

**NATURAL ORGANIC MATTER (NOM) IN AQUATIC SYSTEMS:
INTERACTIONS WITH RADIONUCLIDES (^{234}Th (IV), ^{129}I) AND BIOFILMS**

A Dissertation

by

SAIJIN ZHANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Oceanography

Natural Organic Matter (NOM) in Aquatic Systems: Interactions with Radionuclides

(²³⁴Th (IV), ¹²⁹I) and Biofilms

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August 2010

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ABSTRACT

Natural Organic Matter (NOM) in Aquatic Systems:
Interactions with Radionuclides (^{234}Th (IV), ^{129}I) and Biofilms. (August 2010)

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A series of laboratory and field investigations were carried out to elucidate the importance of natural organic matter in aquatic systems, i.e., trace element scavenging (e.g., ^{234}Th) by exopolymeric substances (EPS), formation of biofilms, as well as interactions with ^{129}I .

A method involving cross flow ultrafiltration, followed by a three-step cartridge soaking and stirred-cell diafiltration, was developed for isolating EPS from phytoplankton cultures, especially in seawater media. EPS isolated from a marine diatom, *Amphora sp.* was then subjected to semi-quantitative (e.g., carbohydrate, proteins) and quantitative analysis (e.g., neutral sugars, acidic sugars, sulfate). It appeared that Th (IV) binding by EPS was dominated by the acidic polysaccharides of fraction.

For EPS of biofilms collected from polluted streams, hydrophobic proteins were the most abundant components in EPS, followed by more hydrophilic carbohydrates. However, chemical composition of carbohydrates or proteins, i.e., monosaccharides and

amino acids, respectively, varied with environmental conditions and substrata applied, which suggests that the formation of biofilms on different substrates is regulated by specific properties of microorganisms, environmental conditions and nature of substratum. No correlation between relative hydrophobicity of substratum and development of biofilm was found in this study.

A sensitive and rapid GC-MS method was developed to enable the determination of isotopic ratios ($^{129}\text{I}/^{127}\text{I}$) of speciated iodine in natural waters. At the F-area of the Savannah River Site (SRS), iodine species in the groundwater consisted of 48.8% iodide, 27.3% iodate and 23.9% organo-iodine. Each of these iodine species exhibited vastly different transport behavior in the column experiments using surface soil from the SRS. Results demonstrated that mobility of iodine species depended greatly on the iodine concentration, mostly due to the limited sorptive capacity for anions of the soil. EPS, especially enzymes (e.g., haloperoxidases) could facilitate the incorporation of iodide to natural organic carbon. At high input concentrations of iodate (78.7 μM), iodate was found to be completely reduced and subsequently followed the transport behavior of iodide. The marked reduction of iodate was probably associated with natural organic carbon and facilitated by bacteria, besides inorganic reductants (e.g., Fe^{2+}) in sediments and pore water.

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CHAPTER I

INTRODUCTION: THE IMPORTANCE AND RELEVANCE OF THE RESEARCH

1.1. Introduction

Natural organic matter (NOM) is organic matter in various stages of degradation that originates from plants and animals in the environment. NOM is very important in the movement of nutrients and trace elements in the environment. However, very little is currently known about natural organic matter as it is heterogeneous and very complex in composition. The relative size, shape, and composition of the molecular assemblage of NOM are quite heterogeneous. NOM can vary greatly, depending on its origin, transformation mode, age, and existing environment, thus its bio-physico-chemical functions and properties vary for different sources and different environments. Generally, NOM, in terms of weight, consist of 45-55% of carbon and 35-45% of oxygen (Wikipedia, http://en.wikipedia.org/wiki/Natural_organic_matter). And 10-35% of the carbon is present in the form of aromatic rings (Schwehr et al., 2009 and references therein).

Exopolymeric substances (EPS) excreted from phytoplankton and bacteria in oceans and freshwaters have more unique properties. They are important constituents of natural

This dissertation follows the style of *Marine Chemistry*.

organic matter in aquatic systems and exist in both the dissolved and particulate form, and in the size range of few nm to few μm in length. Dissolved EPS account for up to 50% of semi-labile dissolved organic carbon (DOC) in oceanic waters (Kirchman et al., 2001). These unique properties make EPS an integral factor regulating marine biogeochemical processes, including the cycling of elements (C, N, and S), microbial loop, particle dynamics and metal scavenging. As shown in Fig 1.1, EPS play a crucial role in the formation of transparent exopolymeric particles (TEP) (Leppard, 1995, 1997; Passow, 2002), marine and freshwater gels (Verdugo et al., 2004), as well as biofilms (Decho, 2000), which are all important in regulating sedimentation processes (Underwood and Paterson, 1993; Welker et al., 2002), and biogeochemical cycling and particle dynamics (Passow and Alldredge, 1994; Logan et al., 1995). When EPS in the form of micro- and nano-particles are stained by alcian-blue, it is called transparent exopolymeric particles (TEP). TEP provide a microhabitat for bacteria by forming relatively stable microzones in aggregates and impact all concentration-dependent processes, like nutrient uptake and grazing rates, by allowing the development of chemical gradients. Many studies have found elevated bacterial productivity within aggregates compared to the surrounding seawater (Herndl, 1988; Grossart and Simon 1993, 1998). Observational evidence from Logan et al. (1995) confirmed that the formation of aggregates at the decline of diatom blooms is frequently controlled by the chemical composition of EPS. The studies of Passow and Alldredge (1994) provide a detailed description of the aggregation dynamics during diatom blooms. Aggregation becomes the dominant process in particle dynamics when particle concentrations reach a

critical concentration threshold (Jackson 1990). Besides, EPS also take part in the scavenging of stable and radioactive trace elements. This dissertation will focus on three of those roles of EPS (highlighted in the Figure 1.1), 1) trace element scavenging, 2) formation of biofilms, and 3) cell adhesion during biofilm formation. As a result, I will attempt a comprehensive and systematic understanding of EPS in environments.

EPS are biopolymers produced by living organisms and primarily consist of polysaccharides, proteins, and small amounts of DNA and RNA. Lipids are also present in small quantities in EPS by forming complexes with proteins or polysaccharides (as lipopolysaccharides or lipoproteins; Bhaskar and Bhosle, 2005). A major but defining difference between polymers and biopolymers can be found in their structures (Wikipedia, <http://en.wikipedia.org/wiki/Biopolymer>). Polymers, including biopolymers, are made of repetitive units called monomers. However, biopolymers often also have a well defined structure. The exact chemical composition or the sequence in which these units are arranged is called the primary structure. Many biopolymers spontaneously fold into characteristic compact shapes, such as proteins, which can fold in a number of different ways (sheets, helices, etc). In contrast, most synthetic polymers have a much simpler and more random structure.

Distinct physico-chemical properties of EPS can be attributed to some unique components in their otherwise diverse and complex chemical composition. For example, arabinose in EPS helps in cell aggregation of bacteria (Bhaskar et al., 2005 and references therein) and whereas deoxy sugars like fucose and rhamnose found in EPS from diatoms can help in foaming and flocculation (Zhou et al., 1998). Apart from

monosaccharides, EPS also contain non-sugar moieties like uronic acids that may constitute up to 20-50% of the polysaccharide fraction in marine bacterial EPS (Majumdar et.al., 1999). The acidic groups give an overall negative charge to the polymers and make EPS highly surface-active and facilitate binding with metal ions. On the other hand, proteins and lipids provide more hydrophobicity to EPS.

Apart from EPS, aromatic carbon, e.g., from lignin, is also an important moiety of natural organic matter in aquatic systems, such as humic substances. Humic substances are major importers, exporters and transporters of solutes in soils and natural waters. For example, Moulin and Moulin (2001) reviewed the effects of humic substances on the solubility, speciation, bioavailability and toxicity of radionuclides in aquatic systems. Radionuclides can be transported and chelated by functional groups of humic substances, or they can be covalently bound to carbon or specific moieties. For example, experimental evidence (Moulin et al., 2001; Steinberg et al., 2008a, 2008b) has shown that iodine isotopes, major nuclear waste components, are covalently bound to aromatic constituents by electrophilic substitution.

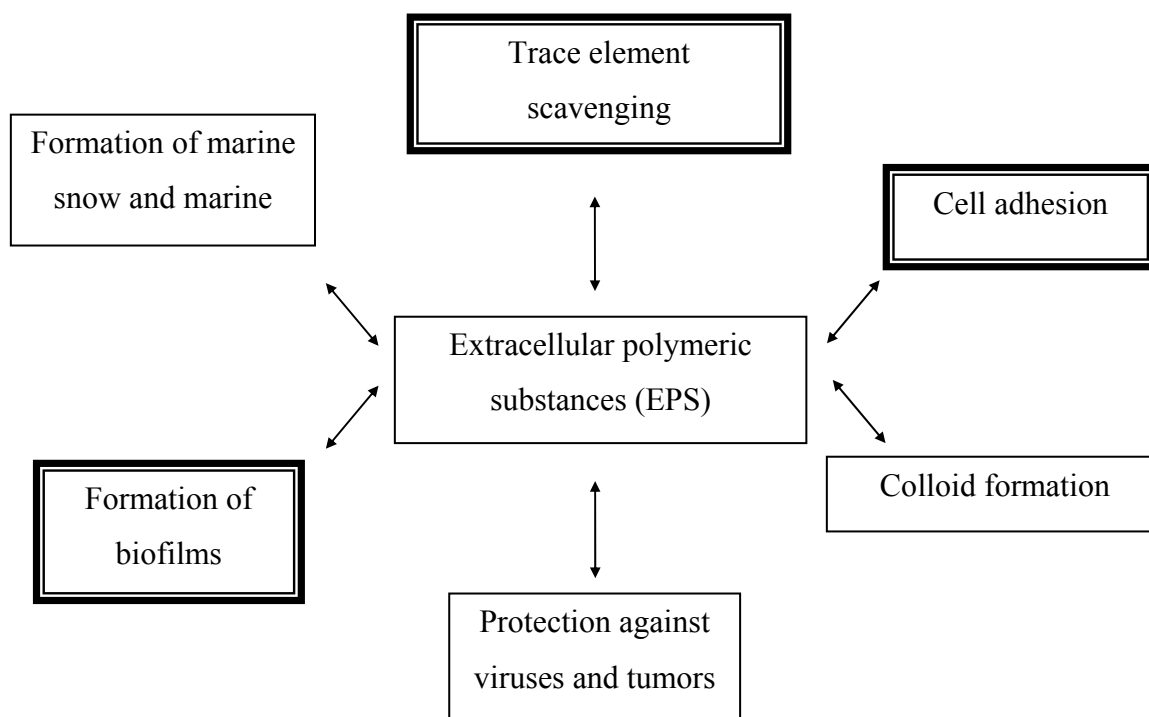


Fig 1.1 Roles of exopolymeric substances (EPS) in aquatic systems (the three double-lined roles are what the dissertation is going to focus on)

1.1.1. ^{234}Th (IV) binding of EPS

^{234}Th (IV) is a naturally occurring radionuclide produced from the alpha decay of uranium-238. Uranium-238 has a near constant concentration in ocean water, which results in a constant production rate of ^{234}Th ($2430 \text{ atoms min}^{-1} \text{ m}^{-3}$). So any departure from the equilibrium is attributed to ^{234}Th (IV) removal to particles due to its highly particle-reactive properties. Therefore, ^{234}Th (IV) has become an important proxy in oceanographic investigations of particle dynamics (e.g., a tracer for particle and colloid

scavenging that might involve spontaneous assembly or aggregation of biopolymers leading to the formation of larger particles). For example, the fluxes of particulate organic carbon can then be determined through the use of $^{234}\text{Th}/\text{POC}$ ratios (Buesseler et al., 2006). $^{234}\text{Th}/\text{POC}$ ratios might be controlled, in parts, by the abundance of exopolymeric substances (EPS) because of their chelating properties and surface activity, and the affinity of Th (IV) to particles. As summarized in Santschi et al. (2006), both experimental (Quigley et al., 2002) and field results (Guo et al., 2002; Santschi et al., 2003; Passow et al., 2006) demonstrate that ^{234}Th (IV) in the ocean is strongly associated with acidic polysaccharide (APS)- rich compounds. Different acidic functional groups in exopolysaccharides, including carboxylate, phosphate and sulfate, have been implicated to contribute to the binding of ^{234}Th (IV) to different EPS compounds (Alvarado-Quiroz et al., 2006). However, very few studies of the chemical composition of EPS have been conducted, at the molecular level, to locate and closely investigate the agents that are responsible for the binding of ^{234}Th (IV) to EPS. One purpose of this study is to understand the relationship between EPS composition at the molecular level and their binding properties to ^{234}Th (IV).

1.1.2. The roles of EPS in the formation of biofilms

Another area where EPS are important and of environmental and health concern is their formation of a matrix of biofilms. A biofilm is a community of microorganisms in which cells adhere to each other on a surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPS). EPS allow a structure that provides enhancement of microbial activities of the

surrounding cells by forming a stable and protective microenvironment (Decho 2000). Biofilms play critical roles in the remineralization of nutrients (Azam, 1998; Azam et al., 1993), primary production in coastal systems (Paerl, 1997) and even sediment stabilization (Paterson, 1995). In recent years, increasing numbers of studies indicate that fecal coliforms can grow and replicate in non-host habitats such as surface waters and sediments (Desmarais et al., 2002; Byappanahalli et al., 2003; Whitman et al., 2006). Under harsh conditions (e.g., antibiotics, toxic metals, chlorination), the structure of biofilms may favor the formation of microsites with specific physicochemical conditions that permit the survival of fecal bacteria (Costerton et al., 1995). The colonization of solid surfaces by microorganisms depends mostly on the production of extracellular substances, i.e., EPS (Czaczyk and Myszka, 2007). Many studies of biofilms in natural aquatic systems have observed that the nature of the substratum can influence the bacterial biomass (Hunt and Parry, 1998), species richness (Baldy et al., 1995; Barbiero, 2000), species succession and colonization patterns (Tank and Dodds, 2003), heterotrophic activity (Romani and Sabater, 2000), and pollutant concentrations in the EPS (Kroepfl et al., 2006), all of which could influence pathogen and indicator survival in a biofilm. However, relatively little is known about the relationship between physicochemical properties of EPS and the nature of the substratum. Here, Buffalo Bayou and White Bayou are chosen as the sites of interest because they are both on the Clean Water Act Section 303(d) List for impaired environments due to chronically elevated bacterial levels. Besides generally accepted non-point source inputs (e.g., storm water run-off, septic tanks and leaking sewer pipes and illegal discharges), non-human sources of these

bacteria, including extended survival and replication of these bacteria in non-host habitats such as biofilms, may also influence the bacterial concentrations. Therefore, this study applies different substrata to investigate their effects on biofilm formation using a chemical approach, e.g., the relationship between chemical composition and EPS production.

1.1.3. The roles of natural organic matter in mobility and transport of iodine species, including ^{127}I and ^{129}I isotopes

Unlike ^{234}Th (IV), the main source of ^{129}I in the aquatic environment is from accidental and purposeful releases, mostly from nuclear fuel reprocessing (Raisbeck and Yiou, 1999; Schnabel et al., 2001). At a number of sites all over the world, accidentally-released ^{129}I has migrated into groundwaters, where the high mobility of iodine and long half-life (16,000,000 years) of ^{129}I has led to a contamination problem. For example, the F-area of the Savannah River Site (Riley and Zachara, 1992) is one such highly contaminated area.

Iodine is a biophilic element which in mammals is concentrated mainly in the thyroid in the form of triiodothyronine (T_3) and thyroxin (T_4), which are responsible for regulation of metabolism. In aquatic environments, iodine mainly exists as iodide, iodate and organic iodine. Each of these iodine species sorbs differently to sediment and therefore moves through the environment vastly differently. Inorganic iodine (iodide and iodate) has been assumed to be the dominant and also the most mobile species in groundwater (Schwehr and Santschi, 2003), but little is actually known about organo-iodine in groundwater. Organo-iodine formation could significantly modify its transport

and bioavailability. The importance of organo-iodine in the chemical speciation of iodine has recently been established for fresh (Krupp and Aumann, 1999, Oktay et al., 2001; Schwehr and Santschi, 2003; Santschi and Schwehr, 2004; Schwehr et al., 2009) and marine surface waters (Wong and Cheng, 2001; Santschi and Schwehr, 2004; Schwehr et al., 2005a, 2005b). However, little is known on the importance of organo-iodine species in the chemical speciation scheme of ^{129}I in groundwater, particularly on how microbiological and chemical factors affect their mobility and properties. I hypothesize that in surface (e.g., soil) and subsurface environments (e.g. groundwater sediment), the transformation of ^{129}I from an inorganic to an organic form might be mediated by microbial processes. The most recent advances in research concerning microbial-iodine interactions have been contributed by Amachi et al. (2001, 2003 and 2005a, 2005b). Different bacteria which can accumulate, oxidize or methylate iodine have been isolated from various environments. Microorganisms might bind iodine in a kinetically fast reaction into labile nitrogen-rich compounds (i.e. proteins with aromatic moieties) over short time scales (days to weeks) and then evolve into more refractory aromatic compounds (humic acids) over longer time scales. Phenol and alpha-methyl carbonyl groups are hypothesized to be most reactive toward iodine, based on what is known from basic organic chemistry (Warner et al., 2000; Steinberg et al., 2008a, 2008b). Therefore, special attention has been paid to speciation of ^{129}I at a contaminated site and the mechanisms of transport in the aquatic system.

1.2. Hypothesis and objectives for the binding of ^{234}Th (IV) with EPS

1.2.1. Hypothesis

Acidic polysaccharides (e.g., uronic acids, sulfates and phosphates) in extracellular polymers excreted by phytoplankton are the reactive agents that bind with ^{234}Th (IV) in marine environments.

1.2.2. Objectives

- Establish an efficient method for large-scale EPS isolation from aqueous samples as well as an effective method to purify and fractionate EPS.
- Develop an understanding of the relationship between EPS composition at molecular level and its binding properties to ^{234}Th (IV).

1.3. Hypothesis and objectives for the roles of EPS in the formation of biofilms

1.3.1. Hypothesis

Chemical and physical properties of EPS in biofilms will be affected by the nature of the substratum and environmental conditions.

1.3.2. Objectives

Investigate chemical and physical properties of natural EPS in biofilm collected from Buffalo Bayou and White Oak Bayou in order to understand the relationships between physico-chemical properties of EPS and substratum.

1.4. Hypothesis and objectives for the roles of natural organic matter in mobility and transport of iodine species, including ^{127}I and ^{129}I

1.4.1. Hypothesis

Iodine mobility through sediment is highly dependent on its speciation as well as its corresponding concentration. Organo-iodine mobility can be enhanced or retarded depending on the organic moiety, whereas inorganic iodine mobility is largely dependent on its oxidation state.

1.4.2. Objectives

- To develop a sensitive and rapid method for determination of isotopic ratios of speciated iodine in order to assess the distribution of ^{129}I and stable ^{127}I in environmental systems.
- Determine the mobility of iodine species, including iodide, iodate and organo-iodine using columns loaded with sediments from Savannah River sites, thus understand the environmental consequence of radioactive contamination of ^{129}I .

CHAPTER II
APPLICATION OF CROSS-FLOW ULTRAFILTRATION FOR ISOLATING
EXOPOLYMERIC SUBSTANCES FROM A MARINE
DIATOM (*AMPHORA SP.*)*

2.1. Overview

The increasing recognition of the roles that exopolymeric substances (EPS) play in the aquatic environment necessitates obtaining sufficient quantities of purified EPS for exploration of its physical, chemical and biological properties, as well as for quantitative and structural analysis of its composition. For this purpose, three preconcentration/purification techniques, i.e., 1) ethanol precipitation, 2) stirred-cell ultrafiltration, 3) cross-flow ultrafiltration, followed by stirred-cell diafiltration, were compared for their effectiveness in quantitatively isolating EPS from laboratory cultures of *Amphora sp.* The results showed that the classical ethanol precipitation method was not effective in isolating and concentrating EPS from this seawater culture medium. Stirred-cell ultrafiltration appeared best for harvesting EPS from this diatom. However, because of its limitations in terms of time and volume, cross flow ultrafiltration needed to be first applied, along with some necessary improvements, followed by a three-step cartridge soaking and stirred-cell diafiltration. After cartridge soaking, the yields of the

*Reprinted with permission from “Application of cross-flow ultrafiltration for isolating exopolymeric substances from a marine diatom (*Amphora sp.*)” by Saijin Zhang and Peter H. Santschi, 2009. *Limnology and Oceanography: Methods*, 7, 419-429, Copyright [2009] by the American Society of Limnology and Oceanography, Inc.

two ultrafiltration methods were comparable. Two different fractions were obtained from EPS of *Amphora sp.* by anion exchange chromatography and were characterized respectively. While these purified fractions had similar molecular weights of 1000 kDa, their monosaccharide composition was different. In conclusion, cross-flow ultrafiltration followed by stirred-cell diafiltration with additional cartridge washing turned out to be the optimal method for EPS separation, based on time, cost, and yield.

2.2. Introduction

Exopolymeric substances (EPS) secreted from phytoplankton and bacteria in the ocean are colloidal polysaccharide-rich polymers that play a crucial role in the formation of marine gels, marine snow and biofilms, as well as in colloid and trace element scavenging (Hoagland et al., 1993; Costerton, 1995; Myklestad, 1995; Verdugo et al., 2004). Diatoms are well known for releasing polysaccharide-rich EPS during all phases of their growth (Hama and Handa, 1983; Sundh, 1989; Williams, 1990). The production and structural characteristics of EPS vary with species, with a production ranging from 1 mg/L to 27.5 mg/L (Myklestad, 1974; Brouwer et al., 2006). Fucose and galactose are sugar monomers that figure prominently in the polysaccharide composition of EPS from diatoms (Myklestad, 1995), while galactose is absent in EPS from *Coscinodiscus nobilis* (Percival et al., 1980) and fucose being absent in EPS from *Nitzhia* (Allan et al., 1972). The composition of EPS from *Amphora sp.* has, however, not been reported before. *Amphora* is a common benthic microalga found in coastal benthic and pelagic environments (Welker et al., 2002; Kasim and Mukai, 2006; Facca and Sfriso, 2007).

Benthic microalgal communities are important primary producers in a wide variety of shallow coastal areas (Cadee and Hegeman, 1974; Colijn and De Jonge, 1984; Cahoon and Cooke, 1992; Barranguet, 1997; Nelson et al., 1999). Up to 40% of the photosynthetically fixed carbon is lost by benthic and pelagic diatoms as an extracellular release (Fogg, 1983; Smith and Underwood, 1998; Taylor and Paterson, 1998). Furthermore, a positive correlation between sediment colloidal carbohydrate concentrations (as a proxy of EPS) and benthic diatom biomass has been reported in many studies (Underwood and Paterson, 1993; Fabiano and Danovaro, 1994; Underwood et al., 1995; Welker et al., 2002). Therefore, a comprehensive understanding of EPS from *Amphora sp.* will advance our knowledge of the roles benthic diatoms play in releasing EPS, particle dynamics and sedimentation processes.

Exopolysaccharides are composed principally of monosaccharide residues and their derivatives, but can also be covalently linked to proteins (e.g., glycoproteins and proteoglycans) and lipids (e.g., lipopolysaccharides). Because of the large number of possible monomers and the multiple types of inter- and extra-polysaccharidic linkages, it would be a great challenge to derive the exact chemical composition and conformation for these types of polysaccharides. Repetitive structural features and repeating units form the basis of the structural classification of polysaccharides. Structural variations within the repeating units and the sequence of the units can be controlled by the microorganisms, which release them at particular times in their life cycle, and in response to environmental stressors. For example, under harsh environmental conditions (e.g., high concentrations of antibiotics, toxic metals, chlorination), the structure of EPS

released by fecal bacteria may favor the formation of microsites with specific physicochemical conditions that permit the survival of fecal bacteria (Costerton et al., 1995). Compositional factors can influence ion selectivity and other chemical properties (Bhaskar and Bhosle, 2005). For example, the presence of sugars like arabinose in EPS helps cell aggregation in bacteria assemblages (Efrat et al., 2004), whereas deoxy sugars like fucose and rhamnose found in diatom EPS help to enhance foaming and flocculation (Zhou et al., 1998). The higher yield of uronic acids and other acidic moieties, including sulfate esters, pyruvates and acyl groups, gives an overall negative charge to the polymer, thereby imparting binding and adsorptive properties to the polymer (Decho, 1990). In order to understand the role exopolysaccharides play in marine systems, it is important to characterize EPS and thus clarify the composition of EPS in relation to their functional properties. Recently, many advanced analytical techniques have been used to characterize polysaccharides, such as GC/MS, FTIR, HPLC, NMR, etc. (Chauton et al., 2003; Lim et al., 2005; Nordmark et al., 2005). Some of these techniques, e.g., NMR, require relatively large (e.g., 30mg or more) amounts of analytes. However, current isolation and analysis methods have difficulties with harvesting and purifying sufficient quantities of EPS from phytoplankton cultures. Ethanol precipitation, the method most often used for EPS isolation, (Schepetkin et al., 2005; Wang and Xia, 2005; Kim et al., 2006; Lbarburu et al., 2007), is ineffective for many cultures due to the large quantities of ethanol required for large-scale isolation of EPS from aqueous media such as seawater. Currently, membrane technology is the preferred method to desalt and concentrate macromolecules from complex matrices. This technology can be found in

most modern food processing applications (e.g., Kamada et al., 2002; Wang et al., 2002; Li et al., 2005). Applications of cross-flow ultrafiltration techniques in marine systems for sampling natural colloids have greatly increased, and their recent progress has been reviewed (Carlson et al., 1985.; Benner et al., 1997; Dai et al., 1998; Guo et al., 2000; Guo and Santschi, 1997; Guo et al., 2007). These studies can provide the foundation for the utilization of cross-flow ultrafiltration to EPS sampling and purification. However, most of the EPS characterization literature is from EPS grown on biofilms, as with biofilms, it is easier to get sufficient material for chemical characterization. A comparison with the literature on EPS extraction and purification from aqueous culture media shows that the newer ultrafiltration procedure improved yield and purity.

The objective of this study is to develop a suitable protocol for large-scale EPS isolation from seawater medium by comparing three different techniques, 1) the classical ethanol precipitation method, 2) stirred-cell ultrafiltration, and 3) cross-flow ultrafiltration, with 2) and 3) followed by stirred-cell diafiltration. *Amphora sp.* was used as a model species in the study. Since fresh EPS biopolymers have molecular masses of tens to hundreds of kDa (e.g., Decho, 1990; Zhang et al., 2008), as opposed to more degraded material (e.g., Alvarado-Quiroz et al., 2006), membrane molecular weight cut-offs of 10kDa or less are perfectly adequate for isolation and separation purposes. The retention characteristics of EPS, as well as their chemical characteristics, were studied to examine the validity of ultrafiltration technology based on the recovery of EPS extraction and their chemical signatures in anion exchange chromatography and size exclusion chromatography. As a result, cross-flow ultrafiltration followed by stirred-cell

diafiltration was proven to be an effective technique to isolate EPS from a large volume of seawater or phytoplankton culture. The large-scale isolation method will enable researchers to recover large amounts of EPS from cultures or water samples with minimal effort and cost. Furthermore, this new technique will be useful in comprehensive understanding of EPS in aquatic systems by allowing isolation of enough material for molecular-level analysis (e.g., NMR, GC- or LC MS) and examinations of their physical, chemical and biological properties. This will advance our understanding of the roles of EPS in the formation of marine snow, particle dynamics, trace metal scavenging and sedimentation processes.

