research

As with any piece of research, those questions that are answered give rise to new areas of possible enquiry and investigation. The research described in this thesis is no different.

8.2.1 Electrodialysis

Many questions and future research directions present themselves on the subject of ED, the central theme of this research. In terms of the isolation of marine NOM, it is unclear why the recovery of TOC varies by 12% even between samples from the same site. The process of RO/ED may have an impact on the recovery of TOC. As was discussed in Chapter 2, DOM can be lost through adsorption onto the ED membrane if there is an insufficient amount of competing Cl^- ions for the charged sites on the membrane. Some of this DOM may be recovered by employing a NaCl / NaOH wash of the ED stack. Other processes may impact the percent TOC recovered including whether

current is continuous or pulsed and temperature of the sample during processing (higher temperature may result in increased loss of TOC). Determining which parameters significantly impact the recovery of TOC requires changing one parameter at a time in the ED process and observing its effect on recovery of TOC. The rigorous cleaning process of the ED membrane described in Chapter 2 could also be improved, further enhancing percent recovery. For example, whereas a NaOH wash was used along with a NaCl wash, one or the other may have been sufficient. Understanding what type of NOM is adsorbed to the membrane may also aid in this effort.

While the samples processed during the two cruises have relatively high concentrations of DOM and >99% of the conductivity has been removed, they still contain significant amounts of salts. The marine samples require further ED to remove the remaining salts. Experiments are under way to determine the best method to achieve near-complete salt removal while recovering most of the remaining TOC. A better understanding of the parameters affecting percent recovery will enhance the usability and success of the RO/ED method and establish it as the new standard.

8.2.2 Bioavailability

Future work should examine the question of whether the bioavailability, and consequently the chemistry of the DOM, is affected by any one of the processes to which it is subjected, namely reverse osmosis to concentrate the DOM, electrodialysis to remove ash, and freeze-drying.

Ideally, a method should be developed that allows the bioavailable fraction for DOM to be separated from the rest of the sample and analyzed independently by CZE,

NMR, and MS. Size-exclusion chromatography could be used to separate the DOM into size fractions, each of which could then be tested for bioavailability. The fraction(s) found to be the most bioavailable would then be analyzed independently by CZE, NMR, and MS. Some researchers have already applied ESI-MS to see how bulk DOM changes as it is taken up by bacteria.

It was assumed in this work that ash content does not have an effect on bioavailability. This hypothesis should be tested to ensure that this is the case. Samples are routinely tested for BOD by water management agencies around the world, so such knowledge is of importance.

Lastly, NOM samples analyzed by CZE are effectively separated on a charge-to-surface area basis. The separated sample could be collected and analyzed by other methods such as ESI-MS, linking charge-to-surface area with elemental composition.

8.3 Concluding Remarks

The use of RO/ED as a method for the isolation of freshwater and marine NOM is expected to yield new insight into the chemistry of NOM. Because the RO/ED method is chemically mild, the quality of the NOM is not expected to change during processing. NOM isolated by RO/ED yields superior percent recoveries for marine NOM, so that classes of NOM previously missing are also isolated. Superior percent recovery and chemically unaltered NOM will allow for a more comprehensive study of its chemical and physical properties and its interaction with the environment. Because NOM is part of the global carbon cycle, the elucidation of its properties is timely, allowing for a better understanding of how its chemistry in different reservoirs may be altered by changing

conditions of climate and land use. These changes have varying degrees of impact on water quality and, ultimately, on human health.