2.3. Materials and methods

2.3.1. Culturing of Amphora sp.

Bacillariophyceae *Amphora sp.* (CCMP1389) was bought from CCMP (National Culture Collection of Marine Phytoplankton). It was originally collected from Flax Pond, near the Stony Brook Marine Lab, Long Island, New York. In this experiment, *Amphora sp.* was cultured in a 2 L flask with 1 L of autoclaved f/2-Si medium (CCMP medium recipe, salinity: 30 psu) at a temperature of 21° C. For each batch, 10 flasks were used to culture *Amphora sp.* in order to harvest 10 L of culture solution for experimental use. Dual lamp fluorescent lighting (Incubator 818, Precision) provided uniform illumination with one cycle per day (e.g. 12h/12h). The status of *Amphora sp.* was monitored by measuring Chlorophyll a concentrations (Yentsch 1963). EPS was collected when *Amphora sp.* was at the stationary phase. Stationary phase was reached on the ninth day

of culturing. In this study, we harvested *Amphora sp.* in the twelfth day allowing the culture more time at stationary phase. Additionally, to prepare for blank samples, another 10 flasks filled with the same autoclaved medium was “cultured” for the same time.

2.3.2. Extraction of EPS

Amphora sp. cultures and blank samples were centrifuged (2694g, 30 minutes) followed by pre-filtering through GF/B, GF/F and 0.45 μm polycarbonate membranes (in series). The filtrate was collected to allow for monitoring the progress of the EPS extraction methods.

Cell lysis during centrifugation and filtration releases cytosolic contents, like proteins, lipids and nucleic acids, into the culture solution, which will compromise the purity of the original exopolymeric substances that were released. In this experiment, nucleic acid concentration was used as an indicator of cell lysis during centrifugation and filtration. Briefly, before the culture was collected for centrifugation, 1 mL of the original culture was filtered through 0.45 μm polycarbonate membranes, where cell lysis was considered to be negligible. Nucleic acid concentration was estimated by measuring the UV light absorbance at 270 and 290 nm (Karklinya et al., 1989). The concentrations in the culture subjected to centrifugation and filtration was then measured at the same time and under the same conditions for nucleic acid determination. The difference in nucleic acid concentration between the two cultures was then used to evaluate cell lysis.

2.3.3. Ethanol precipitation

As the classical method to extract water-soluble polysaccharides, ethanol precipitation was used as a reference method. In our laboratory experiments, three volumes of a

mixture of 95% ethanol and 5% methanol were added to 1 volume (1 liter) of pre-filtered culturing solution, and the solutions was stirred homogenously. The mixture was kept overnight in the refrigerator. After that, the mixture was centrifuged at 2694g for 30 minutes and the supernatant discarded. The precipitate that evolved was first dissolved with 100 mL of nanopure water and then the solution was centrifuged (2694g, 30 minutes) again. The supernatant was kept, and the procedure repeated six times until all the precipitate was completely dissolved. 1 L of blank sample was extracted using the same procedure.

2.3.4. General procedure for stirred-cell ultrafiltration

1 L of pre-filtered culture was ultrafiltered using an Amicon 8200 stirred-cell (Millipore) and 5kDa polyethersulfone membrane, at a working pressure of 45-50 psi. After ultrafiltration, 300 mL of nanopure water was needed to completely remove the salts. 25~30 mL of retentate remained and was stirred for 30 minutes. Another 25 mL of water was used to wash the membrane by stirring 1 hour at the end, which was then combined with the retentate. The final solution was then ready for further analysis. 1 L of blank culture was used for procedural blank estimation.

2.3.5. Calibration of membrane

1.6 mg of 10 kDa fluorescent-tagged dextran (Sigma) in 1 L of nanopure water was used to check and calibrate the stirred-cell system by going through the general procedure described above. Dextran was quantified by establishing a calibration curve from 0, 16, 32, 48, 64 $\mu\text{g/mL}$ on a spectrophotometer at 478 nm. Mass balance between originally added dextran and retentate dextran was calculated to estimate the

effectiveness of the system. Additionally, dextran was used to test the Anion Exchange Chromatography system in order to assess possible changes in charge properties after stirred-cell ultrafiltration.

2.3.6. Testing salt effects

As changes in ionic strength, pH, and seawater cations can modify the behavior and speciation of aquatic macromolecules, the recoveries of 1.6mg dextran in 1 L of distilled water and 1 L of permeate from stirred-cell ultrafiltration (< 5 kDa) were tested for possible salt effects. In addition, EPS harvested from ethanol precipitation were dissolved in 1 L of permeate from stirred-cell ultrafiltration to examine the recovery of EPS during the stirred-cell ultrafiltration process. In parallel, another EPS aliquot that was dissolved in nanopure water was prepared to go through the same procedure. Uronic acid concentrations in the initial feeding solution and in the final solution, respectively, were measured to estimate the effect of seawater chemistry on the recovery of EPS during stirred-cell ultrafiltration.

2.3.7. Cross flow ultrafiltration system

A spiral wound 1 kDa SOC 1812 cartridge (Separation Engineering, Inc), and a Teflon diaphragm pump head (ColeParmer) equipped with Teflon fittings and tubings, were used to minimize the sorptive losses and any possible contamination of the apparatus (Guo and Santschi 1996; Wen et al.1996; Guo et al., 2000, 2001). After each run, the cartridge was subjected to a cleaning procedure with detergent (Micro-90), 0.05 M NaOH, and 0.05 M HCl, respectively. Each solution was recycled for at least for 20 minutes. Between each solution, ~20 L of nanopure water was flushed through the

ultrafiltration system. After the full chemical cleaning, a final 20 L of nanopure water was flushed through the system. All the operations were carried out at 30 psi and at 50-60% pump power efficiency. Additionally, the cartridge was stored in 2% NaN₃ to prevent bacteria growth in-between uses.

2.3.8. General procedure for cross-flow ultrafiltration

In order to meet the demand of greater mass production of EPS, cross-flow ultrafiltration was used to partly desalt and concentrate EPS from *Amphora sp.* from large volumes. A general procedure for obtaining purified EPS from 8 L of culture solution is summarized in a schematic diagram (Figure 2.1). The procedure consists of a) *Amphora sp.* culturing, b) centrifugation, c) filtration, d) cross-flow ultrafiltration, e) stirred-cell diafiltration, and f) anion exchange chromatography steps. In detail, ultrafiltration of 8 L of pre-filtered culture was carried out until 200-300 mL of retentate remained. After that, the cartridge was rinsed with 200 mL of nanopure water, the pump turned off and the cartridge was soaked in water for 6 hours. After that, the cartridge was rinsed with another 200 mL of water, and this whole process was repeated twice, sometimes after waiting overnight when it was inevitable. Subsequently, the retentate solution and four rinse solutions were combined. The resulting 1 L of solution was then further concentrated by stirred-cell diafiltration with a 5 kDa membrane at the same conditions described in the section of stirred-cell ultrafiltration to obtain 50 mL of concentrated EPS solution. After that, the EPS solution was further purified by fractionation using anion exchange chromatography (see section '*Purification and fractionation of EPS*'). A procedural blank was determined by using 8 L of blank culture

sample.

2.3.9. Calibration of cross-flow ultrafiltration cartridge

Calibration was conducted by using 10 kDa fluorescent-tagged dextran. 12.8 mg of 10 kDa fluorescent-tagged dextran was dissolved in 8 L of pre-ultrafiltered culture solution and subjected to the same general procedure for cross-flow ultrafiltration. Dextran was quantified at 495 nm (the absorbance maximum of dextran shifted from 478 nm to 495 nm when it was dissolved in seawater). Mass balance of dextran between the initial solution and the final solution was calculated to estimate the effectiveness of the system. In addition, anion exchange chromatography (AEC) was used to monitor change in dextran charge distributions after this procedural treatment.

2.3.10. Effect of concentration factors (CF)

Since high CFs provide for better speciation and retention results but could increase coagulation of biomolecules in the retentate (Guo et al., 2007), the effects of different CFs were tested. 8 L of culture solution was concentrated to 1600 mL, 800 mL, 400 mL and 200 mL, respectively, by cross-flow ultrafiltration to achieve a CF of 5, 10, 20, 40. The yields of EPS at different CF were measured to determine the effects of concentration factor (CF) on the recovery of EPS during cross-flow ultrafiltration.

2.3.11. Determination of concentrations of total carbohydrates, uronic acids and proteins

Total carbohydrates (including uronic acids) were estimated by using the anthrone method (Morris 1948; Rastogi 2005), which is considered simpler and more specific for carbohydrates compared with other methods. Uronic acids were determined by using

0.0125 mol/L sodium borate in concentrated sulfuric acid and 3-phenylphenol (Blumenkrantz and Asboe-Hansen 1973). Proteins were quantified using bicinchoninic acid (Smith et al. 1985; Stoscheck 1990).

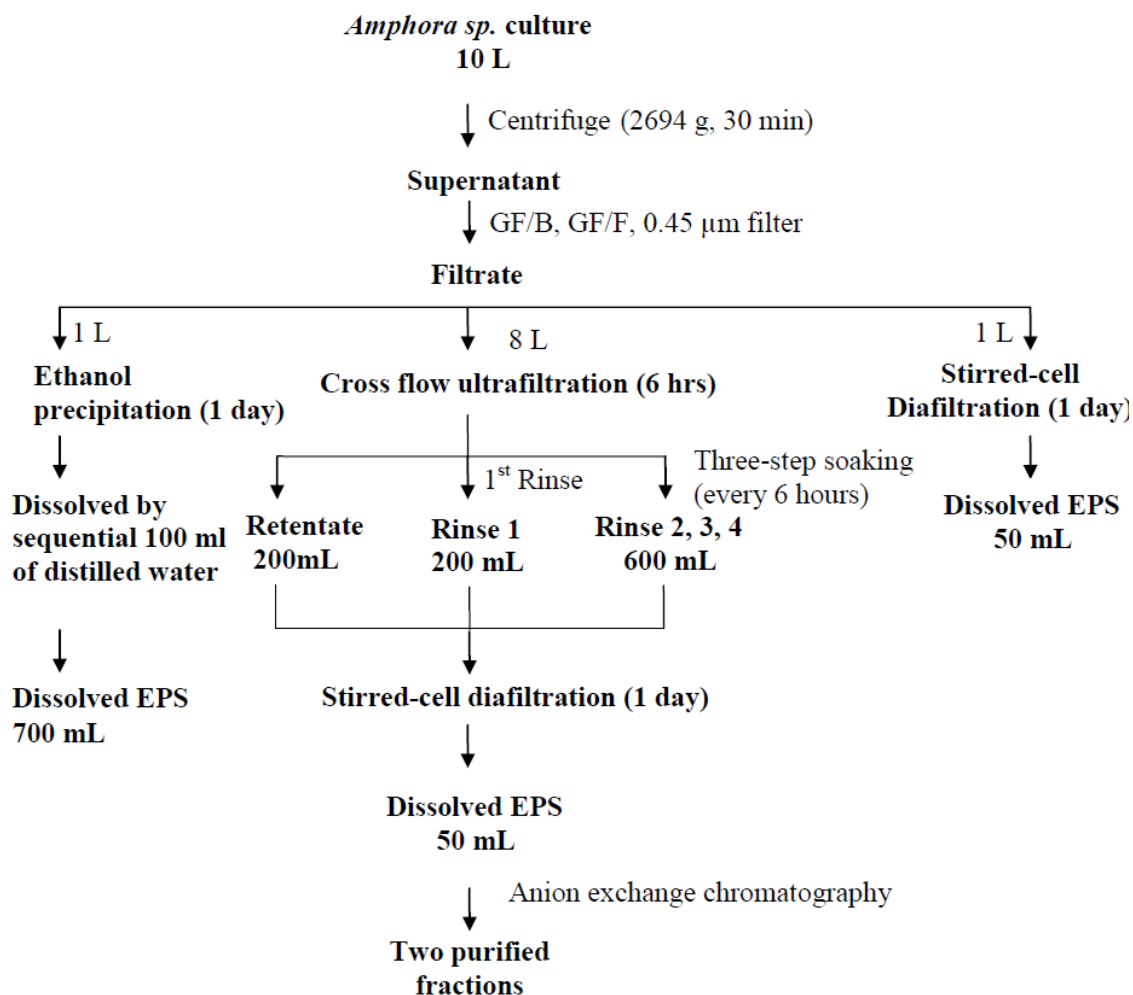


Fig. 2.1 Schematic diagram of the procedures for EPS isolation

2.3.12. Purification and fractionation of EPS - a new anion exchange procedure

A preparative liquid chromatography method was developed to purify and fractionate extracted EPS using a BioSuite Q13 μ m AXC (21.5x150mm) column from Waters. Two different eluents were used for the mobile phase: A: 20 mmol/L tris HCl (pH=8.1) and B: 20 mmol/L tris HCl with 1 mol/L NaCl, where 100% A is changed to 100% B in 70 minutes. EPS from *Amphora sp.* was detected using a fluorescent detector (Waters 474) at excitation $\lambda=285$ nm, emission $\lambda=580$ nm (Ding et al., 2008). The injection volume in the experiment was designed to be 5 mL (approximately 2% of column volume) and the flow rate was 5 mL/min.

2.3.13. Estimation of EPS molecular weight

Size exclusion chromatography was used to measure the molecular weight of EPS. The column used was a Tosoh TSK G-4000PWxl, which was eluted by 10 mmol/L phosphate buffer (pH=6.8 with 0.078 mol/L NaNO₃) at 0.5 mL/min. 150 μ L of sample was injected and detected by a refractive index detector (Waters 410). Polystyrene standards with molecular weights of 8 kDa, 35 kDa, 100 kDa, and 780 kDa were used for the calibration curve, whereby the logarithm of molecular weight was plotted vs. corresponding retention time.

2.4. Results

2.4.1. Cell lysis

Cell lysis can provide large artifacts for EPS collection and purification (Decho, 1990). In order to test for possible artifacts, nucleic acid concentrations were monitored

during all phases of EPS isolation and purification. Results showed that nucleic acid concentrations did not vary after centrifugation and filtration of the cultures, 0.57 ± 0.03 mg/L for initial cultures before centrifugation and filtration and 0.54 ± 0.07 mg/L for cultures subjected to centrifugation and filtration. The minimal cytolysis could be due to the well-developed cell wall of *Amphora sp.*, which helps to prevent occurrence of cytolysis.

2.4.2. Ethanol precipitation of EPS

Even though the isolation of polysaccharides from organisms in most studies is based on ethanol precipitation, in our study, it was difficult to isolate EPS from a culture of *Amphora sp.* using this method due to the large amount of Ca-Mg-carbonate precipitate formed. Simply using HCl to dissolve the carbonates did not result in a visible EPS residue, as ethanol is less effective in acidic solutions to precipitate EPS. Furthermore, using 100 mL nanopure water to dissolve the small amounts of EPS that would coprecipitate with the carbonates led not only to a large loss of EPS, but also to dilution rather than concentration.

Up to 1 g of white precipitate developed when 3 L of a mixture of ethanol and methanol was added to 1 L of pre-filtered culturing seawater solution of *Amphora sp.*, with most of it as calcium and magnesium carbonates, which are not soluble in 70% ethanol. 100 mL of nanopure water was firstly used to dissolve the precipitate. Then the aqueous phase and the remaining precipitate were separated by centrifugation. This procedure was repeated until the precipitate was completely dissolved. As a result, seven 100 mL of solutions were collected and total carbohydrates and uronic acids in the seven

solutions were determined. Their distributions are shown in Figure 2.2. There were no detectable carbohydrates and uronic acids in fractions from the blank sample. Total carbohydrates and uronic acids in solution 1 were not estimated because of an occurrence of browning after the addition of sulfuric acid. Moreover, there were no detectable carbohydrates or uronic acid in the retentate of solution 1 after it was subjected to stirred-cell diafiltration. The even distribution of carbohydrates and uronic acids in the solutions 2~7 shown in Figure 2.2 leads to the difficulty in extracting EPS with ethanol, since 700 mL of nanopure water has to be used to dissolve EPS out of the precipitate when treating 1 L of culture solution. In other words, EPS cannot be concentrated in this way, not to mention the huge consumption of ethanol in the mass production of EPS. The yield of EPS from *Amphora sp.* was 1.5 ± 0.2 mg-glucose/L and 2.9 ± 0.8 (n=3) mg-glucuronic acid/L, by summing the seven solutions. The yield of uronic acids was about twice as much as that of carbohydrates, which may have been caused by an underestimation of uronic acids in the total carbohydrates method using the anthrone reagent. This explanation is further verified by the fact that 60mg/L glucuronic acid was found to be equivalent to only 14 mg/L glucose-equivalents.

2.4.3. Calibration of stirred-cell ultrafiltration

Our results from using 10 kDa dextran in stirred-cell ultrafiltration systems showed undetectable amounts of dextran in the permeate. Thus, high recovery rates of 96.8 ± 2.8 % (n=3) were achieved in our experiment when calculating the mass balance of dextran in initial solutions and the corresponding retentates. Additionally, dextran in the initial solution and in the retentate demonstrated a similar signature on anion exchange

chromatography, which means that neither artifacts nor changes in charge occurred during stirred-cell ultrafiltration of the dextran standard.

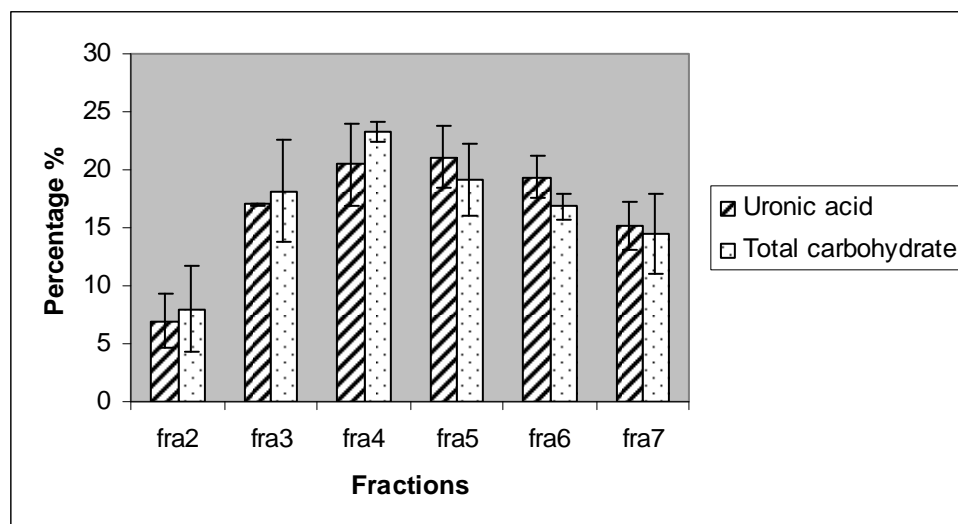


Fig. 2.2 Distribution of total carbohydrates and uronic acids in six fractions from ethanol precipitation (n=3, i.e. 3 repeated ethanol precipitation experiments using cultures from three different batches)

2.4.4. Salt effect on EPS isolation during stirred-cell ultrafiltration

The recovery of dextran and EPS (obtained from ethanol precipitate) in nanopure water and pre-ultrafiltered culture is shown in Figure 2.3. A recovery of approximately 85.0% was obtained for EPS and dextran in the pre-ultrafiltered culture solution, while 95.0% for the same materials dissolved in nanopure water. Neither EPS nor dextran was found in the permeate. So, absorption onto the membrane might account for most loss of EPS. The consistently higher recoveries in nanopure water during stirred-cell ultrafiltration indicate that the aqueous chemistry of seawater could, to some degree,

cause adsorption losses of EPS to the ultrafiltration membrane. As a result, membrane washing is strongly recommended at the end of diafiltration in order to increase the recovery of EPS. However, as for the extraction of EPS, an 85% recovery rate is definitely acceptable and practical. The yield of EPS from *Amphora sp.* using stirred-cell ultrafiltration was 3.1 ± 0.4 (n=4) mg-glucuronic acid L⁻¹, comparable to the yield of ethanol precipitation. Based on the investigation of permeation and retention of EPS, stirred-cell ultrafiltration followed by diafiltration was adopted after cross-flow ultrafiltration of EPS, due to its simplicity and reproducibility.

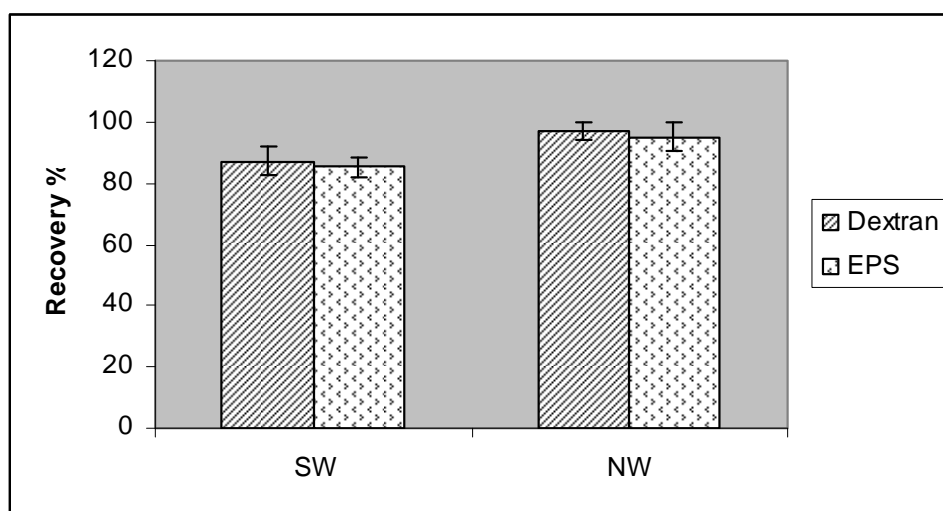


Fig. 2.3 Recovery (n=3) via stirred cell ultrafiltration of dextran and a test EPS isolate dissolved in seawater (SW) and nanopure water (NW)

2.4.5. Calibration of cross-flow ultrafiltration of EPS

The ultrafiltration cartridge was calibrated by using 10 kDa dextran. Dextran recovery was up to $87.0 \pm 3.6\%$ (n=3) at a concentration factor (CF) equal to 20. Additionally, dextran subjected to cross-flow ultrafiltration demonstrated the same anion exchange

chromatographic signature. Therefore, extraction of EPS using cross-flow ultrafiltration was considered practical.

EPS isolation recovery using cross-flow ultrafiltration was very low (i.e., 20~30%) without post-ultrafiltration soaking, regardless of concentration factor (CF: 10~40). However, after 3 extra soakings were introduced into the procedure at the end of ultrafiltration, the isolation recovery of EPS dramatically increased resulting in cross-flow ultrafiltration followed by stirred-cell diafiltration becoming comparable to stirred-cell ultrafiltration, with a yield of 2.8 ± 0.2 (n=4) mg-glucuronic acid L⁻¹. The distribution of exopolysaccharides in terms of uronic acids in the retentate and the four washing solutions is shown in Figure 2.4. It shows that, at a CF of 40, only 15.9% of EPS remained in the retentate and 6.6% of EPS was rinsed off without soaking, while 77.5% EPS was washed out by soaking. Therefore, the loss of EPS on the membrane cannot be neglected but can be recovered by soaking.

Various concentration factors (5, 10, 20 and 40) were investigated to evaluate the effect of concentration factor, CF, on the isolation efficiency of EPS by comparing stirred-cell ultrafiltration. As it turned out, the value of the CF did not significantly impact the yield. The yields were $91.6 \pm 2.8\%$ of those using the stirred cell technique, regardless of CF.

2.4.6. Soaking interval

6-hour and 3-hour soaking times were applied to cross-flow ultrafiltration of EPS. The uronic acids distribution is shown in Figure 2.4. Even though the soaking interval was different, the distribution pattern of uronic acids was similar. A major amount was

washed out during the third rinse. It is obvious that the 3-hour soaking interval would have required more soakings, and would have resulted in longer laboratory processing times and increased distilled water use for rinsing, and thereby much more work for stirred-cell diafiltration that was subsequently used. However, the overall yield of EPS for 3-hour soaking was $2.9 \text{ mg-glucuronic acid L}^{-1}$, which was within $2.8 \pm 0.2 \text{ mg-glucuronic acid L}^{-1}$, a yield for 6-hour soaking. Because of lower water use and shorter processing times, a 6-hour soaking time was adopted.

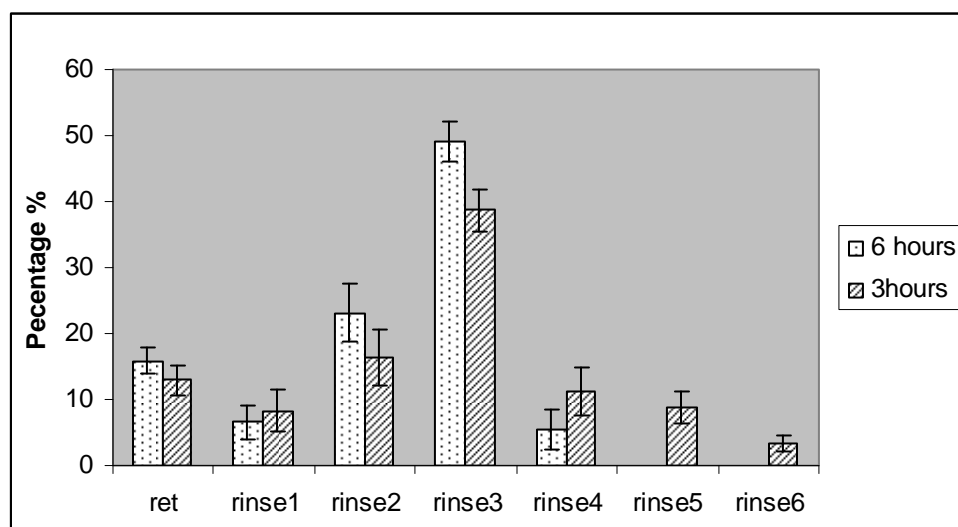


Fig. 2.4 The distribution of EPS in terms of uronic acids in the retentate and washing solutions when different soaking intervals (3- hour and 6- hour) were applied to cross-flow ultrafiltration (CF=40, n=3). Note: rinse 1 is pre-soaking rinse and rinses 2-6 are post-soaking rinse

2.4.7. Purification and fractionation of EPS from *Amphora* sp.

Anion exchange chromatography was used to purify and fractionate extracted EPS. The separation mechanism of anion exchange chromatography is based on the ionic

interactions between negatively charged solutes in the mobile phase and a charged ion-exchange group contained on the stationary phase. EPS of *Amphora sp.* harvested from the three isolation methods demonstrated similar chromatographic characteristics, as shown in Figure 2.5. In other words, the three isolation procedures appear to produce the same exopolysaccharides. Their retention times were 55 (fraction 1) and 66 minutes (fraction 2), respectively. Compared to ethanol precipitation, cross-flow ultrafiltration and stirred cell diafiltration not only were relatively artifact-free, but also effectively concentrated EPS from a large-volume culture.

In order to further validate the composition of the two fractions appearing in the anion exchange chromatography, total carbohydrates and uronic acids were individually determined in fractions, eluted from the anion exchange chromatographic column and collected every 2 minutes. The distributions of carbohydrate and uronic acids determined by their corresponding colorimetric methods were in good agreement with the anion exchange chromatogram of EPS isolates using fluorescence detection, which means that the two fractions separated by anion exchange column are both exopolysaccharides. The monosaccharides in the exopolysaccharide fraction 1 were analyzed to be glucuronic acid, fucose and galactose. As to fraction 2, glucuronic acid was the major component, followed by galactose and fucose (Zhang et al., 2008).

The protein content in the three EPS extracted by the three procedures was also determined by BCA colorimetric method to be below the detection limit of 0.02 g L^{-1} . Size exclusion chromatography was adopted to further validate the uniformity in molecular weight distribution, as well as to determine the molecular weight distribution

of the two different exopolysaccharide fractions. As shown in Figure 2.6, both fractions have a single molecular weight distribution at a retention time around 11.8 minutes, equivalent to a molecular weight of approximately 1000 kDa.

2.5. Discussion

When cross-flow ultrafiltration was used for EPS separation, however, recoveries of EPS were very low (20%~30%), regardless of concentration factor, when membranes were not soaked. Because no EPS was found in the permeate, it appeared that EPS was lost by sorption onto the cartridge due to fouling by EPS and other natural organic compounds. Therefore, a three-step cartridge soaking procedure, followed by diafiltration, was adopted to recover EPS from the cartridge, a procedure that greatly increased the efficiency of EPS isolation, resulting in a yield 2.8 ± 0.2 mg-glucuronic acid/L, comparable to that of stirred-cell ultrafiltration. The length of the individual soaking interval did not impact the overall yield of EPS isolation. 3-hour soaking can remove most of the adsorbed EPS if repeated 5 times, while 6-hour soaking only required 3 repeated soakings. Therefore, the 6-hour soaking method is believed to be the optimal procedure as EPS had to be diluted to a lesser extent. Additionally, the value of the concentration factor was not crucial for EPS isolation yield, since EPS fouling on the membrane could be washed out by extra soakings.

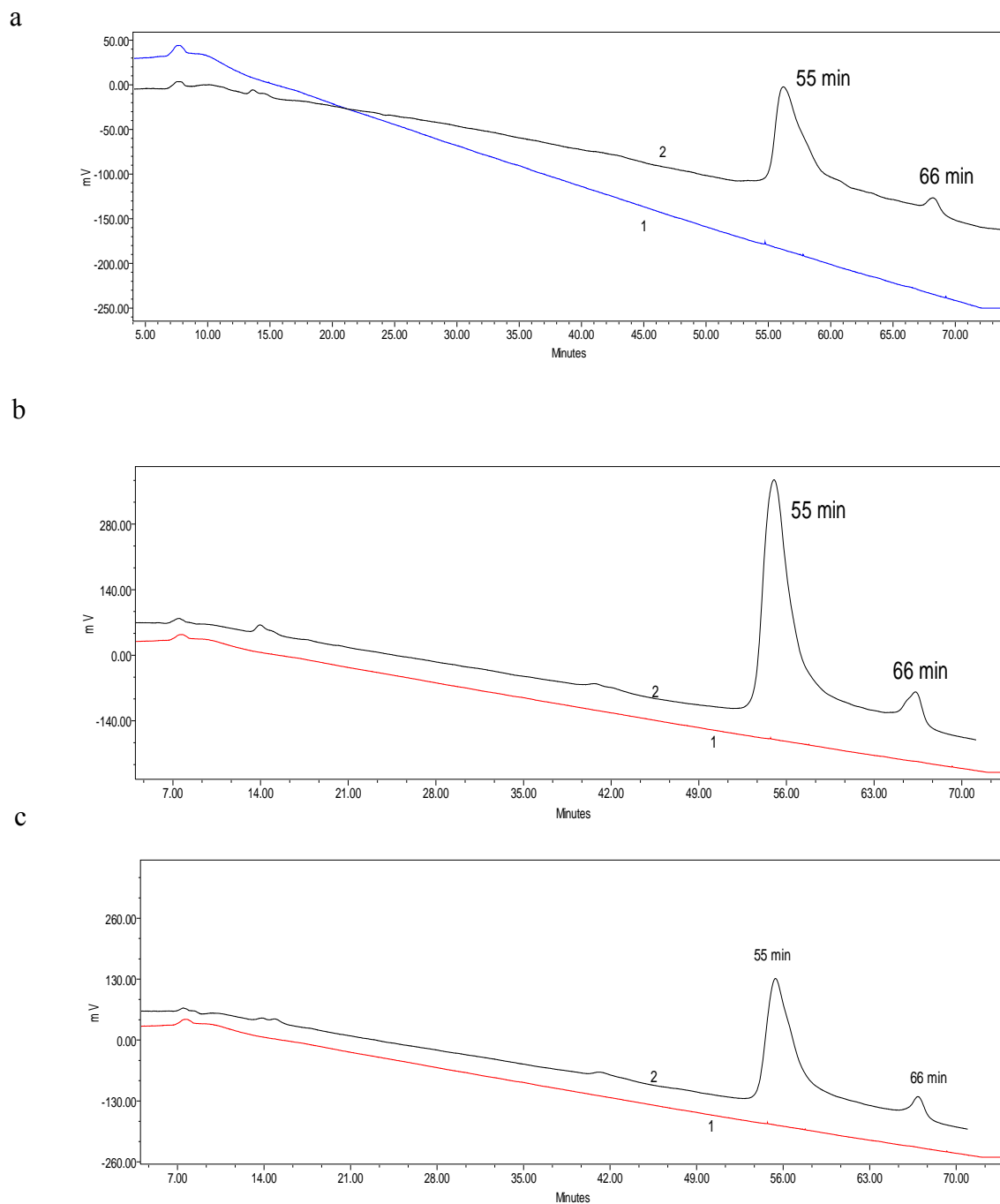


Fig. 2.5 Anion exchange chromatograms of EPS from ethanol precipitate (a), stirred-cell diafiltration (b) and cross-flow ultrafiltration (c) (1. Blank, 2. EPS from *Amphora sp.*)

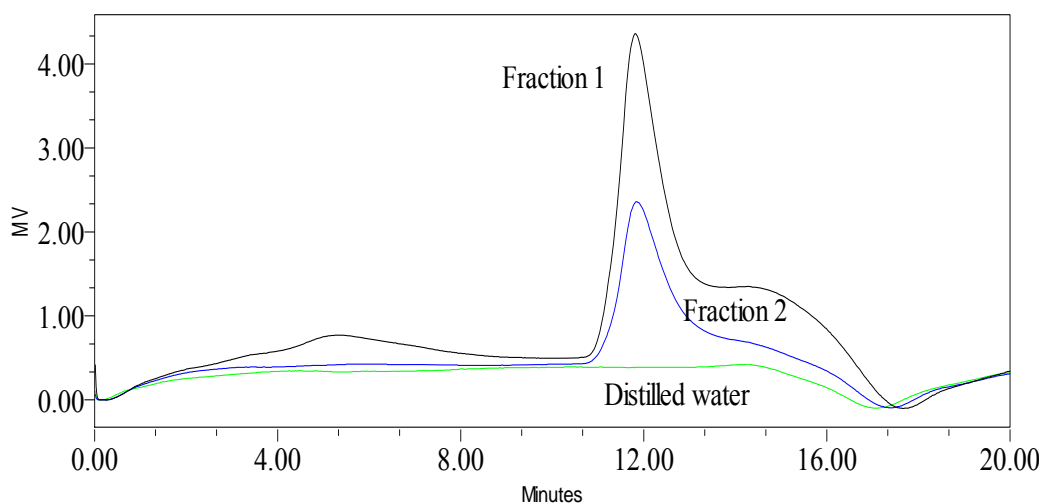


Fig. 2.6 Size exclusion chromatograms of the two exopolysaccharide fractions obtained from anion exchange chromatography of EPS from *Amphora sp.*

The fact that the recovery rate of EPS during ultrafiltration can be greatly increased by soaking indicates good reversibility (on time scales of hours to days) of the interaction of EPS with the membrane (“fouling”) under the operating conditions. Membrane fouling during cross flow ultrafiltration has been increasingly realized as a problem (Yiantsios and Karabelas, 1998; Ye et al., 2005a, 2005b; Katsoufidou et al., 2007). The fouling mechanisms of EPS are not entirely clear, but it appears that cake development on the membrane, i.e., deposition of particles larger than the membrane pore size onto the membrane surface, can explain our results. Self-assembly of DOM induced by EPS and ionic strength promote aggregation of DOM during ultrafiltration and thus fouling of EPS on the membrane. Thus, EPS could be recovered by appropriate backwashing.

The anion exchange chromatography (AEC) procedure allows the detection of

potential artifacts, and serves the purpose of quality control tool. More importantly, it also allows the characterization of individual biopolymers rather than that of a mixture, which is common for some of the other methods. In this study, anion exchange chromatography was used to purify and fractionate crude EPS harvested from the three techniques. EPS from *Sagittula stellata* have been reported by Ding et al. (2008) to have marked fluorescence properties at a given wavelength (e.g. excitation $\lambda=285$ nm, emission $\lambda=580$ nm), a property that was used to monitor self-assembly of DOM polymers and the formation of marine microgels. Here, we further confirmed the application of this fluorescence characteristic of the EPS by measuring the concentration of total carbohydrates and uronic acids in chromatographic fractions collected every two minutes. The two fractions determined by fluorescence detection were uronic acid-containing carbohydrates.

Additionally, the similar characteristics on anion exchange chromatography of EPSs isolated from the three techniques indicated that no detectable artifacts and little loss of exopolysaccharides were caused by cross-flow ultrafiltration followed by stirred-cell diafiltration, which allowed the application of cross-flow ultrafiltration for large-scale isolation of EPS. Two extracellular polysaccharides (fraction 1 and fraction 2) separated by anion exchange chromatography had similar molecular weights of 1000 kDa, but different monosaccharide composition. Fraction 1 was mainly composed of glucuronic acid, fucose, and galactose, while glucuronic acid was the major component of fraction 2, which had much less galactose and fucose. In other words, the second fraction held more acidic functional groups (-COOH) than the first fraction, which is consistent with the

chromatography since more negatively charged compounds would more strongly interact with the charged ion-exchange group contained on the stationary phase.

Based on the results of these experiments, the optimal method for the isolation of EPS from phytoplankton cultures or natural waters, based on time, cost, and yield, is cross flow ultrafiltration, followed by stirred-cell diafiltration with additional cartridge washing. The new procedure thus allows one, with only 10 liters of media, to obtain sufficient amounts of EPS material (10-30 mg, preferably 50mg) for a full chemical characterization at the molecular level (i.e., NMR, GC-MS, HPLC, ATR-FTIR, etc).

2.6. Conclusions

Through controlled experiments, three techniques, i.e., 1) ethanol precipitation, 2) stirred-cell ultrafiltration, or 3) cross-flow ultrafiltration, were compared for their effectiveness to quantitatively isolate exopolymeric substances (EPS) from the marine diatom *Amphora sp.* Both ultrafiltration techniques were followed by stirred-cell diafiltration.

It was shown here that ethanol precipitation techniques, when applied to diatom cultures, are not only impractical, but also lead to loss of EPS, as addition of ethanol dilutes the medium too much. It was also shown that only through a combination of cross-flow ultrafiltration and stirred-cell ultrafiltration, followed by overnight soaking, EPS can be nearly quantitatively recovered. Finally, it was shown that through the application of anion exchange chromatography one can obtain individual EPS compounds for further chemical characterization. Ethanol precipitation was not effective

due to ethanol consuming and significant formation of co-precipitates (Ca and Mg carbonates), which diluted the EPS compounds of interest. The effect of the seawater medium on the retention of EPS during stirred-cell ultrafiltration resulted in the loss of EPS onto the membrane and a lower recovery, i.e., 85% using a seawater medium and 95% using nanopure water. Backwashing was introduced to recover EPS from the membrane at the end of diafiltration, resulting in an improved recovery. Thus, this procedure was optimal for harvesting EPS from *Amphora sp.* in terms of recovery of EPS isolation. However, it would be impractical to ultrafilter 5 liters or more culture solution or natural water using only this small-volume stirred-cell ultrafiltration method. Therefore, cross-flow ultrafiltration was applied as an initial separation technique from large volumes of water.

CHAPTER III
CHEMICAL COMPOSITION AND ²³⁴Th (IV) BINDING OF
EXTRACELLULAR POLYMERIC SUBSTANCES (EPS) PRODUCED BY THE
MARINE DIATOM *AMPHORA SP.**

3.1. Overview

In order to chemically characterize strongly Th (IV)-binding exopolymeric substances (EPS) from a single organism, “particulate” (i.e., p, attached) and “dissolved” (i.e., d, nonattached) EPS from a lab culture of the marine diatom *Amphora sp.* were isolated by centrifugation, followed by alcohol precipitation or ultrafiltration. Both dissolved EPS and particulate EPS were mainly composed of carbohydrates, with only a small fraction of proteins. Individual fractions that were further separated by anion exchange chromatography (AEC) contained EPS with significantly different compositional characteristics and molecular weights. The particulate fraction was composed of two different glucans, p1 and p2, but with the same molecular weight of 25 kDa, while more than 90% of the dissolved fraction was composed of two different acidic polysaccharides, f1 and f2, with a similar molecular weight of 1000 kDa. While both f1 and f2 fractions contained the neutral monosaccharides fucose and galactose, as well as glucuronic acid, they were in a different mole ratio: f1 in a ratio of 1:1.1:1.6, and f2 as 1:0.8:2.8. In addition to glucuronic acid, both f1 and f2 fractions contained relatively high

*Reprint with the permission from “Chemical composition and ²³⁴Th(IV) binding of extracellular polymeric substances (EPS) produced by the marine diatom *Amphora sp.*” by Saijin Zhang, Chen Xu, Peter H. Santschi, 2008. *Marine Chemistry*, 112, 81-92, Copyright [2008] by Elsevier. Doi: 10.1016/j.marchem.2008.05.009.

concentrations of sulfated polysaccharides, with a sulfate content of 9.7% in f1, and 18.2% in f2. The difference between total acid polysaccharide concentrations, as determined by the Alcian blue method, and the actual concentrations of EPS can be explained by the relative amounts of sulfate and glucuronic acid. Dissolved EPS, and fractions f1 and f2 that were labeled with ^{234}Th (IV), all showed peaks at isoelectric points (pH_{IEP}) of about pH 3 during isoelectric focusing, indicating that Th (IV) binding by EPS was dominated by the acidic polysaccharides in f1 and f2. The strong binding of ^{234}Th (IV) to these acidic polysaccharide-rich EPS compounds enables us to locate and closely look at the agents who are responsible for binding of ^{234}Th (IV), which is relevant for a better understanding of the oceanographic applications of $\text{POC}/^{234}\text{Th}$ ratios to particle and organic carbon dynamics in marine systems.

3.2. Introduction

Organic substances, such as exopolymeric substances (EPS), are released from phytoplankton cells during all phases of growth, with extracellular polysaccharides comprising up to 80–90% of the total extracellular release (Myklestad, 1974). EPS are well known for their roles in formation of transparent exopolymeric particles (TEP) (Leppard, 1995, 1997; Passow, 2002), marine gels (Verdugo et al., 2004), and biofilms (Decho, 2000), which are important in regulating sedimentation processes, biogeochemical cycling and particle dynamics in the oceans by bypassing the microbial loop. As micro- and nano-particles, TEP provide a microhabitat for bacteria by forming relatively stable microzones in aggregates and impacting all concentration-dependent

processes, like nutrient uptake and grazing rates. Many studies have found elevated bacterial productivity within aggregates compared to the surrounding seawater (Herndl, 1988; Grossart and Simon, 1993, 1998). Observational evidence from Logan et al. (1995) confirmed that the formation of aggregates at the decline of diatom blooms is frequently controlled by the chemical composition of EPS. The studies of Passow and Alldredge (1994) provide a detailed description of the aggregation dynamics during diatom blooms. Aggregation becomes the dominant process in particle dynamics when particle concentrations reach a critical concentration (Jackson, 1990). In addition, EPS also take part in the scavenging of stable and radioactive trace elements, such as Th (IV), due to their chelating and surface-active properties.

^{234}Th (IV) is a naturally occurring highly particle-reactive radionuclide produced from alpha decay of Uranium-238. It is used as a proxy in oceanographic investigations of particle dynamics and the determination of particulate organic matter fluxes, which are, at steady state, equal to “new production”, through the use of $^{234}\text{Th}/\text{POC}$ ratios (Buesseler et al., 2006). $^{234}\text{Th}/\text{POC}$ ratios might be controlled, in part, by the abundance of exopolymeric substances (EPS) because of their chelating and surface activity and the affinity of Th (IV) to particles. As summarized in Santschi et al. (2006), both experimental (Quigley et al., 2002) and field results (Guo et al., 2002; Santschi et al., 2003; Passow et al., 2006) demonstrate that Th (IV) in the ocean is strongly associated with acidic polysaccharide (APS)-rich compounds. Different acidic functional groups, like carboxylate, phosphate and sulfate, have been implicated to contribute to the binding of Th (IV) to different EPS compounds (Alvarado-Quiroz et al., 2006).

In order to better understand the roles of EPS in particle dynamics of aquatic systems, it is important to characterize, at the molecular level, EPS that have been harvested and purified from experimental systems. The purpose of this study is therefore to characterize EPS from a common marine benthic diatom, *Amphora sp.*, which as characterized in the literature to release large amounts of EPS. Part of the limitation of this research was to find a diatom that could be cultured to obtain sufficient amounts of EPS to carry out a full chemical characterization of its composition. After an initial screening of different species, *Amphora sp.* was selected to be suitable for such an investigation, in order to relate its composition to its binding properties to ^{234}Th (IV). *Amphora* is a common genera found in coastal communities (Welker et al., 2002; Kasim and Mukai, 2006; Facca and Sfriso, 2007) and can be found in both benthic and pelagic environments. Benthic microalgal communities are important primary producers in wide variety of shallow coastal areas (Cadee and Hegeman, 1974; Colijn and De Jonge, 1984; Cahoon and Cooke, 1992; Barranguet, 1997; Nelson et al., 1999). Up to 40% of the photosynthetically fixed carbon is lost by pelagic diatoms as an extracellular release (Fogg, 1983; Smith and Underwood, 1998; Taylor and Paterson, 1998). Furthermore, they release EPS in proportion to their biomass. For example, a positive correlation between sediment colloidal carbohydrate concentrations (as a proxy of EPS) and benthic diatom biomass has been found in many studies. (Underwood and Paterson, 1993; Fabiano and Danovaro, 1994; Underwood et al., 1995; Welker et al., 2002; Underwood and Paterson, 2003). Even though the behavior of benthic diatoms do not represent that of pelagic diatoms, it is likely that the mechanism of binding of their EPS with ^{234}Th can

be extrapolated to oceanic species as physico-chemical properties of EPS are considered to be the major factors in regulating their binding with ^{234}Th (IV). Thus, we expect that this study will shed light on the binding of ^{234}Th with EPS and its relationship to the molecular level composition of EPS, regardless of the exact source of EPS.

3.3. Materials and methods

3.3.1. Isolation of EPS from Amphora sp.

Bacillariophyceae *Amphora sp.* (CCMP1389) was bought from CCMP (National Culture Collection of Marine Phytoplankton). This diatom species was originally collected from Flax Pond, near the Stony Brook Marine Lab, Long Island, New York. In our experiments, it was cultured in a f/2-Si medium (CCMP medium recipe) with a salinity of 30 ppt at a temperature of 21 °C (Incubator 818, Precision) with one light cycle per day (e.g. 12 h/12 h). The status of *Amphora sp.* was monitored by measuring Chlorophyll a (Yentsch, 1963). After 12 days of incubation when *Amphora sp.* reached the stationary phase, the culture solution was centrifuged at 2694 g for 30 min for subsequent experiments. The culture was then separated into two fractions: pellet (cell) and supernatant. EPS isolated from the pellet was called “particulate EPS” (attached EPS). The isolation procedure for processing the pellet, as described below, is based on Kushner et al. (1992) and Hung et al. (2005). 100 mL of nanopure water was first added to the pellet. The pellet then was extracted overnight by stirring, allowing for the equilibrium to be reached between the particle surface and the water. The residue was eliminated by centrifugation and the EPS released into the supernatant was precipitated

by adding 3 volumes of alcohol (95% ethanol and 5% of methanol). The solution was allowed to stand overnight in the refrigerator. The precipitate was collected by centrifugation, followed by re-dissolution in 100 mL of nanopure water. This ethanol precipitation was repeated twice. The final solution containing particulate EPS was used for further purification and characterization.

The procedure for isolation of dissolved EPS from the supernatant of the culture was based on a novel procedure, described in detail by Zhang and Santschi (2009). It included cross-flow ultrafiltration followed by stirred-cell diafiltration with additional cartridge washing. In short, 8 L of the supernatant was pre-filtered through GF/B, GF/F and 0.45 μm poly-carbonate membranes (in series) before feeding it to a crossflow ultrafiltration system that used a spiral wound 1 kDa SOC 1812 cartridge (Separation Engineering, Inc). The supernatant was ultrafiltered at a pressure of 30 psi and at a power efficiency of 50-60% until 200–300mL of retentate remained. Then, the cartridge was rinsed with 200 mL of nanopure water, the pump was turned off, and the cartridge was soaked in nanopure water for 6 h. After soaking, the cartridge was rinsed with another 200 mL of water, and the soaking procedure was repeated twice. Subsequently, the retentate solution and four rinse solutions were combined. The resulting 1 L of solution was then further diafiltered by using an Amicon 8200 stirred-cell ultrafiltration system with a 5 kDa polyethersulfonemembrane at a working pressure of 45–50 psi. At the end, 25~30 mL of retentate remained and was stirred for 30 min. After decanting the retentate, another 25mL of water was used to wash the membrane by stirring for 1 h. The wash

water was then combined with the retentate. The final solution containing dissolved EPS was used for further purification and characterization.

3.3.2. Colorimetric determination of carbohydrate, uronic acid and protein concentrations

Carbohydrate concentrations were estimated by using the anthrone method (Morris, 1948), with glucose as a standard. Uronic acids were determined by using the method of Blumenkrantz and Asboe-Hansen (1973), with glucuronic acid as a standard. The protein content in EPS was measured by using bicinchoninic acid (BCA), and was based on Smith et al., (1985) and Stoscheck (1990), with BSA (Bovine Serum Albumin) as a standard.

3.3.3. Purification and fractionation of EPS

Extracted EPS obtained from previous step (2.1) was further purified and fractionated by using an anion exchange (AEC) — preparative liquid chromatography system, with a BioSuite Q13 μ m AXC (21.5 \times 150 mm) column. The injection volume was 5 mL. The sample was eluted at a constant flow rate of 5 mL/min with a gradient program from 0 to 1 M NaCl in 20 mM Tris-HCl buffer (pH= 8.1, Sigma-Aldrich) in 70 min. EPS from *Amphora sp.* was detected using a fluorescent detector at an excitation wavelength of $\lambda=285$ nm, and emission of $\lambda=580$ nm (Ding et al., 2008).

3.3.4. Estimation of EPS molecular weight

Size exclusion chromatography (SEC) was used to measure the molecular weight of EPS. The column used was a Tosoh TSK G-4000PWxl (300 \times 7.8 mm). The mobile phase was 0.078 M NaNO₃ in 10 mmol/L phosphate buffer (pH= 6.8) at a flow rate of 0.5

mL/min. 150 μ L of sample was injected and detected by fluorescence detection (excitation $\lambda=285$ nm, emission $\lambda=580$ nm). Polystyrene standards with a molecular weight of 8 kDa, 35 kDa, 100 kDa, and 780 kDa were used for the calibration curve, whereby the logarithm of molecular weight was plotted against corresponding retention time.

3.3.5. Determination of neutral sugars using GC-EI-MS

Gas chromatography-flame ionization detection (FID) was used for analyzing neutral sugars. However, mass spectra were used instead of FID detection, in order to increase the accuracy of determination. The procedure was based on Hung et al., (2001, 2005), with some slight modifications. 100 μ L of purified EPS solution (0.1 mg/L) was added to a hydrolysis tube containing 2 mL of 0.1 mol/L hydrochloric acid (HCl), and was then hydrolyzed at 150 $^{\circ}$ C for 1 h. After hydrolysis, water and HCl in the sample were removed by a stream of nitrogen. Then, 0.05 mol/L NaOH and 20 nano moles of myo-inositol (as an internal standard) were added to the dry sample. 10 mg of sodium borohydride (NaBH_4) was used to reduce neutral sugars at 60 $^{\circ}$ C for 1 h. Acetic acid was added drop by drop to remove excess NaBH_4 until there was no bubble produced. The reduced solution was dried by a stream of nitrogen. 1 mL of methanol was used to remove borohydrate produced in the previous process, after which the sample was dried using a nitrogen stream. This procedure was then repeated three times. The final residue was dried at 100 $^{\circ}$ C for 15 min. After that, 0.5 mL of pyridine and 0.5 mL of acetic anhydride were added to the residue and reacted at 100 $^{\circ}$ C for 1 h. After cooling, 1 mL of distilled water was added to the mixture and the mixture was shaken for a short while.

1 mL of dichloromethane was then added to extract the mixture, and this extraction procedure was repeated once more. The two dichloromethane layers were combined and dried under a nitrogen stream. The final residue was then dissolved by 100 μ L of dichloromethane, and 1 μ L of the solution was injected into a GC-MS (Polaris Q GC/MS) at a carrier rate of 1.0 mL/min. The column used here was a DB 1701 (30 m \times 0.25 mm ID). The temperature program was set as follows: the initial temperature was set at 120 $^{\circ}$ C for 1.0 min, and then the temperature was increased to 180 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min and held for another 1.0 min. The column was heated to 190 $^{\circ}$ C at a rate of 0.3 $^{\circ}$ C/min and held for 5 min. In the final cleanup step, the column was heated to 260 $^{\circ}$ C at a rate of 20 $^{\circ}$ C/min for another 2 min. To obtain a mass spectrum, a full scan mode (50–650) was chosen, and the temperature of the ion source was set at 280 $^{\circ}$ C. Neutral monosaccharides in EPS were identified according to their corresponding retention times and mass spectra, which were determined using monosaccharide standards. Quantitative determination was carried out using their corresponding calibration curves.

3.3.6. Determination of neutral sugars and uronic acids using High Performance Liquid Chromatography (HPLC)

Due to the time-consuming treatment for neutral sugars and uronic acids analysis using gas chromatography (Jones and Albersheim, 1972), an HPLC procedure has been developed to measure the composition of polysaccharides including neutral monosaccharides and uronic acids (Whitfield et al., 1991; Gremm, 1997). Purified EPS had to be first hydrolyzed using the same hydrolysis procedure as described in Section

(2.5). A dried hydrolyzed residue was added by 25 μL of deoxy-ribose (400 $\mu\text{mol/L}$, as an internal standard) and 975 μL of distilled water. Two 100 μL of the resulting mixture were injected each for the determination of neutral monosaccharides and uronic acids, respectively, using different chromatographic elution conditions. A CarboPac PA10-4 mm column (4 \times 250 mm), an electrochemical detector with a gold working electrode, and an ISAAC reference electrode were employed. The eluent used for analyzing neutral monosaccharides was 3 mM NaOH (made from 50% W/W NaOH solution), while two mobile phases were used for the determination of uronic acids at a ratio 40:60 (A: 15 mM NaOH, B: 100 mM NaOH with 250 mM NaAc). Both flow rates were set at 1.0 mL/min. The detector setting was: E1=0 V, E2=0.65 V, E3=-0.65 V, t1=400 ms, t2=200 ms, t3=200 ms, ts=100 ms.

For this work, we compared the results from GC-MS with those from HPLC in order to better apply HPLC for our future characterization of polysaccharides. Some parameters used to better judge an analytical method, like reproducibility, recovery, and detection limit, were measured to evaluate the optimal efficiency of the method.

3.3.7. Determination of sulfate in EPS using ion chromatography

A given amount of dissolved EPS was hydrolyzed in 1 mol/L of HCl for 6 h and dried under a nitrogen stream (Dodgson and Price, 1962). The residue was re-dissolved by 1 mL of distilled water. After being filtered through a 0.22 μm syringe filter, 20 μL of the final solution was injected into ion chromatography and eluted, at a flow rate of 1.5 mL/min, by a mixture of 1.8 mmol/L sodiumcarbonate and 1.7 mmol/L sodiumbicarbonate, and then quantified by a conductivity detector from Dionex. An

IonPac AS4A-SC column (4×250mm), with an IonPac AG4A-SC guard column (4×50 mm), was used in this study.

3.3.8. Total acid polysaccharide concentration determination using Alcian blue

Alcian blue is widely used as a stain to estimate the total concentration of acid polysaccharides (APS) and transparent exopolymeric particles (TEP) in particulate samples (Alldredge et al., 1993; Passow and Alldredge, 1995; Hung et al., 2003a, 2003b; Santschi et al., 2003). This method has recently been extended to the measurement of APS in filter-passing water samples (Thornton et al., 2007). Our knowledge about the chemical composition of EPS from *Amphora sp.* provides the opportunity to compare molecular-level compositional results with those obtained from the Alcian blue staining method of Thornton et al. (2007). Briefly, 1 mL of Alcian blue was added to 5 mL of sample with a given concentration of APS, after pH was adjusted to 2.5 using glacial acetic acid. The sample was then vigorously mixed and filtered through a syringe filter containing a surfactant free cellulose acetate (SFCA) membrane with a pore size of 0.2 μm (Nalgene). The first 5mL was discarded and the final 1 mL was used for absorbance measurement in order to minimize the impact of the filter. Both gum xanthan and λ -carrageenan were used for calibration, respectively. Data are presented as gum xanthan equivalents or λ -carrageenan equivalents (mg X eq. /L or mg λ -carrageenan eq. /L).

3.3.9. ^{234}Th (IV) binding to EPS

Purified ^{234}Th (IV), extracted from a ^{238}U solution (Quigley et al., 2002; Alvarado-Quiroz et al., 2006) was added to the EPS solution. The mixture was neutralized by NaOH and allowed to equilibrate for 30 min. After removing the unbound ^{234}Th (IV) by

diafiltration using a 1 kDa membrane, EPS spiked by ^{234}Th (IV) was subjected to isoelectric focusing (IEF) separation in order to determine the pH_{IEF} of ^{234}Th (IV) binding ligands. For a detailed procedure, the reader is referred to Alvarado-Quiroz et al. (2006). In brief, Th-labeled EPS was loaded onto an IPG strip (GE healthcare immobiline™ Drystrip, pH 3-10, 11 cm), in addition to a 140 μL of rehydration solution (mixture of urea, carrier ampholytes and detergent Triton X). The loaded strip was then allowed to re-swell overnight. Subsequently, the strip was then placed in an electrophoresis apparatus (Amersham Biosciences, Multiphor II Electrophoresis System) and ran for 17.5 h. After IEF, the pH gradient of the strip was re-calibrated every 1 cm. The strip was then cut into 11 pieces, and each piece of 1 cm in length was extracted by 1% SDS overnight. After extraction, ^{234}Th (IV) activity of each fraction was analyzed by liquid scintillation counting (Beckman Model 8100 Liquid Scintillation Counter).

3.4. Results

3.4.1. Determination of standards by HPLC

3.4.1.1. Neutral sugars

Figure 3.1 shows HPLC chromatogram of seven neutral sugars. Their corresponding detection limit, reproducibility and recovery are listed in Table 3.1. In this study, 3 mmol/L of NaOH was used as mobile phase, instead of 20 mmol/L of NaOH (Bhaskar et al., 2005; Grossart et al., 2006), in order to separate xylose and mannose.

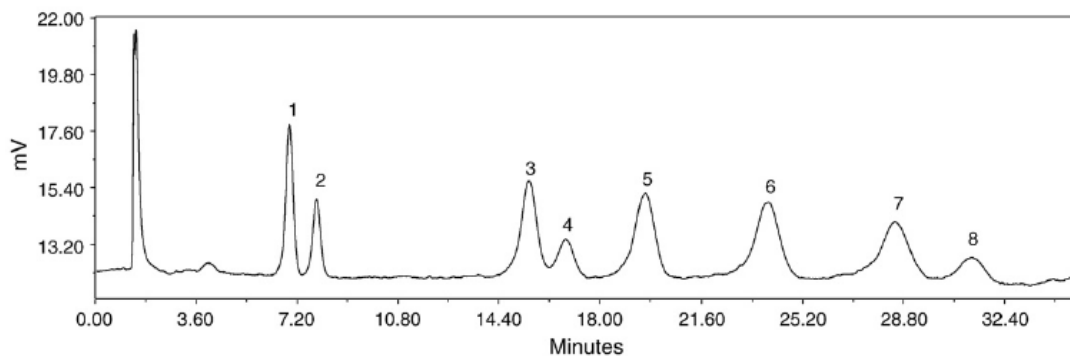


Fig. 3.1 HPLC chromatogram of seven neutral sugars. 1. L-fucose, 2. 2-deoxy-D-ribose (internal standard), 3. L-rhamnose, 4. D-arabinose, 5. D-galactose, 6. D-glucose, 7. D-xylose, 8. D-mannose

Table 3.1 Detection limit and recovery of neutral sugars

| Sugars | Detection limit (n mol) | Reproducibility (RSD, %, Conc.=4 μ mol/l, n=6) | Recovery % (Conc.=30 μ mol/l) |
|-----------|-------------------------|--|-----------------------------------|
| Fucose | 0.013 | 1.4 | 75.6 |
| Rhaminose | 0.035 | 2.4 | 77.3 |
| Arabinose | 0.064 | 1.9 | 73.9 |
| Galactose | 0.044 | 3.2 | 79.3 |
| Glucose | 0.053 | 3.3 | 82.1 |
| Xylose | 0.064 | 2.8 | 73.9 |
| Mannose | 0.194 | 4.9 | 70.7 |

Note: The relative standard deviation (RSD) is the standard deviation divided by the mean.

3.4.1.2. Uronic acids

Figure 3.2 shows HPLC chromatogram of two uronic acids. Their corresponding detection limit, reproducibility and recovery are also listed in Table 3.2. Only two

individual uronic acids, glucuronic acid and galacturonic acid, were analyzed in this study. Even though there is no mannuronic acid standard available on the market, our previous analyses did demonstrate the method has the ability to separate the three uronic acids.

Table 3.2 Detection limit and recovery of uronic acids

| Uronic Acid | Detection limit (n mol) | Reproducibility (RSD, %, Conc.=5 $\mu\text{mol/l}$, n=5) | Recovery % (Conc.=5 $\mu\text{mol/l}$) |
|-------------------|-------------------------|---|---|
| Galacturonic acid | 0.086 | 12.9 | 87.2 |
| Glucuronic acid | 0.052 | 7.1 | 72.4 |

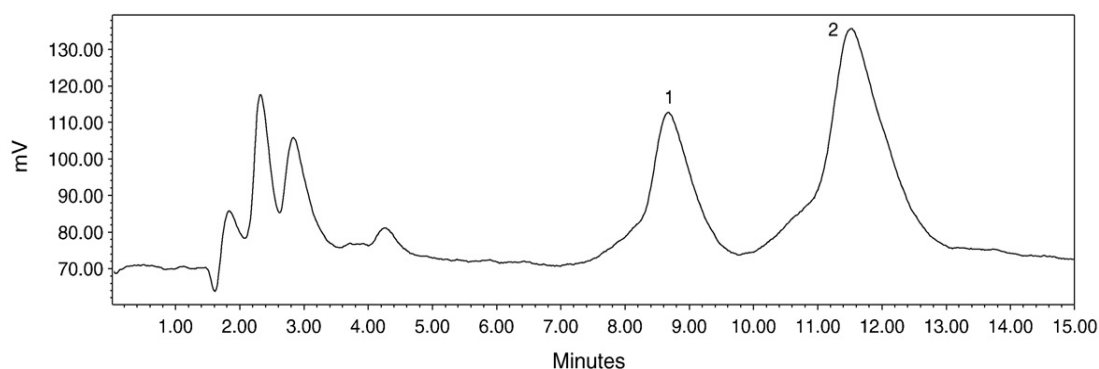


Fig. 3.2 HPLC chromatogram of uronic acids. 1. D-galacturonic acid, 2. D-glucuronic acid (peaks before 4 min are neutral sugars)

3.4.2. Characterization of EPS from *Amphora* sp.

The yields for dissolved EPS are 2.1 ± 0.3 mg-glucose equivalents/L and 2.8 ± 0.2 mg-glucuronic acid equivalents/L. *Amphora* sp. also produces particulate EPS with a yield of 0.5 ± 0.15 mg-glucose equivalents/L. Neither uronic acids nor sulfate are found in

particulate EPS. The chemical composition of different fractions in the EPS of *Amphora sp.* is shown in Table 3.3. Obviously, uronic acids are the main components in dissolved EPS. Sulfated polysaccharides are another important component in the dissolved EPS, with sulfate accounting to 11.5% of dry weight. However, in particulate EPS, carbohydrates are only a small portion of the total dry weight, which is mainly made up of SiO₂. The relatively low carbon content in particulate EPS by elemental analysis confirmed that result. A significant difference in the C/N ratio between dissolved EPS and particulate EPS, with 32 for the former and 7 for the latter, could account for their different roles during the cell growth.

Additionally, proteins in both particulate EPS and dissolved EPS are below the detection limit (20 mg BSA equivalents/L), which means that proteins account for less than 2.1% in particulate EPS and less than 12.6% in dissolved EPS. The result that extracellular polymeric substances from *Amphora sp.* are dominated by carbohydrates followed by a very minor fraction of proteins is consistent with other studies on EPS from diatoms (Hoagland et al., 1993; Sdrigotti et al., 1994; Staats et al., 1999).

Table 3.3 Chemical composition and elemental analysis of EPS from *Amphora sp.*

| EPS | Carbohydrates % | Uronic acids % | Proteins* % | Sulfate % | Carbon % | Nitrogen % | Hydrogen % |
|-------------|--------------------|-------------------|----------------|--------------|-------------|---------------|---------------|
| Dissolved | 31.5±1.5 | 47.5±2.5 | <12.6±2.1 | 11.5±0.2 | 37.7 | 1.37 | 6.27 |
| Particulate | 4.0±1.4 | 0 | <2.1±0.2 | 0 | 5.17 | 0.83 | 2.85 |

*) Protein percentages were calculated according to the detection limit 20 mg BSA eq./L.

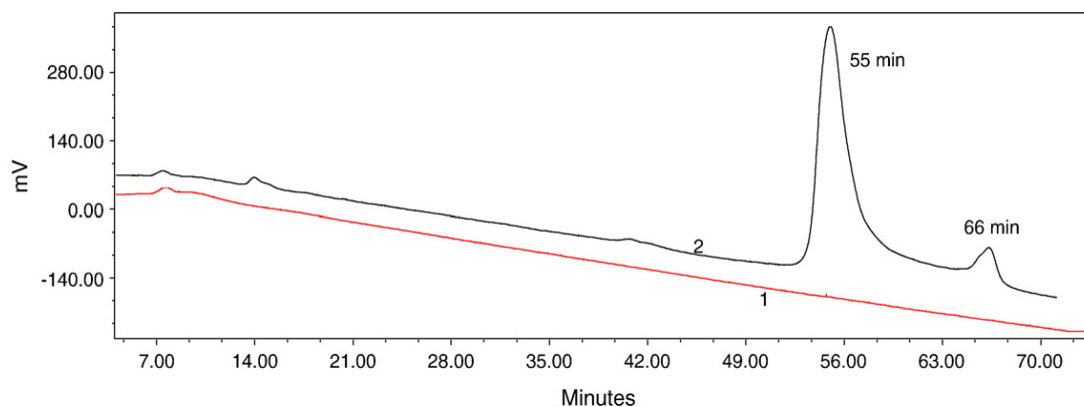


Fig. 3.3 Anion exchange chromatogram of dissolved EPS from *Amphora sp.* using fluorescence detection (Fluorescence Detection: excitation $\lambda=285$ nm, emission $\lambda=580$ nm. 1. Blank, 2. Dissolved EPS, the fraction at the retention time of 55 min is called f1 and the one at the retention time of 66 min is called f2) (Zhang and Santschi, 2009)

Two fractions were distinguished by anion exchange chromatography from dissolved EPS of *Amphora sp.* and were called f1 and f2 (Figure 3.3, Zhang and Santschi, 2009). The molecular weight of the two fractions, f1 and f2, had a similar molecular weight of 1000 kDa, but contained a different sulfate content, i.e., $9.7\pm 0.5\%$ for f1 and $18.2\pm 0.9\%$ for f2. Two polysaccharides (p1 and p2) were separated from particulate EPS by anion exchange chromatography (Figure 3.4), and qualitatively verified by the anthrone method of carbohydrate (Figure 3.5), had a similar molecular weight of 25 kDa (Figure 3.6). The HPLC chromatograms of the four polysaccharidic fractions, analyzed by HPLC, are shown in Figures 3.7–3.10. The two polysaccharides from dissolved EPS of *Amphora sp.*, f1 and f2, had a similar composition, primarily composed of fucose, galactose and

glucuronic acid. However, the mole ratio of the three components for f1 was 1:1.1:1.6, while it was 1:0.8:2.8 for the polysaccharide f2. The determination of neutral sugars from the polysaccharides f1 and f2 was further confirmed by the application of GC-EI-MS. The results of GC-EI-MS were shown in Figures 3.11–3.13 and the mole ratio of fucose to galactose was 1:1.2 for the polysaccharide f1 and 1:0.7 for the polysaccharide f2, perfectly matching the results from HPLC.

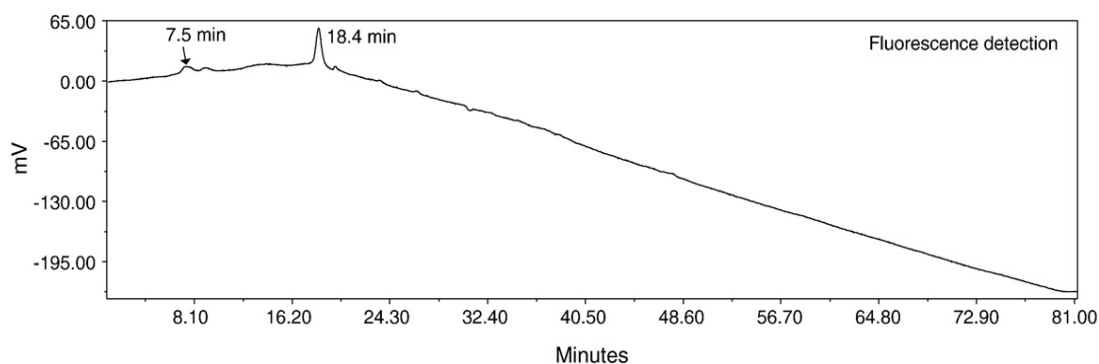


Fig. 3.4 Anion exchange chromatogram of particulate EPS from *Amphora sp.*

(Fluorescence detection: excitation $\lambda=285$ nm, emission $\lambda=580$ nm. The fraction at the retention time of 7.5 min is called p1 and the one at the retention time of 18.4 min is called p2)

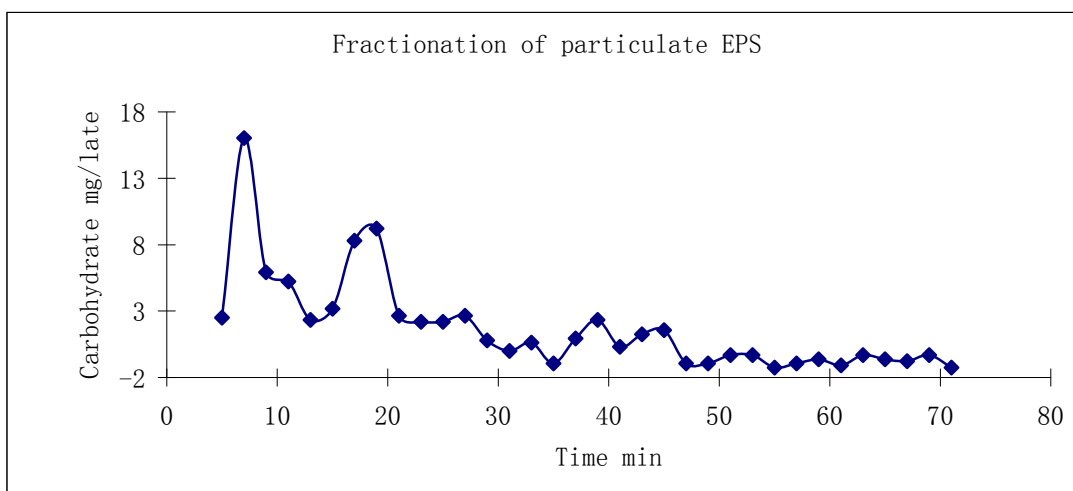


Fig. 3.5 Concentration of carbohydrate in each chromatographic fraction (time) of particulate EPS

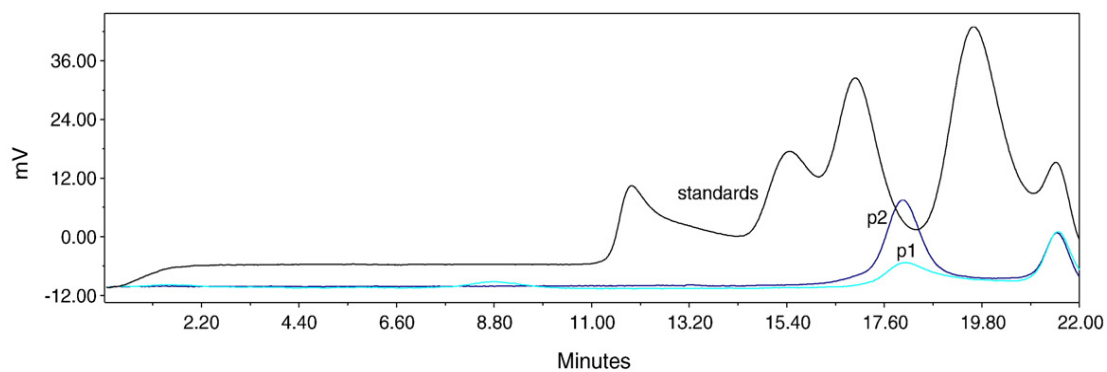


Fig. 3.6 Distribution of molecular weight of the two fractions, p1 and p2, of particulate EPS (peaks at 21~22 min were caused by non-ionic interactions)

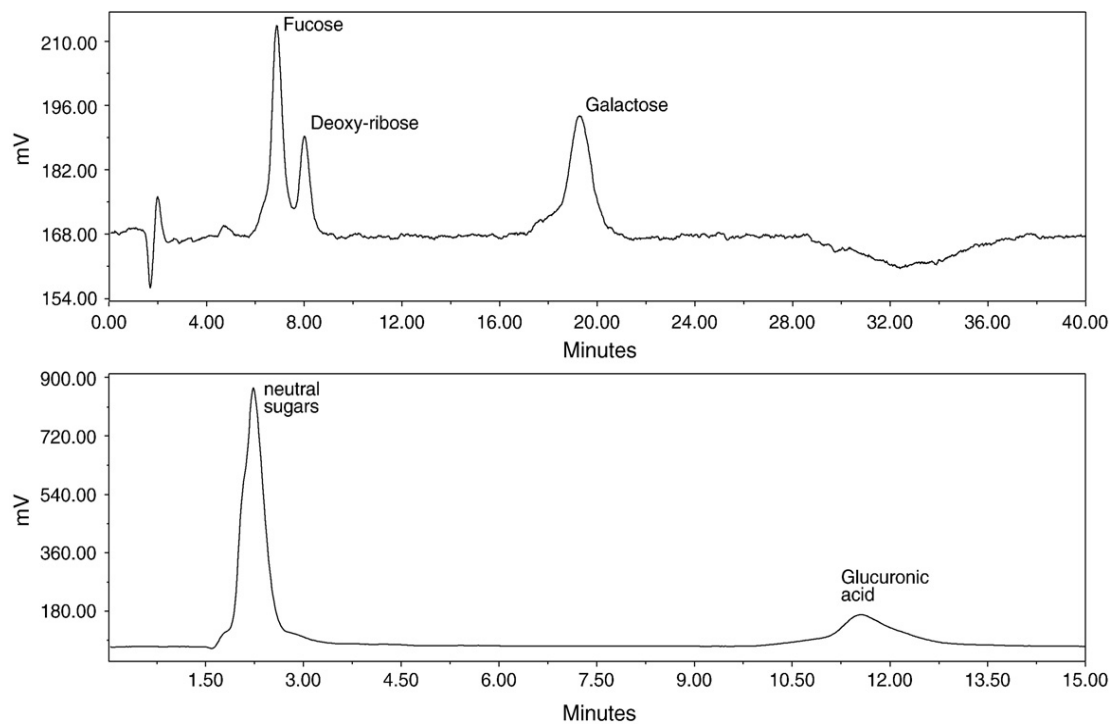


Fig. 3.7 HPLC chromatograms of polysaccharide fl

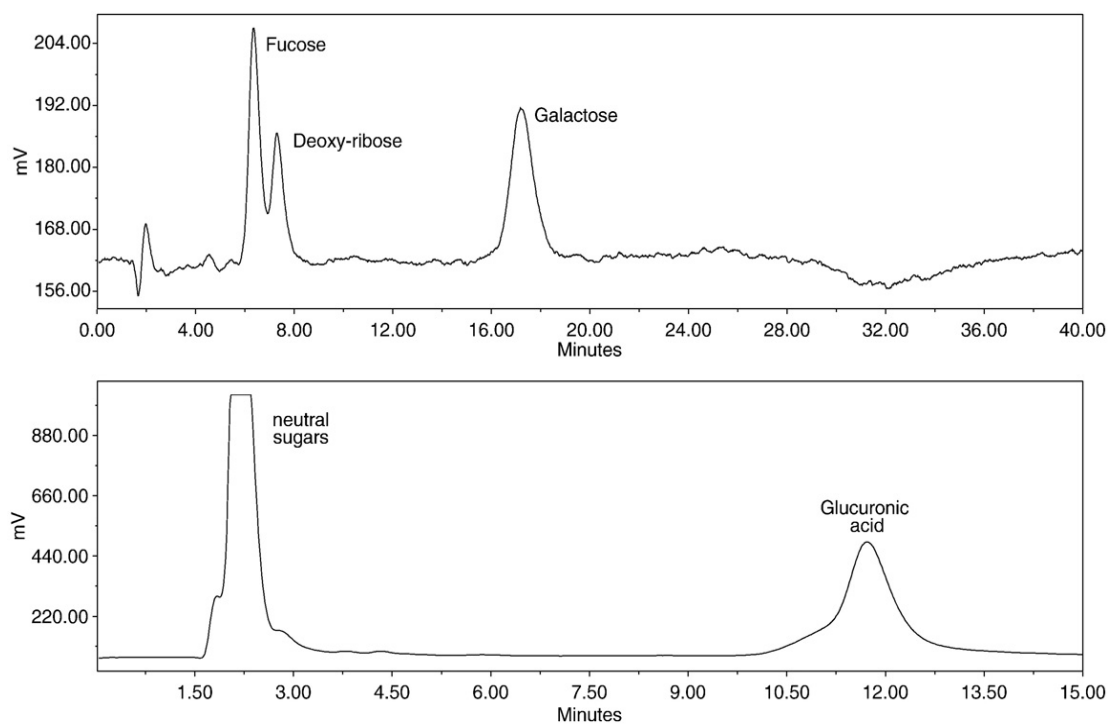


Fig. 3.8 HPLC chromatograms of polysaccharide f2

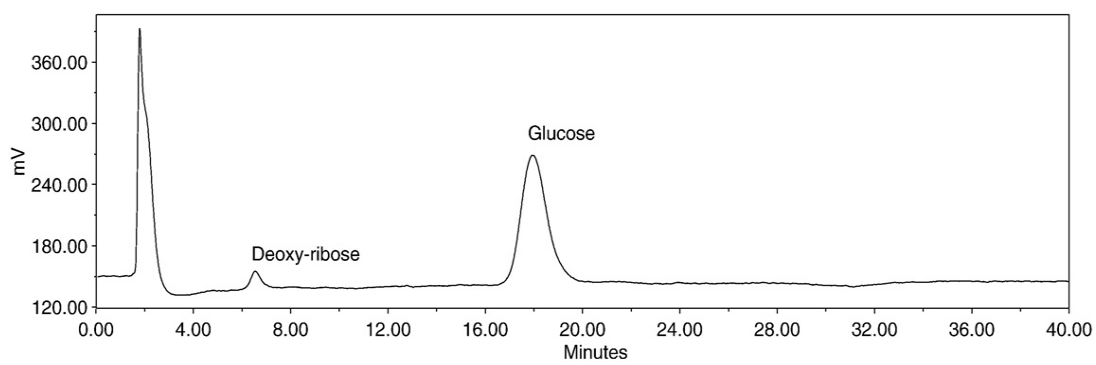


Fig. 3.9 HPLC chromatogram of polysaccharide p1

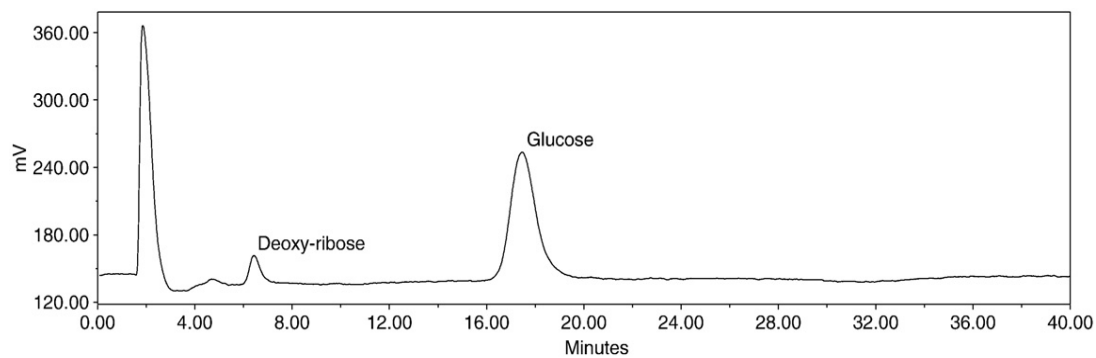


Fig. 3.10 HPLC chromatogram of polysaccharide p2

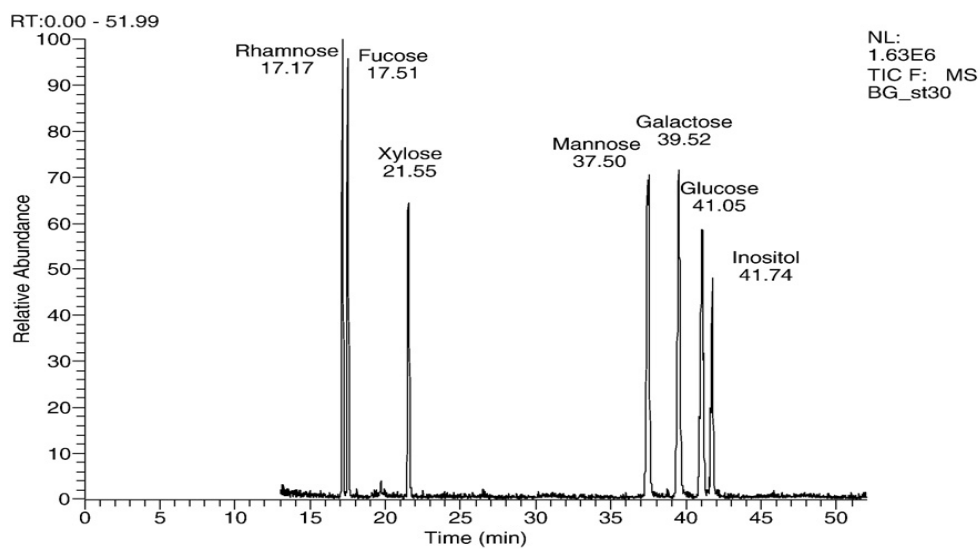


Fig. 3.11 GC-MS chromatogram of neutral sugars

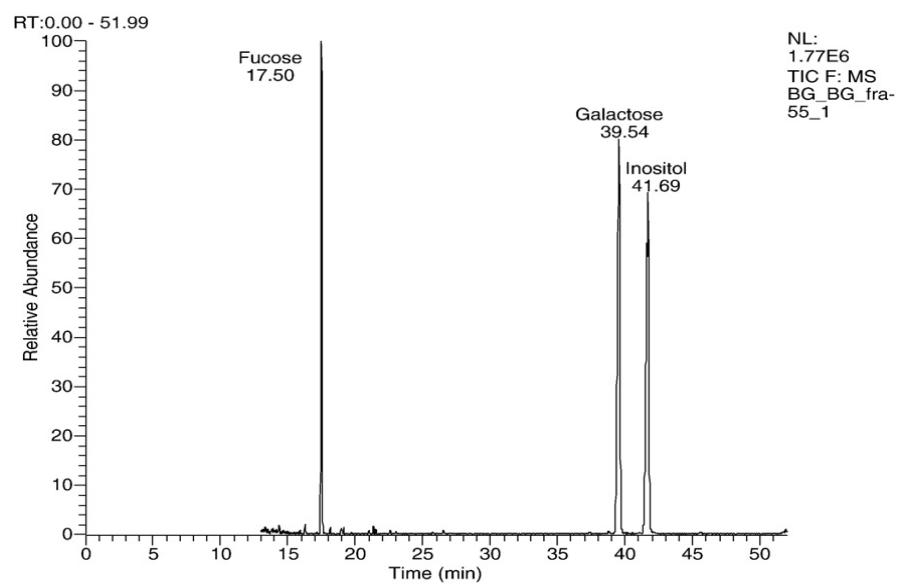


Fig. 3.12 GC-MS chromatogram of polysaccharide f1

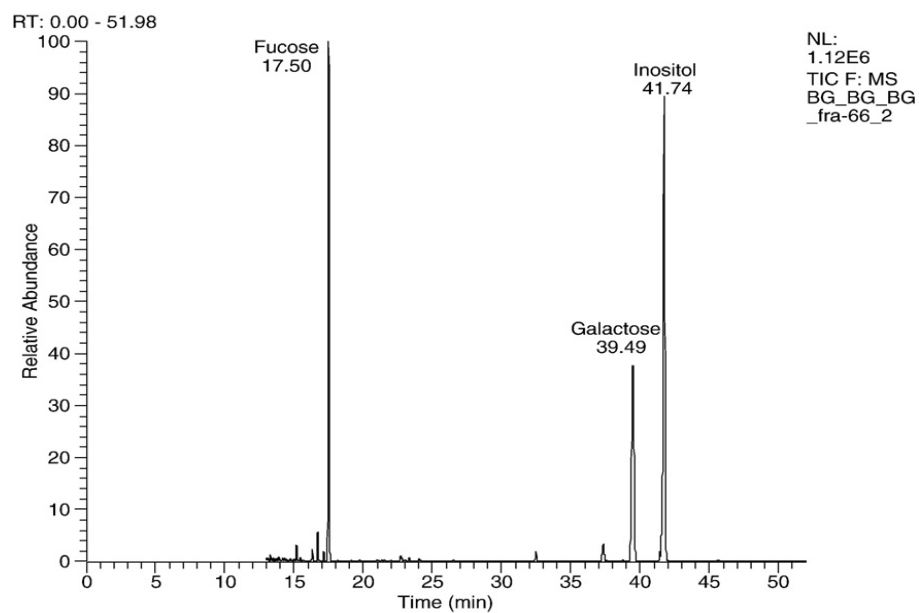


Fig. 3.13 GC-MS chromatogram of polysaccharide f2

3.4.3. Comparison of molecular-level composition with that of Alcian blue method

The content of acid polysaccharides (APS) in EPS solutions from *Amphora sp.*, measured by Alcian blue, is compared to the chemical composition, i.e., uronic acids and sulfate in Table 3.4. The APS concentration, expressed in gum xanthan units, i.e., as mg X eq. /L, is significantly higher than the actual EPS concentration. However, if one expresses the APS concentration in " λ -carrageenan equivalent" units, the total amounts are much closer. The factor of 3 from the ratio of slopes of the two standard compounds (data not shown), i.e., 2 mg/l of λ -carrageenan was determined to yield 6 mg X eq. /L. For EPS f2, the agreement is within the error of the measurement, i.e., 60 vs. 55 mg/L, which can be explained by a relative high sulfate content of 18.2%, and a similar ratio of acidic groups (sum of sulfate and glucuronic acid) vs. neutral sugars in λ -carrageenan (3:2) and EPS f2 (4:2). However, for EPS f1, a lower sulfate content of 9.7% and a ratio of 2:2 make the agreement a bit worse. For dissolved EPS, the agreement is worst, i.e., 584 vs. 309 mg/L, which might be a consequence of other components in the EPS such as proteins and nucleic acids. The results demonstrate the importance of using the appropriate standard for the Alcian blue method (Hung et al., 2003b).

3.4.4. ^{234}Th (IV) binding of EPS from *Amphora sp.*

In order to better understand the role of EPS and polysaccharide binding to Th (IV), the ^{234}Th binding behavior of three EPS fractions, i.e., dissolved EPS (without purification by AEC), polysaccharide f1 and polysaccharide f2, was investigated by using isoelectric focusing (IEF) electrophoresis. This procedure separated them using their different isoelectric points, pH_{IEP} . The results are shown in Figure 3.14, in terms of

percentage of ^{234}Th in each fraction vs. corresponding pH. All three fractions show the highest Th activity at a pH of 2.9 ± 0.4 . The similar binding characteristics of the three materials likely indicate that glucuronic acid (as well as sulfated polysaccharides), which were common in all three fractions, are responsible for Th (IV) binding to EPS.

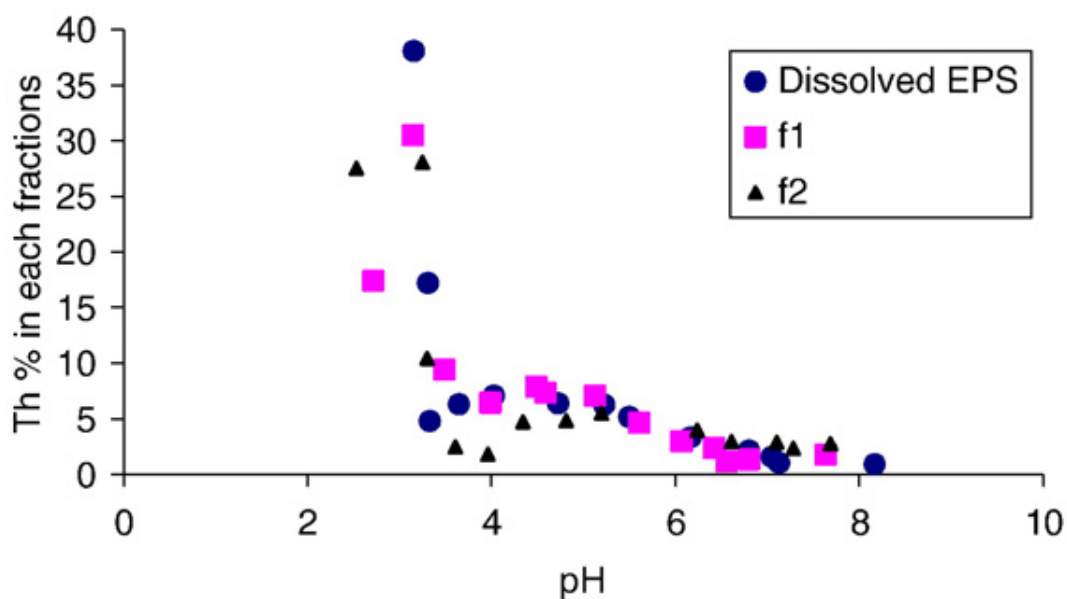


Fig. 3.14 ^{234}Th (IV) distribution on IEF showing that most ^{234}Th is concentrated at a pH_{IEP} of 3, which is the isoelectric point of uronic acids and sulfated polysaccharides (f1 and f2 are the two fractions of the dissolved EPS)

Table 3.4 Determination of acidic polysaccharides (APS) concentration of EPS

| EPS | APS1 (mg X eq. /L) | APS2 (mg λ - carrageenan eq./L) | Sulfate (mg/L) | Glucuronic acid (mg/L) | Mole ratio of Sulfate/ Glucuronic acid | Actual EPS concentration (mg/L) |
|---------------|--------------------------|---|-------------------|---------------------------|---|---------------------------------------|
| Dissolved EPS | 926 | 309 | 67 | 293 | 0.46 | 584 |
| f1 | 645 | 215 | 29 | 148 | 0.39 | 297 |
| f2 | 164 | 55 | 11 | 40 | 0.55 | 60 |

3.5. Discussion

The amounts and composition of extracellular polysaccharides in solution are profoundly affected by nutrient status and vary with species. Myklestad (1995) reported that severe N and P-limitation favors the release of carbohydrates. In addition, increase in the N/P ratio of the medium increases the production of extracellular polysaccharides in laboratory cultures. However, in our study, *Amphora sp.* cultured in f/2-Si medium with rich nutrients also released significant amounts of exopolysaccharides, which were analyzed to contain acidic polysaccharides. Rhamnose, fucose and galactose were generally found to be prominent in diatom polysaccharides (Myklestad and Haug, 1976; Magaletti et al., 2004). Furthermore, many studies have reported sulfates, uronic acids and other neutral sugars, e.g. glucose and xylose/mannose in EPS from diatom, like *S. costatum* (Bhaskar et al., 2005), *C. clostreium* and *N. salinarum* (Staats et al., 1999; de Brouwer et al., 2006). However, the chemical composition of EPS from *Amphora sp.* had not been characterized previously. Our laboratory experiments not only showed that there was a major difference between particulate EPS and dissolved EPS in terms of yield, composition and chemical structure of carbohydrates, which are caused by their different sources, but also that, contrary to other diatoms, EPS from *Amphora sp.* is only composed of two neutral polysaccharides (fucose and galactose), and does not contain any other neutral sugar. The yield of dissolved EPS was four times higher than that of particulate EPS, based on the carbohydrate determinations. 47.5% uronic acids in freeze-dried dissolved EPS are in a good agreement with 48.8% calculated according to the mole ratio of the monosaccharide composition in f1 (fucose: galactose: glucuronic acid:

1:1.1:1.6), considering that the polysaccharide f1 was the major component of dissolved EPS. Therefore, we can conclude that the dissolved EPS from *Amphora sp.* is composed of more than 90% polysaccharides. Evidence from the elemental analysis of carbon and hydrogen also supports this conclusion, since 37.70% of C and 6.27% of H in dry dissolved EPS was close to their corresponding contents in neutral sugars (40% of C and 6.7% of H) and uronic acids (37% of C and 5.2% of H). 31.5% of carbohydrates, as determined by the spectrophotometric anthrone method, however, seemed to underestimate the carbohydrate content in the dissolved EPS, which could be caused by differences in response factors between glucose, galactose, fucose and glucuronic acid. In fact, our experimental results showed galactose, fucose and, especially, glucuronic acid would be underestimated if one were to use glucose as a standard in the colorimetric anthrone method. Polysaccharide f2 was a minor component in the dissolved EPS from *Amphora sp.* and was composed of fucose, galactose and glucuronic acid in a mole ratio of 1:0.8:2.8. Even though the difference is significant, and might suggest some difference in the chemical structure, both f1 and f2 were large molecules with the same molecular weight of 1000 kDa.

The acidic exopolysaccharides of high molecular weight are considered multifunctional in many cases involving many important biological processes. Their molecular architecture appears to be genetically determined and related to environmental triggers and aquatic feedbacks.

Compared to dissolved EPS, particulate EPS mainly consist of neutral carbohydrates. Its two fractions, p1 and p2, are both glucans, which implies that the particulate EPS

originated from structural polymers of the cell wall (Storseth et al., 2004). Their molecular weights are similar, i.e., 25 kDa for both. Thus, further experiments should be carried out to confirm the hypothesis that their compositional difference lies in their 3D architecture and monosaccharide linkage of the two fractions.

Alcian blue staining, as a widely-used method to semi-quantitatively determine the TEP concentration, was evaluated in this study because our knowledge of the exact chemical composition of EPS in the solution could help to improve the Alcian blue staining method in future studies. Different acidic groups, like uronic acids and sulfates, contribute differently to the staining of Alcian blue (Hung et al., 2003b). Therefore, the type and ratio of acidic groups in the TEP as well as the choice of an appropriate standard or reference compound could greatly affect the accuracy of method.

Considering the significance of dissolved EPS in particle dynamics, our laboratory experiments that were carried out to examine ^{234}Th binding behavior of EPS provide information on binding and structure at the molecular level. This is also the first time that ^{234}Th -binding dissolved EPS was compared to its corresponding purified individual components fractionated by AEC in order to investigate the specific role of exopolysaccharides in ^{234}Th binding. The coincidence of the pH_{IEP} distribution of dissolved EPS, f1 and f2 on IEF strips tracked by ^{234}Th indicates that the two acidic exopolysaccharides dominated the ^{234}Th binding of dissolved EPS. In addition, it also implies that the three compounds having an approximate isoelectric point at $\text{pH}=3$ must be negatively charged at neutral pH and thus would migrate to lower pH values. Laboratory and field studies have suggested that different functional groups, such as

carboxyl, phosphate, or sulfate, contribute to the aggregation of EPS (Santschi et al., 2003; Alvarado-Quiroz et al., 2006; Passow et al., 2006). The analysis of the chemical structure of EPS from *Amphora sp.* revealed that, in this case, glucuronic acid and sulfate in the two acidic exopolysaccharides, were the main chelating biomolecules for ^{234}Th (IV). Given that *Amphora sp.* is a representative species of benthic diatoms (Welker et al., 2002; Facca and Sfriso, 2007) and the crucial roles benthic diatoms play in the organic carbon flux of the coastal oceans (Cadee and Hegeman, 1974; Fogg, 1983; Colijn and De Jonge, 1984; Cahoon and Cooke, 1992; Barranguet, 1997; Smith and Underwood, 1998; Taylor and Paterson, 1998; Nelson et al., 1999), our study therefore provides further molecular level insight into the roles of EPS as metal chelators and scavenging agents of thorium and organic carbon from the ocean.

3.6. Conclusions

This study provides a molecular-level understanding on the interactions of ^{234}Th (IV) with EPS of *Amphora sp.*, which could greatly benefit the applications of ^{234}Th (IV) in the study of particle dynamics. Based on the molecular composition of particulate EPS and dissolved EPS we found that they reflect the different sources of these biopolymers. Particulate EPS originates from compounds used in the formation of cell wall of *Amphora sp.* and contains two glucans, p1 and p2, with the same molecular weight of 25 kDa. In comparison to the particulate EPS, dissolved EPS are multifunctional and facilitate the growth and survival of the organism. Over 90% of the dissolved EPS from *Amphora sp.* was composed of exopolysaccharides. The two exopolysaccharides from

dissolved EPS, f1 and f2, had the same molecular weight of 1000 kDa, and the same chemical composition. However, f1 had the main monosaccharide building blocks of fucose, galactose and glucuronic acid in a mole ratio of 1:1.1:1.6, while f2, it was 1:0.8:2.8. The HPLC analysis of neutral monosaccharide composition was confirmed by GC-EI-MS, which gave the same results. Sulfate was another acidic group in the two polysaccharides, 9.7% in f1 and 18.2% in f2, together with glucuronic acid to dominate the interaction of ^{234}Th (IV) with dissolved EPS. These results thus provide molecular level insights not only into the binding of ^{234}Th (IV) to EPS in the ocean, but also provides crucial information to the frequently observed relationships between ^{234}Th (IV) binding to suspended matters in the ocean and their uronic acid, sulfated polysaccharide, or the TEP content determined by the Alcian blue method.

CHAPTER IV
SUBSTRATA EFFECTS ON THE CHEMICAL CHARACTERIZATION OF EPS
IN BIOFILM FORMED IN WHITE OAK BAYOU AND BUFFALO BAYOU
(HOUSTON TX)

4.1. Overview

Biofilms are common phenomena in the environment. They can grow on all types of surfaces, such as soils, medical equipment, implants, plastics, metals, rocks, etc. EPS in biofilms not only serve as a matrix for biofilm growth by holding cells together, but also serve as sorption sites for inorganic and organic solutes such as nutrients and trace elements. Biofilms were grown on four substrata (brick, glass, plastic and wood) at the two Houston bayous, White Oak Bayou and Buffalo Bayou, TX. After four weeks of growth, biofilms were collected and subject to EPS extraction by steaming and EDTA techniques. Significantly elevated concentrations of organic phosphorus and proteins as well as DNA were observed in EDTA extracted samples. Proteins were the most abundant components in EPS of all the collected biofilms, followed by carbohydrates, except for the EPS in biofilms from the glass substrata. In addition, analyses of carbohydrates and proteins, i.e., as monosaccharides and amino acids, respectively, indicate that the molecular chemical composition of biofilms that formed was in response to different environmental conditions on the different substrata. No correlation between hydrophobicity of substratum and development of biofilm was found in this study.

4.2. Introduction

A biofilm is a community of microbial cells, including algae and heterotrophic microorganisms, embedded in a mucus layer composed of extracellular polymeric substances (EPS) (Decho, 2000). According to Hall-Stoodley et al. (2004), the formation of a biofilm on a surface involves three stages. It begins with the spontaneous deposition of a conditioning film of high molecular weight humic or glycoproteinaceous matter, after which, single bacterial cell can attach to a surface. The cell attachment is mainly dictated by weak, reversible electrostatic interactions between the bacterial cell and the surface (e.g., depending on Van der Waals' forces, hydrogen bonding, and the relative hydrophobicity of both bacterial cells and the surface). The second stage involves biological and chemical responses from prokaryotic and eukaryotic organisms, such as the production of EPS. The final stage of biofilm formation is known as development, in which the biofilm is established and may mainly change in shape, size and chemical composition.

EPS are primarily composed of polysaccharides and proteins, and in some case, nucleic acids and lipids (Flemming and Wingender, 2001). EPS not only serve as a matrix for biofilm growth by holding cells together, but also serve as sorption sites for inorganic and organic solutes such as nutrients. The enrichment of inorganic and organic nutrients facilitates cell growth in biofilms. In addition, this matrix protects the cells within it and facilitates communication among them through biochemical signals. Some biofilms have been found to contain water channels that help distribute nutrients and signaling molecules (Karatan and Wetnick, 2009). Therefore, the colonization on solid

surfaces by microorganisms has been proposed to depend mostly on the production of extracellular substances, i.e., EPS (Czaczyk and Myszka, 2007).

Buffalo Bayou and White Oak Bayou are two of the main waterways flowing through Houston TX USA. They deliver up to 60 million gallons of freshwater daily to Galveston Bay (Rifai, 2006) and are both on the Clean Water Act Section 303(d) list for impaired environments due to chronically elevated bacteria levels as determined from total counts of culturable bacteria. Each of these bayous is a stream that also receives industrial and waste water effluents. In dry weather, wastewater treatment plants (WWTP) and industrial effluents supply 60 MGD (million gallons per day) (>95%) of the surface water flow (Rifai 2006). In addition, both of the two streams are channelized to different extents (Buffalo Bayou has natural soil/sediment beds and banks and White Oak Bayou is concrete lined) and the lower segments of each bayou are tidally influenced. Both sampling sites are polluted equally by highly elevated nutrients, > 4 mg/L NO₃-N, >5 mg/L NH₄-N, and >1.5 mg/L PO₄-P (Brinkmeyer et. al., 2008). Dissolved organic carbon at the sampling site of White Oak Bayou (600 μM) is 30 % higher than that in Buffalo Bayou (Brinkmeyer et al., 2008). Buffalo Bayou has seven times higher concentration of total suspended solids (TSS) than White Oak Bayou. Many artificial wastes, such as glass, plastic, wood and concrete are found at both streams and thus could be potential substrata for biofilm formation.

Many ecotoxicological and environmental studies investigated the effects of substrata on community structure and function of biofilms. Danilov and Ekelund (2001) reported that the nature of the substratum could considerably affect patterns of colonization of

algae. Compared with pieces of PVC and pieces of wood, glass turned out to be the most favorable substrata for algae settlement. However, plastics were found to be most favorable artificial substratum for biofilm in Cattaneo and Amireault's study (1992). In the study of Kropfl et al. (2006), natural biofilms were simultaneously grown on granite, polished granite, andesite, polycarbonate, and plexi-glass substrata. It was found that the polycarbonate substratum had the highest biofilm production and abundance of algae, while bacterial activity was similar among the different substrata.

However, very little is known about the relationship between physico-chemical properties of EPS and the nature of the substratum. Therefore, in this study, the molecular level composition of EPS, i.e., the monosaccharide and amino acid composition was investigated to improve our understanding of the effect of the potential substrata (glass, plastic, wood and concrete) on the formation of natural biofilms.

4.3. Materials and methods

4.3.1. Growing of natural biofilm on different substrata

Natural biofilms were grown on different substrata for 4 weeks from July 2008 to August 2008 at White Oak Bayou (TCEQ station 16646 near N Freeway 45 and Katy Freeway 10 at N29°46'58", W95°22'30") and Buffalo Bayou (TCEQ station 11351 near Shepherd and Allen Pkwy/Kirby Dr. at N29°46'34", W95°24'36"), Houston, TX, USA. Bricks (concrete, 7.5 × 15.5"), glass pieces (window glass, 11 × 14"), plastic pieces (acrylic, 8 × 12") and wood (birch plywood, 11 × 14") that were thoroughly cleaned

beforehand by water, were placed on the stream beds of selected sites to allow biofilm growth. Plastic and glass surfaces were attached to the top of concrete bricks with screws before being submerged onto stream beds. While the sampling was not replicated, the subsequent chemical analyses were carried out in replicates.

4.3.2. Extraction of EPS from biofilms

Extraction methods have been reported to affect not only the yields of carbohydrates and proteins (Zhang et al., 1999), but also the characteristics of EPS, such as EPS extracted by chemical reagents (NaOH and EDTA) present significantly different fingerprints on high-pressure size exclusion chromatography (Comte et al., 2007). In order to have a comprehensive understanding of EPS in biofilms, two extraction methods were used to extract EPS of biofilms from substrata submerged in the two streams.

Steaming extraction: Biofilms were mechanically stripped off from substrata using disposable sterile spatula and then were suspended in 15 mL of water and heated at 80 °C for 10 minutes. After being centrifuged at 8000 g for 10 minutes, the supernatant was collected and filtered for analysis while the solution was still hot (Brown and Lester, 1980).

EDTA extraction: mechanically stripped biofilms were suspended in a mixture of 7.5 mL water and 7.5 mL of 2 % EDTA. The content was put in a refrigerator (4 °C) for 3 hrs and stirred every 30 minutes. After that, it was centrifuged at 8000 g for 20 minutes. The supernatant was collected and filtered for analysis (Brown and Lester, 1980; Liu and Fang, 2002).

4.3.3. Determination of organic phosphate

Generally, organic phosphate content was measured by subtracting the dissolved inorganic phosphorus from the measured total phosphorus (Solorzano and Sharp, 1980, Murphy and Riley, 1962). For dissolved phosphorus, a mixed reagent was prepared by combining 25 mL of ammonium molybdate solution (30 g/L), 62 mL of sulfuric acid (15% V/V), 25 mL of ascorbic acid (55 g/L) and 12 mL of potassium antimonyl-tartrate (1.36 g/L). It was diluted by nanopure water (1:5) before use. 1.0 mL of each extraction solution and phosphate standards (0.25-5.0 ppm) were separately added to test tubes (10 × 100 mm) containing 3.0 mL of diluted mixed reagent. The solution was swirled for 20 min. A reagent blank was determined in the same manner. The solutions were then measured by spectrophotometry at $\lambda=885$ nm in 1 cm pathlength cells. Total phosphorus was measured as follows: 5 mL of extraction solution was added to a glass vial (20 × 57 mm) containing 0.1 mL of 0.17 M MgSO_4 . The solution was evaporated to dryness at 95 °C. The vial was then covered by aluminum foil and transferred to a furnace and baked at 450 °C for 2 h. After cooling, 1.5 mL of 0.75 M HCl was added and heated at 80 °C for 10 min without a cover. Then 3.5 mL of nanopure water was added and the solution heated again for an additional 10 min. After cooling, the sample was transferred to a 10 ml of centrifuge tube. The volume was then brought to 5 mL with nanopure water. The sample was then treated using the same procedure as that for reactive phosphorus.

4.3.4. Colorimetric determination of carbohydrates, uronic acids, proteins and DNA

Carbohydrate concentrations were estimated by using the anthrone method (Morris, 1948; Zhang and Santschi, 2009), with glucose as a standard. Uronic acids were

determined according to the method of Blumenkrantz and Asboe-Hansen (1973), with glucuronic acid as a standard.

The protein content in the steam extraction solutions was measured using a modified Lowry Protein Assay Kit (Pierce USA, 23240,) because of its high sensitivity. The procedure followed the protocol provided by the manufacturer. The protein content in the EDTA extraction solutions was measured using the bicinchoninic acid (BCA) (Smith et al., 1985, 1990) with Bovine Serum Albumin (BSA) as a standard, as EDTA would interfere with the measurement of protein in the Lowry method.

DNA concentration was determined by reacting DNA with diphenylamine (Burton 1956, Holme and Peck, 1998). Briefly, a diphenylamine reagent was prepared by dissolving 1.5 g of diphenylamine into 100 ml of acetic acid, followed by the addition of 1.5 ml of concentrated sulfuric acid. This reagent was stored in the dark. Acetaldehyde (0.5 ml of 1.6 %) was added to the reagent on the day of the experiment. 2 mL of the reagent was added to 1 mL of extraction solution. The absorbance was then measured after 18 hours, by spectrophotometry at the wavelength of 600 nm in a 1 cm cell. Deoxyribose was used as a standard. The concentration range was from 0.3 to 13.4 µg/mL. This method is specific for DNA. Carbohydrates, uronic acids, proteins, amino acids as well as nucleic acids do not interfere with the measurement when present at concentrations as high as 1 mg/mL.

4.3.5. Determination of hydrolysable monosaccharides and amino acids

Neutral sugars and uronic acids were analyzed by GC/MS and HPLC, respectively. (Zhang et al., 2008).

Seventeen amino acids were determined by derivatization with *o*-phthaldialdehyde (OPT) with a modification from Lindroth and Mopper (1979) and Duan and Bianchi, (2007). In brief, 1 ml of 6 M HCl (containing 0.5% phenol) was added to a 10 ml of ampule containing 1 ml of extracted solution. The ampule was sealed under an N₂ atmosphere and then hydrolyzed at 110 °C for 24 hrs. Then the hydrolysis solution was dried under N₂ stream. The residue was dissolved in 1 ml of nanopure water. This resultant mixture was ready for amino acids analysis after being filtered through a 0.22 µm syringe filter. 20 µL of the filtered mixture solution was mixed with 100 µL of OPT reagent at room temperature and reacted for 2 min, then 40 µL were injected onto Alltech Alltima C₁₈ column (250 × 4.6 mm). Eluent A, 0.05M sodium acetate with 5 % tetrahydrofuran (pH was adjusted to 5.5 with acetic acid), and eluent B, 80 % methanol were used as mobile phases. A gradient begin with 30 % of eluent B, ramped to 70 % of eluent B over 40 min, and finally to 100 % of eluent B at 60 min, kept for 15 min. Then eluent B was decreased back to 30 % for preparing for next injection. The equilibrium step at least took 15 min.

The extraction and characterization of EPS were summarized in Figure 4.1.

4.3.6. Experimental determination of model biopolymer removal to representative substrata

In a separate experiment, representative model biopolymers, such as hemoglobin, pullulan (a polysaccharide), lipopolysaccharide, carrageenan IV and xanthan, were used to investigate the removal of these compounds to representative substrata by incubating them in glass or plastic containers for 28 hrs.

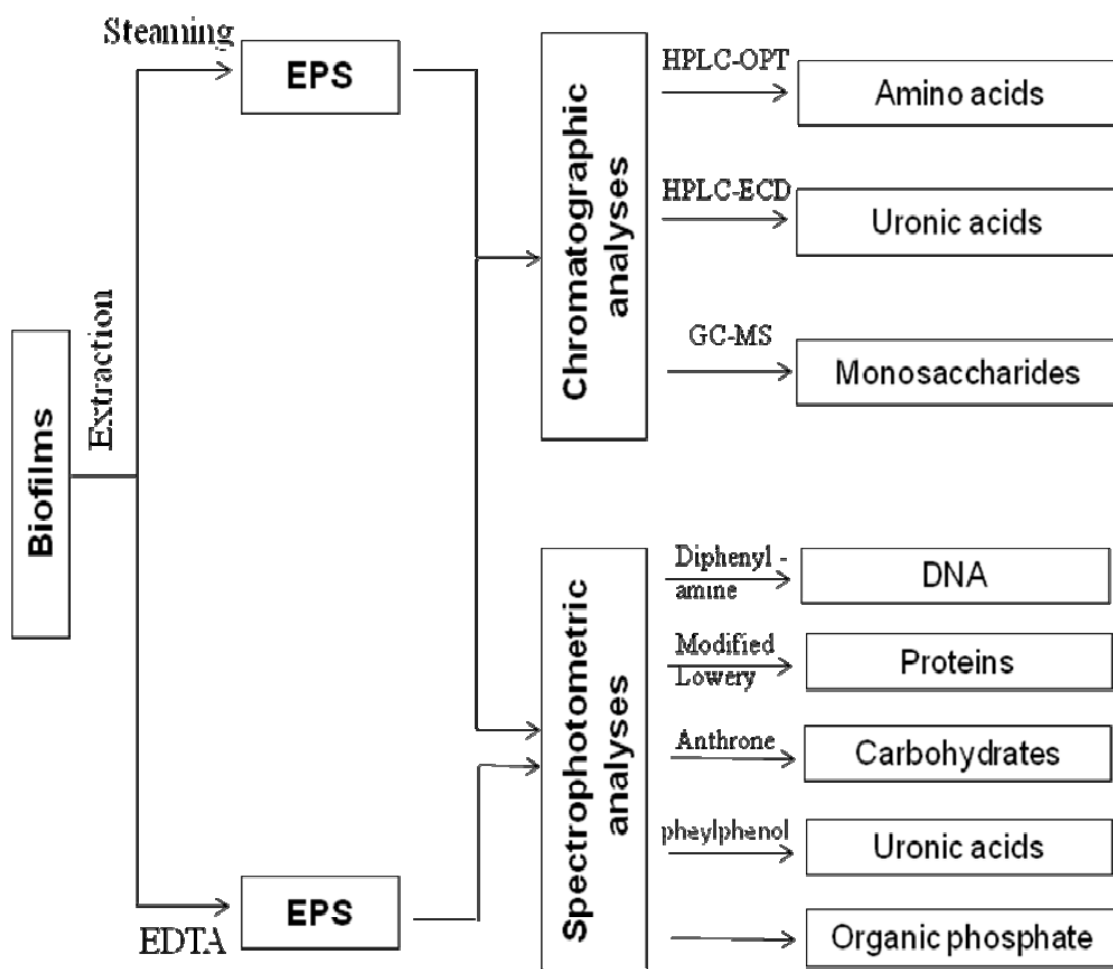


Fig. 4.1 Diagram for EPS extraction and characterization

4.4. Results

After four weeks of growth, biofilms on the four substrata at each site were visible, with the exception of the wood surface from White Oak Bayou. The biofilm development on that sample was limited and did not allow a full characterization of the biofilm because of insufficient amounts of EPS.

4.4.1. Comparison of extraction methods, using EDTA and using steaming, for quantifying the chemical composition of biofilms at the two bayous

EPS of biofilms on different substrata from the two sites were extracted with two methods, steam and EDTA. The concentrations of organic phosphorus, proteins, uronic acids and carbohydrates of EPS are presented in Table 4.1 (White Oak Bayou) and Table 4.2 (Buffalo Bayou), respectively. The EDTA extraction consistently produced a much higher yield of all the measured components in EPS from all biofilms on different substrata in the two sites, especially for DNA, organic phosphorus, uronic acids and proteins. At White Oak Bayou site, among these three compounds, the efficiency for extracting DNA was 2-7 times higher. For uronic acids it was higher by a factor of 3-5, for organic phosphorus a factor of 1.6-5.6, for proteins a factor of 1.3-3.1. However, the yield of carbohydrates did not significantly increase when using EDTA. At Buffalo Bayou, the difference caused by the two extraction methods was more distinct, such as the concentration of DNA in the biofilm on the brick surface, which increased from 0.4 to 13.5 $\mu\text{g/g}$, i.e., by a factor of 33. Generally, DNA increased by a factor of 18-33, phosphorus by 4-30, uronic acids by 6-18, proteins by 2-8, and carbohydrates by 1.3-5 times. EDTA, as a strong chelating agent, sequesters metal ions, such as Ca^{2+} . Despite

distinctly different yields of measured compounds for the two extraction methods, they consistently show that carbohydrates and proteins are the two primary components of EPS from biofilms, regardless of substratum or sampling site.

4.4.2. Effect of substratum on formation of biofilm in terms of chemical composition of EPS

The composition of EPS varied with substratum and sampling site (Table 4.1 and Table 4.2). At White Oak Bayou, proteins were the most abundant compounds in EPS, followed by carbohydrates. EPS of biofilm grown on the glass surface had lowest concentration of uronic acids, proteins and carbohydrates. Whereas there were slight differences in concentrations of measured compounds for the EPS of biofilms from the brick and plastic substrata.

As a contrast, at Buffalo Bayou, the highest concentration of uronic acids, proteins and carbohydrates were found in extracted EPS from the biofilm on the glass substratum. Moreover, the concentration of carbohydrates was higher than that of proteins. Plastics and wood surfaces had moderate concentrations of the measured compounds. Brick had the lowest amounts of biopolymers in these biofilms.

4.4.3. Effect of substratum on monosaccharide composition of EPS extracted from biofilms

Considering cell disruption during EDTA extraction, only results from the steaming extractions of EPS will be discussed. Six sugars were analyzed and shown in Figures 4.2 and 4.3. In White Oak Bayou, EPS in biofilms from the glass and plastic substrata had a similar monosaccharide distribution. Galactose and glucose were the two main

monosaccharides, and the other four sugars were comparable. While glucose and mannose were the most abundant monosaccharides in EPS from biofilm on the brick surface.

The pattern at Buffalo Bayou, however, was completely different. Galactose was prominently present in the EPS from biofilms on the glass and plastics surfaces, especially on the glass surface, while it was absent in EPS from biofilms on the brick and wood substrata. In addition, in all EPS from the two bayous, uronic acids were present in the form of galacturonic acid.

4.4.4. Effect of substratum on the hydrolysable amino acids of EPS extracted from biofilms

Seventeen L- amino acids were determined in this study (presented in Figures 4.4 and 4.5). At White Oak Bayou, 13 amino acids appeared in the EPS from the biofilm on the plastic substratum. L- leucine, L-threonine, L-glycine and L-serine were the main four amino acids, while L-Glutamic acid, L-asparagines, L-glutamine, L-methionine and L-lysine were absent. L-lysine, L- Leucine, L-alanine and L-glycine were the main three amino acids in the EPS from the biofilm on the brick substratum. Only two amino acids were missing. They were L-histidine and L-glutamine. Biofilm grown on the glass had EPS mainly composed of L-glutamic acid, followed by L-glycine, L-alanine.

At Buffalo Bayou, the EPS in the biofilm from the glass substratum distinguished itself with a dramatically different distribution of amino acids. L-glutamic acid was the most prominent amino acid in this EPS.

Table 4.1 Concentrations of DNA, organic phosphorus, uronic acids, proteins and carbohydrates of EPS from biofilms on three substrata at White Oak Bayou

| Substrata | DNA μg deoxyribose eq./g | | Organic PO ₄ μg/g | | Uronic acids μg/g | | Proteins μg/g | | Carbohydrates μg/g | |
|-----------|-----------------------------|------|---------------------------------|-------|----------------------|-------|------------------|--------|-----------------------|--------|
| | Steam | EDTA | Steam | EDTA | Steam | EDTA | Steam | EDTA | Steam | EDTA |
| Brick | 32.6 | 63.1 | 196.5 | 312.5 | 77.2 | 263.9 | 1781.3 | 4809.9 | 1367.9 | 1433.6 |
| Glass | 3.7 | 23.6 | 11.1 | 62.7 | 19.6 | 91.6 | 170.1 | 533.1 | 266.1 | 305.5 |
| Plastic | 22.2 | 58.2 | 261.6 | 589.3 | 88.6 | 318.7 | 2300.6 | 2902.2 | 1633.2 | 1354.3 |

Table 4.2 Concentrations of DNA, organic phosphorus, uronic acids, proteins and carbohydrates of EPS from biofilms on four substrata at Buffalo Bayou

| Substrata | DNA μg deoxyribose eq./g | | Organic PO ₄ μg/g | | Uronic acids μg/g | | Proteins μg/g | | Carbohydrates μg/g | |
|-----------|-----------------------------|------|---------------------------------|-------|----------------------|-------|------------------|--------|-----------------------|--------|
| | Steam | EDTA | Steam | EDTA | Steam | EDTA | Steam | EDTA | Steam | EDTA |
| Brick | 0.4 | 13.5 | 3.0 | 45.6 | 4.8 | 73.8 | 82.2 | 313.7 | 31.7 | 100.3 |
| Glass | 2.5 | 45.8 | 101.8 | 558.5 | 64.6 | 374.4 | 973.2 | 1883.8 | 1357.8 | 1720.0 |
| Plastic | 1.8 | 48.1 | 27.3 | 119.3 | 18.4 | 291.3 | 344.8 | 1114.7 | 186.9 | 420.0 |
| Wood | 1.3 | 43.2 | 6.8 | 207.1 | 12.4 | 217.6 | 154.6 | 1263.7 | 51.6 | 283.2 |

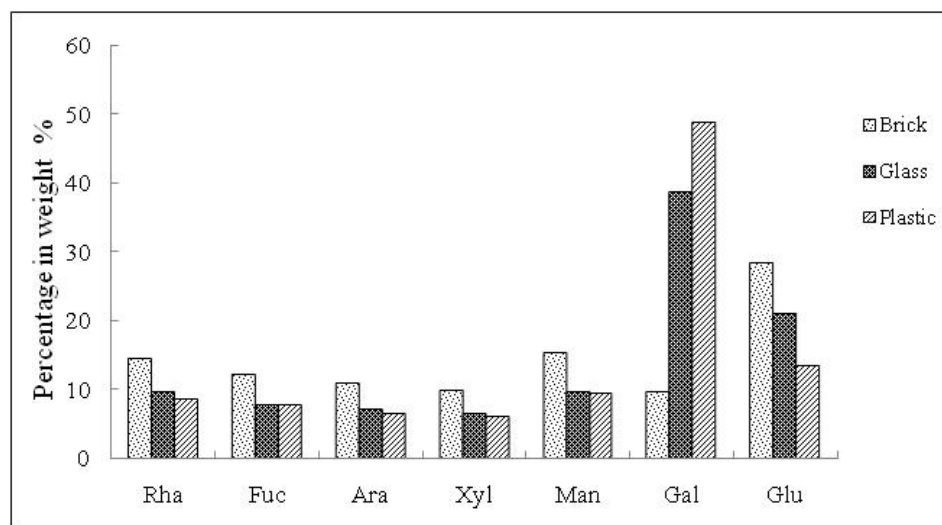


Fig. 4.2 Monosaccharide distribution of biofilms from White Oak Bayou extracted by steam. (Rha: rhamnose, Fuc: fucose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glu: glucose). Note: the relative standard deviations of monosaccharide analyses were <3%

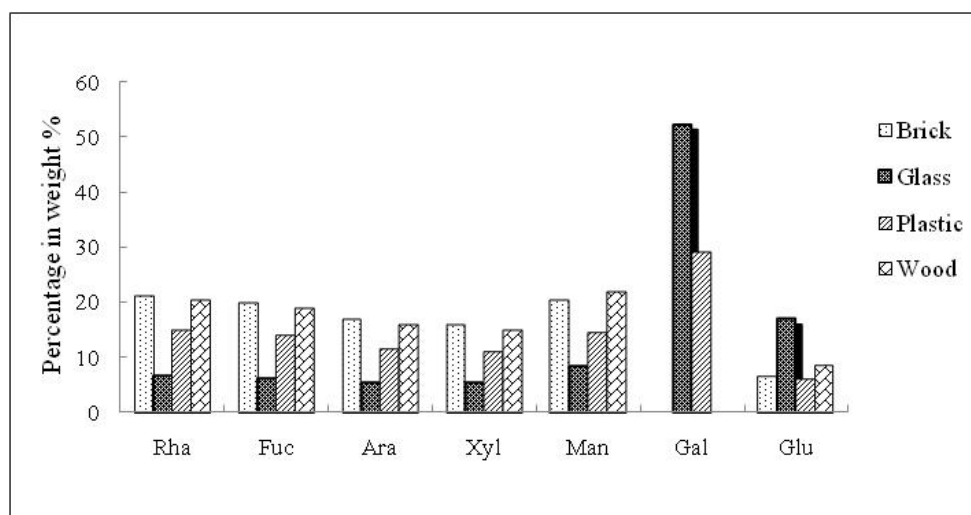


Fig. 4.3 Monosaccharide distribution of biofilms from Buffalo Bayou extracted by steaming

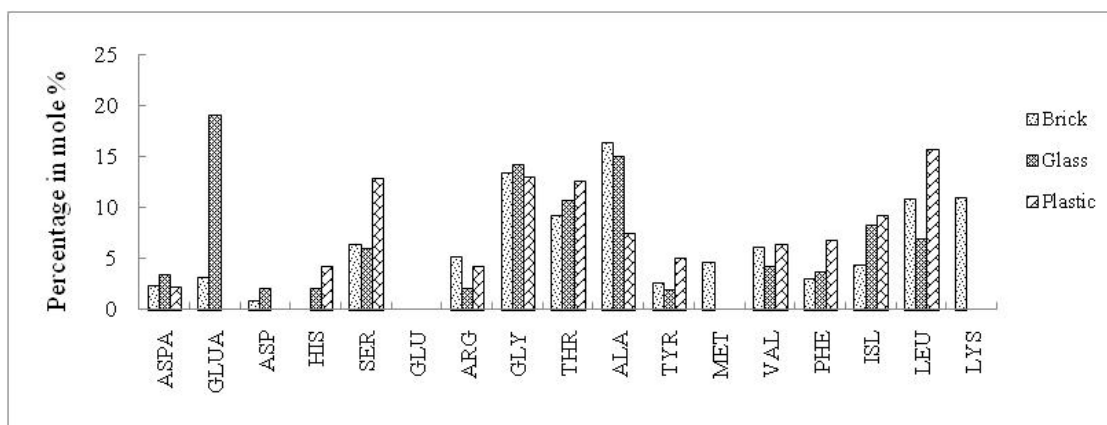


Fig. 4.4 Mole percentages of hydrolysable amino acids in biofilms from White Oak Bayou extracted by steam. (ASPA: aspartic acid; GLUA: glutamic acid; ASP: asparagine; HIS: histidine; SER: serine; GLU: glutamine; ARG: arginine; GLY: glycine; THR: threonine; ALA: alanine; TYR: tyrosine; MET: methionine; VAL: valine; PHE: phenylamine; ISL: isoleucine; LEU: leucine; LYS: lysine). Note: the relative standard deviation of amino acid concentrations were <5%

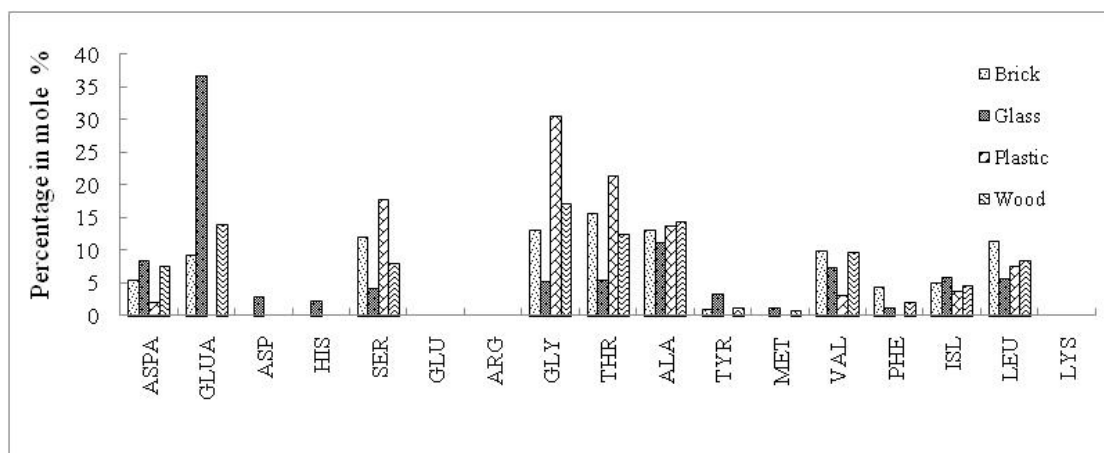


Fig.4.5 Mole percentages of hydrolysable amino acids in biofilms from Buffalo Bayou extracted by steaming

4.4.5. Interaction of biopolymers with substrata, glass and plastic

In a separate experiment, hemoglobin, pullulan, lipopolysaccharide, carrageenan IV, and xanthan were used as representative model compounds to investigate the interactions of the substrate with representative biopolymers with more defined relative hydrophobicities, e.g., hemoglobin is the most hydrophobic biopolymer, followed by neutral polysaccharides and acidic polysaccharides (Xu. et al. 2010), by incubating them in glass or plastic containers for 28 hrs. In this study, interactions between these biopolymers and substrum were analyzed by monitoring the percentage change in the concentration of biopolymers over the corresponding periods, shown in Table 4.3. Generally, these model biopolymers were preferably retained on the glass surface after incubation for 28 hours, except for pullulan. These patterns differ from their relative hydrophobicity, although the plastic surface is usually considered to be more hydrophobic than glass. Complex carbohydrates, like lipopolysaccharide, carrageenan IV

also demonstrated significant interactions with glass and plastic substrata. Therefore, one could conclude from these experimental results that the interactions between biopolymers and substrate are not regulated by the relative hydrophobicity of substrate, but by the ionic properties of the biopolymers or biological processes (e.g., microbial consumption).

4.5. Discussion

Compounds extracted from biofilms, such as DNA, carbohydrates, organic phosphorus and proteins have two sources. They are either released from the polymer matrix or through cell disruption. If the measured compounds are products of autolysis, and have been released from the EPS, a constant relationship should be achieved between carbohydrates and other compounds (Brown and Lester, 1980). However, for all biofilms in this study, the ratios of proteins vs. carbohydrates were elevated when using EDTA extraction, and so were the ratios of organic phosphorus and DNA relative to carbohydrate and so were the ratios of organic phosphorus and DNA relative to carbohydrates. This might suggest that some cell disruption had occurred during EDTA extraction, but could also be a consequence of EDTA being a strong chelator for metal ions such as Ca^{2+} . The presence of Ca^{2+} facilitated the self-assembly of EPS (Chin et al., 1998), and thereby EDTA complexation of Ca^{2+} resulted in enhanced extraction of EPS. The result of increasing yield of uronic acids using EDTA in the present study supports this theory, and is in a good agreement with previous studies, considering uronic acids as an important agent to interact with cations in EPS (Hung et al., 2003a).

Table 4.3 Percentage of change in the concentration of biopolymers when incubated
in glass and plastic containers

| Inoculation time (h) | | 3.5 | 7 | 11 | 21 | 28 |
|--------------------------|---------|----------|-----------|-----------|-----------|-----------|
| | | | | | | |
| Hemoglobin % | Glass | -2.4±1.0 | -4.4±0.9 | - | -6.1±0.8 | -3.1±0.8 |
| | Plastic | -0.2±0.9 | -1.2±2.9 | - | -2.6±10.9 | -0.1±0.3 |
| Pullulan % | Glass | -3.8±6.4 | - | -1.4±2.2 | 2.4±1.4 | - |
| | Plastic | -0.4±4.5 | - | 1.2±1.4 | 2.8±7.8 | - |
| Lipopolysaccha ride % | Glass | 4.6±9.1 | -1.8±0 | -7.4±1.3 | - | -15.5±4.1 |
| | Plastic | 0.0±2.6 | -6.5±11.7 | -11.1±6.5 | - | -9.8±4.1 |
| Carrageenan IV % | Glass | 1.6±6.1 | 4.3±3.8 | -1.1±0 | -7.0±9.1 | -9.8±7.9 |
| | Plastic | 8.6±6.9 | 2.7±3.1 | -1.1±9.2 | -4.9±13.7 | -5.3±4.8 |
| Xanthan % | Glass | 1.7±2.4 | 1.1±3.1 | -8.3±2.4 | -7.2±11.8 | -24.5±5.7 |
| | Plastic | 3.3±0 | 1.1±0 | -6.1±1.6 | -13.3±0 | -24.5±8.1 |

The development of biofilm on a solid surface is known to be a multi-stage process. Attachment of bacteria to a surface initiates a sequence of changes in phenotype, where large suites of genes are differentially regulated (An and Parsek, 2007). Therefore, the cells of a microorganism growing in a biofilm are physiologically distinct from planktonic cells of the same organism. The synthesis of EPS by the attached microorganisms strengthens their binding to a surface. Observed changes in the chemical composition of EPS in biofilms from different substrata suggest that the nature of substratum takes part in regulating the synthesis of EPS during formation of the biofilm. One of the reasons to choose the four substrata (glass, plastic, wood and brick) is that they are common but artificial substrata occurring as waste material in these two bayous. Therefore they are potential surfaces for biofilm growth. Moreover, they represent different properties, for example, brick and wood have rougher surfaces than plastic and glass, while plastic and glass are more hydrophobic than brick and wood. The rougher surfaces could facilitate the attachment of bacteria and provide protection from water currents and predators. Glass was consistently found to be the most preferred surface for algae (Danilov and Ekelund, 2001 and references therein), possibly because glass is a good nutrient source of silicon for diatoms. Similarly, wood provides a source of organic matter for heterotrophic microorganisms. Furthermore, hydrophobic interactions have been reported to be important for the adhesion of biofilm to plastic surfaces (Elhariry, 2008). However, the distinctly different chemical compositional patterns in EPS on the four substrata in the two streams suggest that formation of biofilm is in response to many factors, which include the properties of the substratum, e.g., its relative hydrophobicity

(Silyn-Roberts and Lewis, 2003), charge density, bacterial cell properties (e.g., cell charge), environmental conditions (e.g., ionic strength, Li and Logan, 2004) and microbial consumption. At White Oak Bayou, EPS from the biofilm on the plastic substratum consisted of highest concentrations of polysaccharides, proteins, DNA, organic phosphorus and uronic acids than those on the brick and glass substrata. While at Buffalo Bayou, EPS from the biofilm on the glass substratum had highest concentrations of these biopolymers. In addition, the results from the investigation of the interactions between biopolymers and substrata, e.g., separate laboratory experiments using different model biopolymers as well as field data, cannot explain the interactions of these compounds with glass and plastic substrata at the two sites. Therefore, the formation of biofilms in the study sites might be more dependent on the microorganism community and environmental situations. However, glass was consistently a good substratum for algae, as EPS of biofilms formed on glass surfaces at the two bayous had a ratio of proteins/carbohydrates less than 1 by releasing polysaccharide-enriched EPS from algae, which might be due to the source of silicon in glass for diatoms.

Although carbohydrates are a class of compounds that degrade rapidly in aquatic systems, the distribution of monosaccharides has been found to be a potential candidate for monitoring the changes in microbial community (Dowling et al., 1986; Khandekar and Johns, 1990a). At White Oak Bayou, the high percentage of (arabinose+galactose) in the EPS of biofilm from the glass (45 %) and plastic (55 %) substrata, together with a mannose/xylose ratio of 1.6, strongly indicates a phytoplankton input (Cowie and Hedges, 1984; Khandekar and Johns, 1990b). In contrast, a relatively high percentage of

rhamnose and arabinose in the EPS of biofilm from the brick substratum suggests a relatively high abundance of bacteria in the biofilm. The relatively rough surface of concrete brick could have facilitated the attachment of bacteria and provided protections from grazers. In addition, a relatively low percentage of xylose, less than 10%, minimized any contribution from terrestrial plants on all three types of substrata.

At Buffalo Bayou, EPS in the biofilm from the glass substratum demonstrated a similar monosaccharide distribution as that at White Oak Bayou, having a high percentage of arabinose plus galactose, 57%, as well as a mannose/xylose ratio of 1.6. In addition, a low percentage of rhamnose, arabinose and xylose indicate a major source of EPS from algae. EPS in the biofilm from the plastic surface showed an even distribution of monosaccharides, and thus suggests that the EPS were contributed by both bacteria and algae. In contrast, EPS in the biofilm from the brick and wood substrata had a low percentage of arabinose plus galactose, 17%, which suggests that the source for the biofilm EPS could be from bacteria or vascular plants which are abundantly along the two bayous.

Amino acids are usually not potential indicators for sources of organic matter in environmental investigation due to their high turnover rates in aquatic systems. However, when combined with other compound classes such as sugars or where amino acid sources are very limited in number, amino acids have been used to elucidate the diagenesis and nature of organic matter in marine samples (Muller et al., 1986; Cowie and Hedges, 1992). In this study, EPS in biofilms had a high mole fraction (15-36 %) of hydroxyl acids (serine, threonine) and glycine (13-29 %), especially in the EPS of

biofilm from the plastic substrata from both sites. Those three amino acids were reported to be consistently enriched in diatom cell walls as compared to their contents of cell interiors (Muller et al., 1986; Bhosle and Wagh, 1997). The high content of these structural constituents in the extracted EPS indicates a deposit of highly degraded refractory detrital material occurred during biofilm development. In contrast, a relative low mole fraction of serine (4.1 %), threonine (5.5 %) and glycine (5.4 %) on the glass substratum at the Buffalo Bayou suggests an abundance of diatoms and/or bacteria cells compared to other substrata. Nevertheless, the composition of EPS in biofilms from White Oak Bayou demonstrates a consistent high mole fraction of those three amino acids, even from the glass substratum. This might be a consequence of the considerably lower concentration of total suspension solids (TSS) but relatively high abundance of phytoplankton in this study site. In addition, the predominance of aspartic acid and a high ratio of aspartic acid/glycine were previously reported as a sensitive indicator for the source of terrestrial plant materials (Muller et al., 1986; Bhosle and Wagh, 1997). The relatively low abundance of aspartic acid and low aspartic acid/glycine ratio thus indicates limited terrestrial plant materials in these biofilms.

4.6. Conclusions

Many studies have shown that the extraction method used for biofilms will affect the resulting EPS composition. This study compared yields of DNA, uronic acids, organic phosphorus, proteins and carbohydrates using two methods, EDTA and steaming extraction. As it turned out, EDTA might have caused significant cell disruption as well

as the release of EPS by chelating Ca^{2+} . In addition, in this study, chemical composition of EPS in biofilms was found to vary with substrata. Proteins were the most abundant components in EPS of all collected biofilms, followed by carbohydrates, except the EPS from the glass substrata. The analyses of the distribution of monosaccharides in EPS revealed potential contributors for EPS excretion in biofilms. At White Oak Bayou, the distribution of monosaccharides in biofilm EPS from the glass and plastic substrata were similar, with a high percentage of (arabinose+galactose) and with a mannose/xylose ratio of 1.6, which strongly indicates a phytoplankton contribution for the EPS. While for EPS in biofilm from the brick substratum, a relatively high percentage of rhamnose and arabinose suggests a relatively high abundance of bacteria in the biofilm. There was no visible or quantifiable biofilm on the wood surface at that site that could be characterized. In contrast, at Buffalo Bayou, the distribution of monosaccharides in biofilm EPS from the glass substratum was similar to that in White Oak Bayou. However, EPS in the biofilm from the plastic substratum showed different distribution patterns of monosaccharides, compared with White Oak Bayou. Bacteria and phytoplankton were the major contributors for the EPS. In contrast, the bacterial community was the major contributor to EPS in the biofilms from wood and brick substrata. No significant terrestrial sources were found in EPS of biofilms. Even though the hydrophobicity of the substrata was not significantly correlated with the formation of biofilms at the study sites, it is believed that multiple factors participate in regulating the formation of biofilm, such as substratum nature, hydrological conditions (e.g., TSS), as well as attachment properties of microorganisms.

CHAPTER V
DETERMINATION OF ^{127}I AND ^{129}I SPECIATION IN ENVIRONMENTAL
WATERS USING A NOVEL GAS CHROMATOGRAPHY-MASS
SPECTROMETRY METHOD

5.1. Overview

In aquatic environments, iodine mainly exists as iodide, iodate and organic iodine. The high mobility of iodine in aquatic systems has led to ^{129}I contamination problems at sites where nuclear fuel has been reprocessed, such as the F-area of Savannah River Site, South Carolina, USA. In order to assess the distribution of ^{129}I and stable ^{127}I in environmental systems, a sensitive and rapid method was developed which enables determination of isotopic ratios of speciated iodine. Iodide concentrations were quantified using gas chromatography-mass spectrometry (GC-MS) after derivatization to 4-iodo-N,N-dimethylaniline. Iodate concentrations were quantified by measuring the difference of iodide concentrations in the solution before and after reduction by $\text{Na}_2\text{S}_2\text{O}_5$. Total iodine, including inorganic and organic iodine, was determined after conversion to iodate by combustion at $900\text{ }^\circ\text{C}$. Organo-iodine was calculated as the difference between the total iodine and total inorganic iodine (iodide and iodate). The detection limits of iodide-127 and iodate-127 were 0.34 nM and 1.11 nM, respectively, while the detection limits for both iodide-129 and iodate-129 was 0.08 nM (i.e., 2pCi ^{129}I /L). This method was successfully applied to water samples from the contaminated Savannah River Site, South Carolina, USA and more pristine Galveston Bay, Texas USA.

5.2. Introduction

Iodine is a biophilic and essential trace element that exists as one stable isotope, ^{127}I and 25 radioactive isotopes. ^{129}I is of particular concern due to its extremely long half-life (16,000,000 yr) and because it is perceived to be highly mobile in the environment. The primary source of ^{129}I in the aquatic environment is from accidental and purposeful releases associated with nuclear fuel reprocessing worldwide (Raisbeck and Yiou, 1999; Schnabel et al., 2001). For example, groundwater from F-area at the Department of Energy's Savannah River Site (SRS) in South Carolina, USA is highly contaminated with ^{129}I and other radionuclides (Riley and Zachara 1992). Approximately 7 billion liters of predominantly acidic aqueous waste from nuclear processing facilities were disposed in three un-lined basins from 1955 until 1988. The groundwater still remains acidic, with pH as low as 3.2 in the middle of the plume, increasing to background pH levels of 5 to 6 at the plume fringe. It was found that plutonium and other actinides are mostly bound to sediments beneath the basins and only very low concentrations occur in groundwater. Other more mobile radionuclides, such as ^{129}I , have been detected in this groundwater, at concentrations that are exceeding the primary drinking water limit for this nuclide. The Savannah River flows along a portion of southwestern border of Savannah River Site (Figure 5.1).

field data have shown that the speciation of anthropogenic ^{129}I in the environment can be different from that of stable iodine ^{127}I with iodide/iodate ratios of ^{129}I two times higher than that of ^{127}I along the European coastal area (Hou et al., 2009).

Several methods have been proposed to determine stable iodine species (iodide and iodate) in the literature. Ion chromatography (IC) and high performance liquid chromatography (HPLC) have been used for the direct determination of iodide (Ito 1999; Bichsel and Gunten 1999). However, high levels of chloride in seawater media affect the efficiency of ion separation, and can thus compromise analytical accuracy. Although the salt effect could be avoided by adding chloride to the mobile phase (Schwehr and Santschi, 2003), the limitation of the routine use of the technique is the inadequate sensitivity of detection and difficulty in maintaining the exchange capacity of the column. The voltammetric method, which was used for the determination of inorganic iodine in open ocean waters (Wong and Cheng, 1998), is also limited by its low sensitivity and fouling of the electrodes. Inductively coupled plasma mass spectrometry (ICP-MS) has been successfully applied to analyze iodine species in fresh water, but it is often impractical for seawater samples due to the build-up of salts (Izmer et al., 2003; Brown et al., 2007; Warnken et al., 2000).

The contemporary methods used to measure ^{129}I were recently reviewed by Hou et al. (2009). Only neutron activation analysis (NAA) and accelerator mass spectrometry (AMS) provide the sensitivity required for low level environmental samples ($^{129}\text{I}/^{127}\text{I}$ ratio of $10^{-6}\sim 10^{-10}$ with NAA and $^{129}\text{I}/^{127}\text{I}$ ratios down to 10^{-14} with AMS). Before AMS measurements can be conducted, iodine needs to be separated and purified from a sample

and prepared as an AgI target. As AMS is a relative analytical method, the absolute concentration of ^{129}I is calculated by measuring separately, apart from the $^{129}\text{I}/^{127}\text{I}$ ratio, the content of ^{127}I in the samples. For samples with $^{129}\text{I}/^{127}\text{I}$ ratios higher than $\sim 10^{-10}$, 1000 fold higher amounts of ^{127}I must be added to the sample prior to chemical separation, which may hinder chemical separation by overwhelming the exchange capacity of a resin. Alternatively, liquid scintillation and γ spectrometry can be applied to determine ^{129}I after iodine is separated from other radionuclides. Both methods have very low detection limits, 20 mBq (equivalent to 0.03 nM) for γ spectrometry and 0.3 Bq (equivalent to 0.4 nM) for liquid scintillation counting.

In this study, ^{127}I and ^{129}I were distinguished by the mass of 4- ^{127}I -iodo-N, N,-dimethylaniline (247g/mol) and 4- ^{129}I -iodo-N, N,-dimethylaniline (249 g/mol). Iodide was quantified using gas chromatography/mass spectrometry, after derivatization to 4-iodo-N, N,-dimethylaniline (Mishra et al., 2000). Iodate was quantified by first reducing iodate to iodide, using $\text{Na}_2\text{S}_2\text{O}_5$, then calculating the difference between pre-reduction and post-reduction iodide. Total iodine, including organo-iodine, was determined after being converted to iodate by combustion at 900 °C. Organo-iodide was then calculated as the difference between the total iodide and total inorganic iodide (i.e., iodide and iodate). We applied the new methodology to analyze iodine species including ^{129}I and ^{127}I in SRS groundwater, in order to understand the equilibration mechanism and kinetics of ^{127}I with respect to anthropogenic ^{129}I to provide a rationale for improved remediation strategies.

5.3. Materials and methods

5.3.1. Equipment

GC-MS instrumentation consisted of an autosampler AS3000, Finnigan Trace GC and Polaris Q EI-MS from Thermo. A TR-5MS capillary column (30m×0.25 mm id, 0.25 μm) was used for separation. The injector temperature was set at 220 °C and injections (2 μL) were made in the splitless mode. For each sample run, the oven temperature was held at 90°C for 3 minutes and then increased to 220°C at a rate of 30 °C/min. The GC transfer line was set at 280 °C. The MS ion source temperature was set to 250°C. All the mass spectra were collected in full scan mode. Thermo Xcalibur™ software was used for data acquisition and processing.

A LS 6500 multi-purpose scintillation counter from Beckman Coulter™ was used to measure ¹²⁵I radioactivity in samples. All samples were counted for 10 minutes.

5.3.2. Reagents and standard solutions

Sodium 2-iodosobenzoate reagent was prepared by mixing 400 mg of free benzoic acid (Alfa Aesar, USA) with 3.8 mL of 0.2 M sodium hydroxide on a Touch Mixer (Model 231, Fisher Scientific) and diluted to 50 mL with nanopure water. The solution was filtered through a 0.45 μm polycarbonate membrane. This solution is stable for at least 4 months when stored at ambient temperature.

N, N-dimethylaniline solution was prepared by diluting 20 μL of N,N-dimethylaniline to 10 mL with methanol. Phosphate buffer (pH 6.5) was prepared by dissolving 10 g each of NaH₂PO₄ ·H₂O and Na₂HPO₄ ·7H₂O in 250 mL of nanopure water. Fresh

solutions of 0.01 M of $\text{Na}_2\text{S}_2\text{O}_5$ were daily prepared by dissolving 0.019 g of sodium metabisulfite (Fisher Scientific) in 10 mL of nanopure water.

An internal standard stock solution was prepared by dissolving 25 mg of 2, 4, 6-tribromoaniline in 50 mL of methanol. To generate the working internal standard solution, 50 μL of the stock solution was added to 10 mL methanol.

To prepare an iodide stock solution (1000 mg/L), 65.4 mg of potassium iodide was dissolved in 50 mL of nanopure water. Working solutions of 100 $\mu\text{g/L}$ and 1 $\mu\text{g/L}$ iodide were then prepared and used to generate 1-16 $\mu\text{g/L}$ and 0.1-1 $\mu\text{g/L}$ calibration curves, respectively. An iodate stock solution (1000 mg I eq /L) was prepared by dissolving 84 mg of potassium iodate in 50 mL of nanopure water. Both the iodide and iodate stock solutions were stored in glass vials at 4°C in light-proof containers and are good for one week. Working solutions were freshly prepared daily from the stock solutions.

5.3.3. Sampling

Groundwater samples were collected from the SRS F-area plume in February 2010 at well FPZ6A (Figure 5.1), where ^{129}I contamination had previously been measured at > 100 pCi/L (SRS report, contract number: DE-AC09-08SR22470). Groundwater was filtered through 0.45 μm and 0.2 μm in parallel. Then 1.2 L of 0.45 μm -filtered permeate was divided into three aliquots of 400 mL for fractionation by ultrafiltration using cartridges with pore sizes of 100 k Da, 10 k Da and 1 kDa. Each ultrafiltered fraction consisted of 40 mL retentate and 360 mL permeate. Surface seawater collected from Galveston Bay (Figure 5.2) was filtered through a 0.22 μm membrane immediately after sampling. Galveston Bay is a large estuary located along the upper coast of Texas USA.

It is connected to the Gulf of Mexico and is surrounded by sub-tropic marshes and prairies on the mainland. The water in the Bay is a complex mixture of sea water and fresh water.



Fig. 5.2 Map of Galveston Bay, the sampling site was marked by the triangle

5.3.4. Determination of iodide in aqueous samples

An aliquot of 5 ml sample or iodide standard solution was mixed with 0.5 mL of 1% acetic acid and 1 ml of phosphate buffer in a culture tube (16×150mm). Internal standard (50 μ L), N, N-dimethylaniline solution (50 μ L), and 2-iodosobenzoate solution (0.4 mL) were then added to each tube and shaken on a Touch Mixer for 1 minute. Next, cyclohexane (0.5 mL) was added to the tubes and shaken on a Touch Mixer for 20

seconds. The top cyclohexane layer was removed and placed into an auto sampler vial for GC-MS analysis.

5.3.5. Determination of iodate in aqueous samples

A 5 mL aliquot of sample or standard was mixed with 50 μ L of 1M HCl and 100 μ L of 0.01 M of sodium meta-bisulfite in a culture tube (16 \times 150mm). This solution was heated at 95°C for 30 minutes. Next, 1 mL of phosphate buffer was added and mixed. Internal standard (50 μ L), N,N-dimethylaniline solution (50 μ L), and 2-iodosobenzoate solution (1.0 mL) were added, and the solution was shaken on a Touch Mixer for 1 minute. The solutions were then extracted with cyclohexane for GC-MS analysis, as described in the previous section. Iodate concentrations were then calculated by difference using the iodide concentrations before and after Na₂S₂O₅ treatment.

5.3.6. Determination of iodine in aqueous samples

A 5 mL sample aliquot was mixed with 0.5 mL of 1% acetic acid and 1 mL of phosphate buffer. Then 50 μ L of internal standard and 50 μ L of N, N-dimethylaniline solution were added and mixed on a Touch Mixer for 1 minute. Cyclohexane extraction for GC-MS analysis was performed as described above.

5.3.7. Determination of iodide and iodate in aqueous samples with high concentrations of ¹²⁹I

A Strata SAX SPE column (anion exchange column, Phenomenex) was used to purify samples before measurement by eliminating interferences from inorganic ions and charged organic compounds in samples. The Strata SAX SPE column was conditioned with 3 mL of acetone, followed by 3 mL of methanol and 3 mL of nanopore water. Next,

the Strata SAX SPE was equilibrated with 3 mL of 1 M NaOH and 3 mL of nanopure water. After column conditioning and equilibration, 5 mL of sample that had been filtered through a 0.45 μm polycarbonate membrane was loaded and the flow through solution was collected. The column was then eluted with 5 mL of 1M NaCl. The NaCl eluent and the initial sample flow through solution were combined and brought to a final volume of 10 mL using nanopure water. This solution was then split into two 4.8 mL sub-samples to separately measure iodide and iodate as described above.

5.3.8. Determination of organo-iodine in aqueous samples

The organo-iodine concentration was determined by calculating the difference between total iodine and total inorganic iodine. Total iodine concentration was determined by combustion of aqueous samples. The procedure was based on Schnetger and Muramatsu (1996), but was modified for aqueous samples measurements. Aqueous samples (2.5 mL) were mixed with 20 mg of vanadium pentoxide (vanadium pentoxide acts as a catalyst for the rapid combustion of environmental samples) in a ceramic boat. The boat with the sample was placed into a quartz combustion tube and preheated for 8 minutes at 200°C, after which the temperature of the furnace was increased to 900°C over 10 minutes and then held steady at that temperature for an additional 10 minutes. Oxygen was used as a carrier gas during the combustion at a flow rate of 200-250 mL/min. A glass tube containing 1 mL of nanopure water was used as a receiver. The carrier was directed into the receiver by connecting glass tubing with the tapered end of quartz combustion. After combustion, the glass tubing and the tapered end of the combustion tube were rinsed twice with 0.75 mL and 0.5 mL of nanopure water,

respectively. The rinses were combined with the solution collected in the glass receiver tube (~6 mL final volume) and subjected to the iodate quantification as described above.

The three separate methods necessary for the determination of iodide, iodate, and iodine after their derivatization to 4-iodo-N, N-dimethylaniline and a fourth method to determine organo-iodine after combustion are illustrated in Figure 5.3.

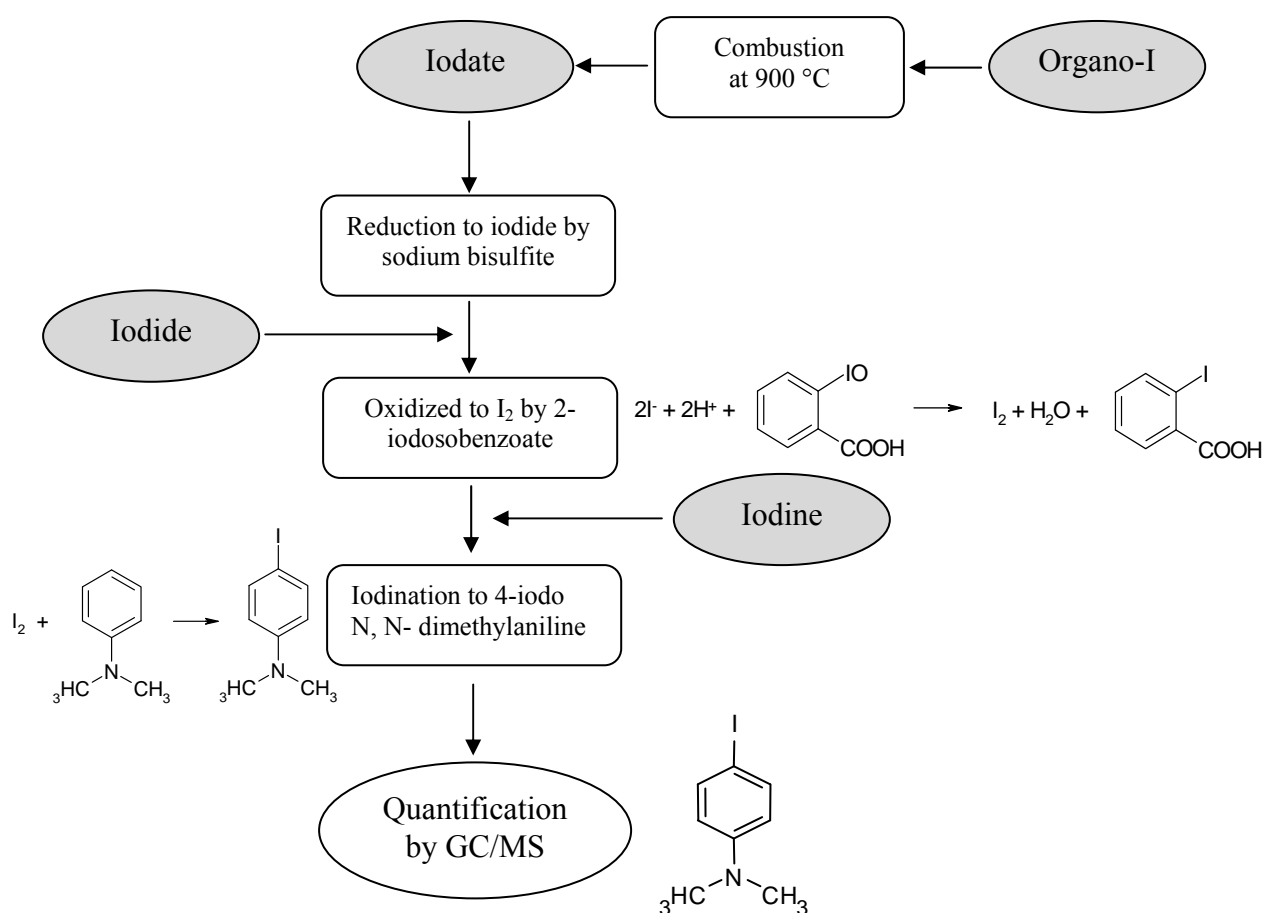


Fig. 5.3 Derivatization flowchart of iodine species to 4-iodo-N, N –dimethylaniline. The four iodine species were highlighted in the shaded circles. Each species was quantified by GC/MS after final derivatization to 4-iodo N, N- dimethylaniline

5.3.9. Chemistry of reactions

The determination of iodide is based on the oxidation of iodide with 2-iodosobenzoate to iodine and subsequent iodination of N, N-dimethylaniline (Figure 5.3). The pH optimum for these two reactions has been verified by Mishra et al. (2000) as 6.4. Moreover, the overall reaction is completed within 1 minute over a pH range from 5-7. A phosphate buffer was used to control the pH of the reactions.

The selectivity of 2-iodosobenzoate, as an oxidizing agent for I species, was validated by an examination of the published redox properties of this compound. At 25°C the redox potential of 2-iodosobenzoate was reported as: 1.21 V at pH 1, 0.53 V at pH 4 and 0.48 V at pH 7 (Shin et al 1996). Therefore, in neutral and weakly acidic solutions, 2-iodosobenzoate oxidizes iodide to iodine without further oxidization to iodate (the redox potential of iodate is >0.8 V) (Hou et al., 2009 and references therein).

Aromatic amines and phenols are exceptional iodination reagents (Shin et al 1996; Mishra et al., 2000). Mishra et al. (2000) proposed that N, N-dimethylaniline could act as an iodine scavenger that forms just a single isomer of the derivative (at the *para* position), because substitution at the two *ortho* positions are impeded by the large dimethylamino group.

5.4. Results

5.4.1. Reduction of iodate to iodide

Iodate was prepared for quantification by a two-step chemical process: 1) reduction to iodide followed by 2) oxidation and derivitization of iodide as described in Figure 5.3.

Researchers have previously used ascorbic acid as a reducing agent to convert iodate to iodide (Mishra et al., 2000), however, in our hands the redox reaction was not successful, possibly because of the high sensitivity of ascorbic acid to light and air. Instead, we used sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) as the reductant at pH 2 (Schwehr and Santschi, 2003; Hou et al., 2001).

An experiment was conducted to determine the optimum concentration of $\text{Na}_2\text{S}_2\text{O}_5$ to use as reductant for iodate at a working concentration of 78.7 nM. As shown in Figure 5.4, addition of 0.2 mM $\text{Na}_2\text{S}_2\text{O}_5$ provided sufficient reducing power to recover nearly all the iodate present.

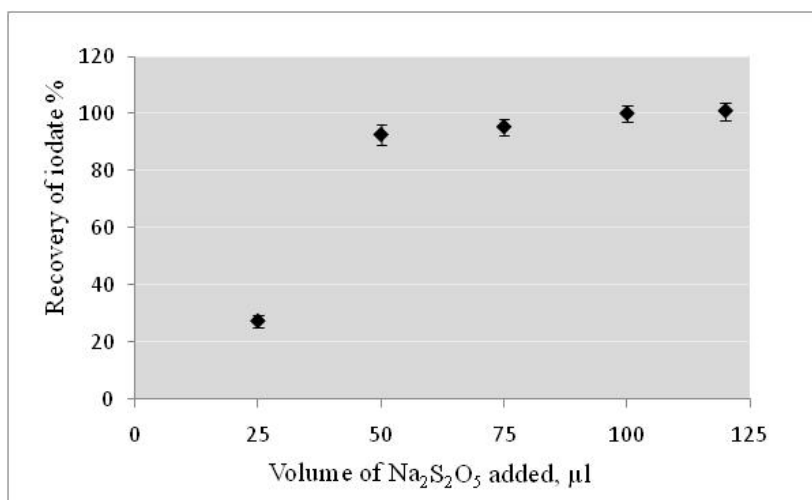


Fig. 5.4 Effect of $\text{Na}_2\text{S}_2\text{O}_5$ concentration on iodate reduction

5.4.2. Evaluation of iodosobenzoate dose for iodate measurement

During the two-step chemical process that we used to enable iodate quantification (Figure 5.3), iodosobenzoate is used as an oxidizing reagent to convert iodide to iodine, however, it can also react with excessive $\text{Na}_2\text{S}_2\text{O}_5$ from the iodate reduction step. Insufficient iodosobenzoate will result in low recovery due to incomplete conversion of iodide to iodine. Therefore, to determine the optimum level of iodosobenzoate, an assay, 0.4, 0.6, 0.8 and 1 mL of iodosobenzoate solution, was tested in a dose experiment in which concentrations of $\text{Na}_2\text{S}_2\text{O}_5$ and iodate were 0.2 mM and 78.7 nM, respectively. Even though three batches of experiments showed that 0.4 mL of iodosobenzoate resulted in an iodate recovery of $91.4 \pm 1.0\%$, 1 mL of iodosobenzoate (i.e., 4.3 mM) was eventually chosen for the assay because it consistently worked both for all ranges of standards and water samples (data not shown).

5.4.3. Distinguishing ^{129}I from ^{127}I

The chromatographic peak of iodinated N, N-dimethylaniline was identified by the retention time and verified by its mass spectrum at full scan (Figure 5.5). ^{129}I was distinguished from ^{127}I by the different mass of their iodinated product, 4-iodo-N, N-dimethylaniline, which are 249 and 247 g/mol, respectively. Therefore, to quantify ^{127}I , the mass range was set to 247 (Figure 5.6 b) and to quantify ^{129}I , the mass range was set to 249 (Figure 5.6 c). The setting of single mass range works analogously to the single ion mode (SIM) of mass spectrometry. However, the post-run setting is more informative and flexible by providing a full scan chromatogram.

5.4.4. Calibration curves

For quantitative GC-MS analysis of iodinated N, N-dimethylaniline, 2, 4, 6,-tribromoaniline was used as the internal standard. The peak area of the internal standard was gained from the full scan chromatogram (Figure 5.6 a). Quantification of iodinated N, N-dimethylaniline (for ^{127}I) was performed by integrating the appropriate peak in the chromatogram using a mass range 247 filter. The ratios of the respective areas for peaks representing 4-iodo-N, N-dimethylaniline and the internal standard, N, N-dimethylaniline, 2, 4, 6,-tribromoaniline, were plotted against the concentrations of iodide. Two calibration curves were obtained (Figure 5.7). One was for a high concentration of iodide, with a range of 1-16 $\mu\text{g/L}$ (7.9-126 nM). The other one was for low concentrations of iodide, with a range of 100 -1000 ng/L (0.8-7.9 nM). Their correlation coefficients are 0.9983 and 0.9982, respectively.

5.4.5. Detection limits

Due to the application of mass spectrometry, the sensitivity of detection was significantly increased. As a result, reagent blanks for the measurement of ^{127}I species could not be overlooked. The reagent blank for iodide was 2.51 ± 0.11 nM, and the detection limit of ^{127}I was 0.34 nM. During the quantification of iodate, the inclusion of iodosobenzoate increased the reagent blank of ^{127}I to 8.74 ± 0.32 nM and the detection limit to 1.11 nM. For the determination of ^{129}I species, no detectable ^{129}I was found in the reagents, which leads to a more sensitive detection limit of 0.08 nM (2 pCi ^{129}I /L).

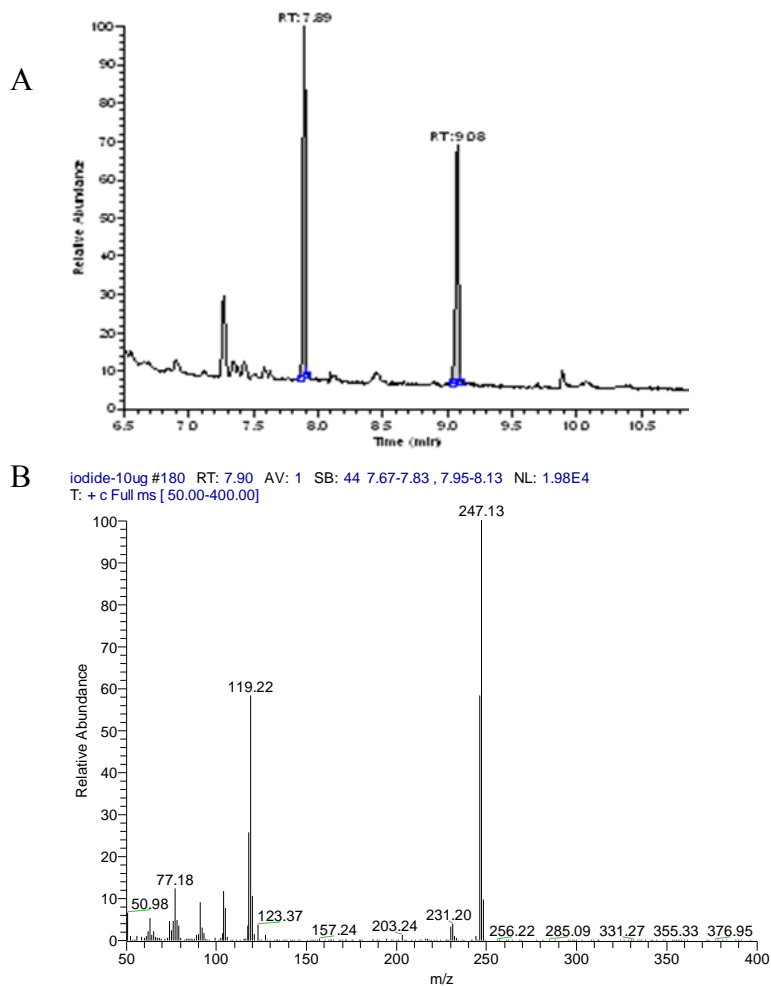


Fig. 5.5 A) Chromatogram of 16 $\mu\text{g/L}$ iodide (5 ml sample) after derivatization to 4-iodo-N, N-dimethylaniline (retention time 7.89 min), and 25 $\mu\text{g/L}$ 2, 4, 6,-tribromoaniline (retention time 9.08 min) used as internal standard. B) mass spectrum of 4-iodo-N, N-dimethylaniline

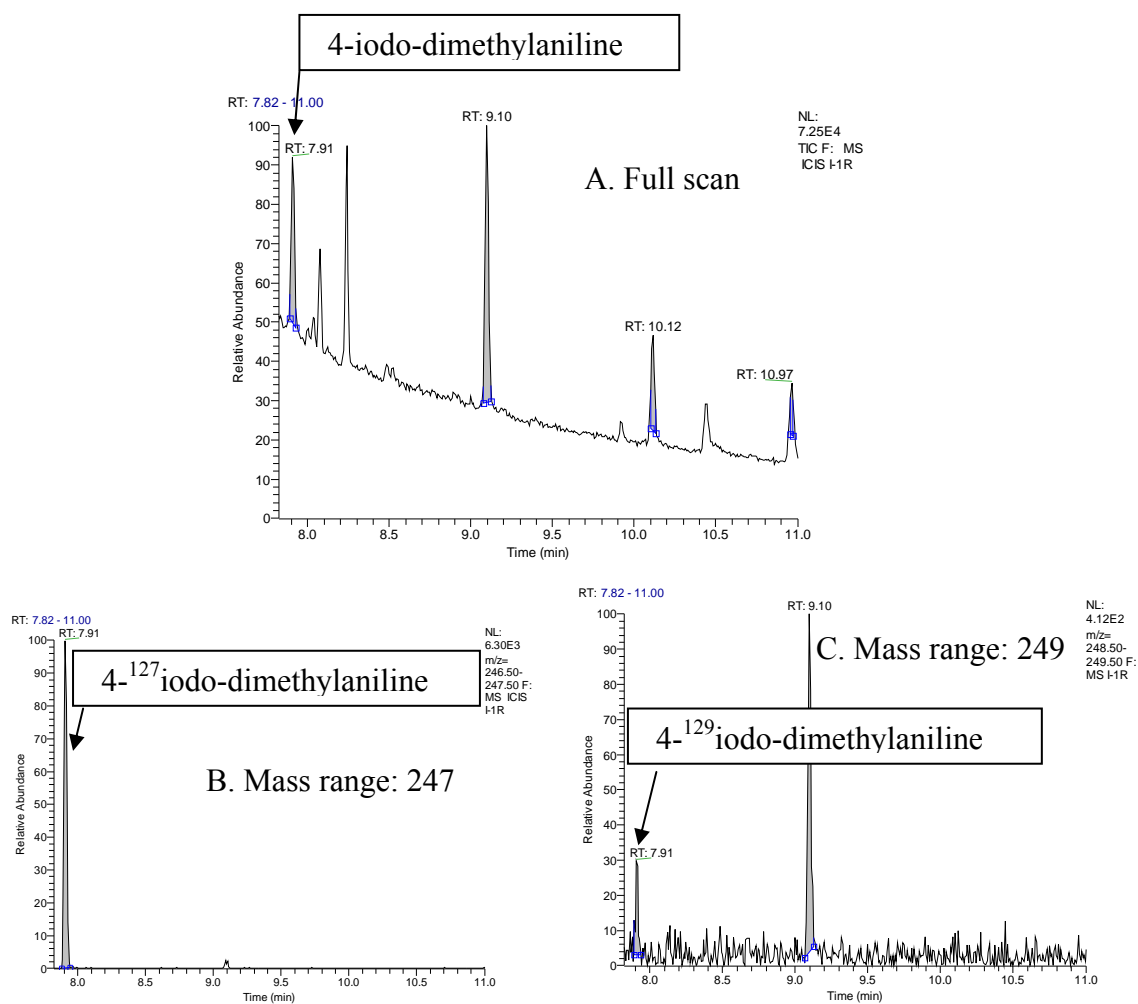


Fig. 5.6 Strategy for distinguishing ^{129}I from ^{127}I . Arrows point to the peak with RT 7.91 that represents 4-iodo-dimethylaniline. A) Full scan of GC chromatogram representing a sample containing 4-iodo-dimethylaniline and the internal standard, 2, 4, 6,-tribromoaniline (RT 9.10), B) GC chromatogram filtered for mass range at 247 for identification of 4- ^{127}I -iodo-dimethylaniline, C) GC chromatogram filtered for mass range at 249 for identification of 4- ^{129}I -iodo-dimethylaniline

5.4.6. *Quality control by standard addition to samples*

For general groundwater and seawater samples with a neutral pH of 7, iodide and iodate could be directly measured with the procedures for aqueous samples described above. Quality control was carried out by monitoring the recoveries of iodide or iodate standards added to the samples. The average recoveries of iodide and iodate were $99.8 \pm 0.9\%$ and $102.6 \pm 1.6\%$, respectively, for near neutral natural samples. However, direct measurement of iodide or iodate in SRS groundwater samples was difficult due to the acidic nature of the F-Area plume (pH 3.5-4). These low pH values altered the chemistry of the groundwater and interfered with the reduction of iodate and subsequent oxidation of iodide to iodine. Therefore, we applied a Strata SAX SPE column for cleanup of interfering compounds in these samples, with specific conditioning and equilibration steps. The average recoveries for the two batches of contaminated samples were $90.5 \pm 1.3\%$ for iodide and $95.1 \pm 0.2\%$ for iodate, respectively.

5.4.7. *Validation of total iodine quantification with known amounts of iodide, iodate, ^{125}I , and thyroxine*

Iodide and iodate (2.5 ml of 78.7 nM) were added to samples and combusted to verify the recovery efficiency of our method. The recoveries were $81.0 \pm 2.1\%$ for iodide and $85.4 \pm 1.3\%$ for iodate. In addition, 100 μL of an ^{125}I standard (0.18 mCi/mL iodide) was diluted to 2.5 mL with nanopure water and combusted. The recovery calculated by the ^{125}I activity was 90.0%. Finally, thyroxine, as a proxy of organic iodine, was mixed with a NIST 2709 reference standard (1:200). A 20 mg sub-sample of the thyroxine mixture was combusted with 20 mg of V_2O_5 . The recovery of thyroxin was $92.7 \pm 3.0\%$.

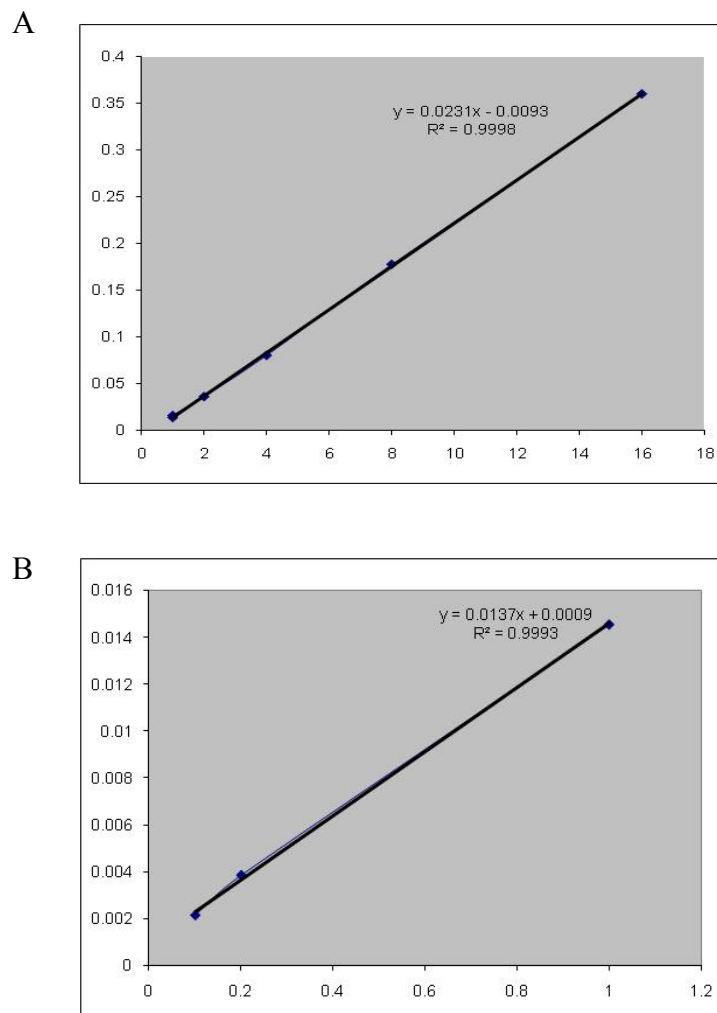


Fig. 5.7 Calibration curves for iodide quantification by derivatization to 4-iodo-N, N-dimethylaniline. A) high iodide concentration range 1-16 $\mu\text{g/L}$ (7.9-126 nM), B) low iodide concentration range 100 -1000 ng/L (0.8-7.9 nM)

5.4.8. Iodine speciation of samples

Iodine speciation in surface water from Galveston Bay was determined and compared with earlier determinations in the same estuary as Schwehr et al., (2005b) which used HPLC methods. The concentration of iodide was 67.6 ± 0.5 nM, 116.7 ± 1.0 nM for iodate and 28.5 ± 0.2 nM for organo-iodine. This result is comparable to that of Schwehr et al. (2005b), who determined 66-116 nM for iodide, 50-111 nM for iodate, and 12-158 nM for organo-iodine for this estuary. Higher concentrations of IO_3^- than I^- are reasonable as there is ample oxygen in the surface waters. Both ^{129}I and $^{129}\text{IO}_3$ were measured as lower than 0.08 nM (i.e., 73 mBq ^{129}I /L).

Size-fractionated samples from SRS F-Area well FPZ 6A were analyzed and their iodine species are shown in Figure 5.8. In all fractions, iodine mainly existed as iodide > iodate > organo-iodine. For ^{127}I , iodide accounted for 45 ~ 57% of total iodine, with a concentration of 95.7 nM in the < 0.45 μm fraction. Iodate and organo-iodine contributed nearly evenly to the remaining iodine total, except the <100 kDa fraction where significantly elevated concentrations of organo-iodine was detected due to an experimental artifact. ^{129}I species showed similar distributions in their respective fractions. The concentration of total ^{129}I was 5.3 nM (133 pCi/L) in the < 0.45 μm fraction. The ratios of $^{129}\text{I} / ^{127}\text{I}$ for iodide, iodate and organo-iodine were similar (0.02~0.03, in all fractions) which is dramatically elevated when compared to values ($^{129}\text{I} / ^{127}\text{I}$ ratios $\sim 10^{-12}$) typically observed in pristine waters (Raisbeck and Yiou, 1999).

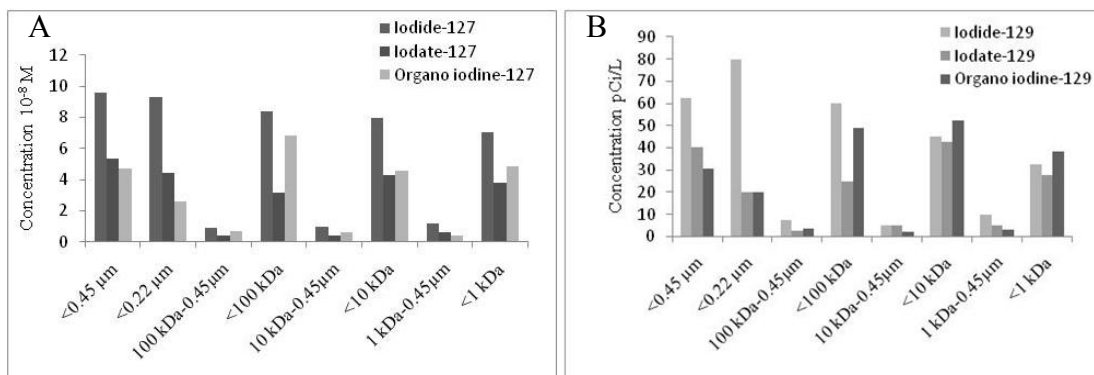


Fig. 5.8 Iodide speciation in samples from well FPZ6A at the Savannah River Site. A) ^{127}I species, B) ^{129}I species

5.5. Discussion

Currently there are no sensitive methods available for the direct determination of iodate, instead iodate is typically reduced to iodide and then measured by HPLC or IC or AMS. This approach can be problematic though, in that sample chemistry can greatly influence the percentage of iodate recovered as iodide. Indeed, the common iodate reductant, $\text{Na}_2\text{S}_2\text{O}_5$, completely failed to yield detectable iodide when applied to groundwater samples collected from a contaminated plume in this study. Waste that was deposited in the F-Area seepage basin at SRS was strongly acidic and significantly changed the chemistry of the downgradient groundwater by leaching cations (e.g., Mn^{2+}) and low molecular weight organic compounds (e.g., organic acids) from aquifer sediments into the aqueous phase. Therefore, pre-treatment of these acidic groundwater samples was required to enable effective iodate reduction by $\text{Na}_2\text{S}_2\text{O}_5$. A strong anion exchange column with a sorbent functional group of $^+\text{R}_3\text{N}$ -, Strata SAX (Phenomenex),

was used to pre-treat the samples after being equilibrated by 1 M NaOH. After equilibration with NaOH, the functional groups of the sorbent were replaced by OH⁻, which would remove Mn²⁺ as well as charged organic compounds (e.g. organic acids by either chemical reaction or anion exchange). These results highlight that the inherent chemical diversity of environmental samples necessitates the validation of iodate reduction prior to iodide oxidation, derivitization, and detection.

The distribution of iodine species in natural waters depends on many environmental factors, including chemical composition, pH, Eh and primary and secondary productivity. Under normal conditions (pH 3-10, Eh < 0.8 V), iodine should theoretically exist in freshwaters as iodide (Hou et al., 2009 and references therein). However, at the F-area of SRS, iodide only accounted for 48.8% of total iodine. The relatively high concentration of iodate (27.3%) and organo-iodine (23.9%) implies that chemical and biological factors, other than pH and Eh, are involved in regulating iodine speciation in the system. In addition, the even distribution of iodine species within each of the fractions examined suggests that organo-iodine is associated primarily with low molecular weight organic moieties. Tremendously elevated but relatively constant ratios of ¹²⁹I / ¹²⁷I for iodide, iodate and organo-iodine (¹²⁹I / ¹²⁷I ratios ~0.03) can be attributed to the spread of ¹²⁹I from nuclear waste into the groundwater where it equilibrated with stable ¹²⁷I. The similar magnitude of ratios of ¹²⁹I / ¹²⁷I for the three iodine species implies that the conversion of iodine species of ¹²⁹I must have been of recent origin, i.e., the conversion occurred within the decadal time frame after it was released to the surrounding groundwater. These consistent ratios of ¹²⁹I / ¹²⁷I also support the application

of using $^{129}\text{I} / ^{127}\text{I}$ ratios as an environmental tracer on time frames of decades (Raisbeck et al., 1995; Santschi and Schwehr, 2004).

5.6. Conclusions

Here we present a novel method for the quantification of iodine species, which distinguishes itself by providing 10-50 fold higher sensitivity than HPLC or IC without compromising accuracy. In addition, this method provides a much simpler and more convenient way to analyze ^{129}I species when compared to currently available techniques such as AMS or neutron activation methods. As such, it is a valuable complementary method to AMS for the analysis of environmental samples that are highly contaminated with ^{129}I , such as groundwater near nuclear reprocessing facilities. However, the detection limit of our method (0.08 nM or 2 pCi ^{129}I /L) does not permit the determination of ^{129}I in more pristine, natural samples. Finally, the primary innovation of this method is that it permits full speciation at ambient concentrations of ^{127}I and ^{129}I , thereby permitting greater mechanistic understanding of the terrestrial biogeochemical fate and transport of radioiodine. At the F-area of the Savannah River Sites, the ratio of $^{129}\text{I} / ^{127}\text{I}$ was found to be extremely high, up to 0.03, comparing with $10^{-6} \sim 10^{-10}$ in natural waters. Furthermore, the constant ratios of $^{129}\text{I} / ^{127}\text{I}$ for each iodine species (iodide, iodate and organo-iodine) implied fully equilibrated behavior in the groundwater. In addition, apart from iodide, iodate and organo iodine contributed 27% and 24% to the total iodine inventory. In contrast, iodate was the primary iodine

component in the water from Galveston Bay due to the abundance of oxygen in the surface water.

CHAPTER VI

CONCENTRATION DEPENDENT MOBILITY AND RETARDATION OF

IODINE SPECIES IN SURFACE SEDIMENT FROM THE SAVANNAH RIVER

SITE

6.1. Overview

Iodine occurs in multiple oxidation states in aquatic systems in the form of organic and inorganic species. This feature leads to the complex biogeochemical cycling of iodine and ^{129}I , which is a major by-product of nuclear fission. ^{129}I is among the top three risk drivers for waste disposal at Savannah River Site (SRS) due to its perceived mobility in the environment, excessive inventory, toxicity, and long half-life (~16 million yrs). To better understand the environmental consequences of radioactive contamination from ^{129}I , we conducted column experiments to investigate the sorption, transport and potential interconversion of iodine species using surface soil from the SRS. This study is unique in that we compared the mobility in groundwaters that had ambient concentrations of iodine species (10^{-8} to 10^{-7} M) to the mobility in groundwaters with artificially elevated concentrations of iodine species (~ 0.1 mM), such as are typically used in most laboratory analyses. Results demonstrate that the mobility of iodine species greatly depends on the iodine concentration used, mostly due to covalent binding of iodine to organic carbon moieties of the surface soil. At ambient concentrations, iodide and iodate were significantly retarded, while at artificially high concentrations of 0.1 mM, iodide traveled along with the water at a retardation factor of ~ 1, which might be

due to the limited sorptive capacity for anions of the surface soil. Appreciable loss of iodide during transport was observed and attributed to iodination of organic carbon, specifically aromatic C. At high input concentrations of iodate (78.7 μM), iodate was found to be completely reduced to iodide and subsequently followed the transport behavior of iodide.

6.2. Introduction

Anthropogenic ^{129}I is found in the environment mainly due to releases from fuel reprocessing facilities, with smaller amounts from atmospheric bomb testing (1945-1970s) and natural production (Santschi and Schwehr 2004 and references therein). Due to its long half-life (16,000,000 yrs), high inventory and high mobility, accidentally-released ^{129}I from fuel reprocessing facilities has migrated into groundwaters, and thus led to contamination problems. For example, the Savannah River Site (SRS) in South Carolina (Riley and Zachara 1992) is highly contaminated with ^{129}I and other radionuclides.

For both stable ^{127}I and ^{129}I isotopes, iodide (I^-), iodate (IO_3^-), and organo-iodine are the dominant forms of iodine in aquatic environment. While in seawater, iodate is the dominant specie due to the relatively high concentration of oxygen, iodide is often the main species in freshwater, as well as coastal and estuarine environments (Santschi and Schwehr, 2004; Hou et al. 2009 and references therein). As a biophilic element, iodine in mammals is mainly concentrated in the thyroid, in the form of triiodothyronine (T_3) and thyroxine (T_4). Other forms of iodine in body tissues are associated with proteins,

polyphenols and pigments. Seaweeds are known to contain water-soluble iodine, composed of iodide, organic iodine and minor amounts of iodate. The distribution of iodine species in soils and sediments depend on soil chemistry, such as pH, redox and organic carbon content. Generally, iodine in soils is found to be associated with organic matter, mainly with humic substances. There is a significant body of laboratory and environmental studies that indicate that iodine can react with natural organic matter (NOM) and become covalently bound (Steinberg et al., 2008a), i.e., to the aromatic carbon on phenolic moieties of NOM.

Iodate and iodide mobility in the subsurface environment has been studied by a number of researchers. Iodate was found to be retarded in the soils to a significantly greater degree than iodide. In batch experiments, iodide distribution coefficients (K_d , the ratio of the concentration of I^- sorbed onto sediments to the I^- concentration in the aqueous phase) are relatively low, $<10 \text{ cm}^3 \text{ g}^{-1}$, while K_d values for iodate are in the range of $1\text{-}1000 \text{ cm}^3 \text{ g}^{-1}$, depending on sediment type and microbial biomass in the sediment (Fukui et al., 1996; Kaplan et al., 2000; Schwehr et al., 2009). This retardation of iodate on soils has been noted to be related to the presence of organic matter in soils. An investigation of the abiotic reaction of iodate with natural organic matter has demonstrated that under mild conditions, IO_3^- could be reduced to iodide and partially converted to organo-iodine by covalently binding to NOM (Steinberg et al., 2008b). In addition, interconversion of iodine species during transport in soils has been noted by laboratory and field experiments. In the field, oxidation of I^- to molecular iodine (I_2) and iodate (IO_3^-) over transport distances of several meters was found in an oxic, Mn(IV)

zone of a sand and gravel aquifer after injection of high (mM) concentrations of iodide (Fox et al. 2010). Additionally, in an Fe-reducing, anoxic zone of the aquifer, injected IO_3^- was rapidly and completely reduced to I^- within 3 meters of transport (Fox et al. 2010). Hu et al. (2005) also found significant conversion of IO_3^- to I^- in their laboratory sediment column experiments.

Even though many studies have been carried out to investigate the mobility of iodine species in the natural environment, few studies were conducted at ambient concentrations of iodine species. For example, Hu et al. 2005 and Fox et al. 2010 (discussed above) applied artificially elevated concentrations of iodine species, i.e., 0.1-1 mM. However, experiments with SRS soils showed that the interactions of iodine species with soils were highly dependent on the iodide concentration added. Iodide distribution coefficients between soil and aqueous phase, K_d , increased with decreasing iodide concentration in the aqueous phase (Schwehr et al., 2009). Nevertheless, batch experiments do not exactly reproduce the behavior in the field and might overestimate or underestimate the retardation in soils or rocks, especially for most subsurface conditions. Therefore, in this study, we designed soil column experiments to investigate the transport and potential interconversion of iodine species at ambient concentrations.

6.3. Materials and methods

6.3.1. Soil sample

A surface soil sample was collected with a 7.6 cm diameter auger from a riparian ^{129}I contaminated zone located in the F-Area of the Savannah River Site, where 7 billion

liters of predominantly acidic aqueous waste from nuclear processing facilities were disposed in three un-lined basins from 1955 until 1988. As a radionuclide with high mobility, ^{129}I has been found in groundwater, at concentrations that exceed the primary drinking water standard for this nuclide (i.e., 2 pCi/L). The soil sample was stored in a zip lock bag under ice and was transferred to a refrigerator at 4 °C in the lab. The soil was characterized as having high content of organic carbon, 108 mg-OC/g dry soil, and low organic nitrogen content of 5.4 mg-ON/g dry soil.

6.3.2. Column experiment

Soil in a moist state was used in order to minimize the experimental artifacts introduced by drying soils, such as changes in the relative hydrophobicity of soils, as well as in the availability and reactive sites on organic matter, and changes in microbial activity that might have profound impact on the speciation and mobility of iodine (Amachi et al., 2005a, 2005b). Moist soil was sieved with a stainless steel sieve (nominal sieve opening: 425 μm ; US standard no. 40) by rinsing it with two volumes of artificial freshwater (ionic strength: 1 mM, pH: 6.95, Smith et al., 2002). The pH of the soil slurry was measured as 5.63 at room temperature. The soil-water slurry was concentrated by removing 80% supernatant after centrifugation at 2000 g for 30 minute. Then, the soil was resuspended into the remaining supernatant.

A Knotes glass column (15mm ID, 100mm length, 18mL volume) was packed with 16 mL of the soil slurry. Then the column containing soil slurry was connected to a peristaltic pump and was equilibrated with the artificial fresh water for 14 days at a rate of 0.18 mL/h, equivalent to approximately 10 pore volumes. After equilibration, prior to

the loading of iodide or iodate, 20 mL of artificial freshwater spiked with tritium (activity 200 kBq/L) was injected onto the column as a conservative tracer to compare the transport behavior of water with that of iodine species under the same column conditions. After finishing the injection of ^3H -spiked freshwater, the column was flushed with non-spiked freshwater until no radioactivity was found in the effluents. Effluents were collected by an automated fraction collector (Waters Fraction Collector) every 99 minutes and 59 seconds, and the radioactivity of the fractions was measured on liquid scintillation counter (Beckman Coulter_{TM} LS 6500).

Iodide solutions spiked by iodide-125 (50 kBq/L, carrier - free) were prepared in artificial freshwater at two concentration levels, 7.87 nM (ambient concentration) and 78.7 μM . Iodate solutions were also prepared in artificial freshwater, but at concentrations of 78.7 nM and 78.7 μM . The equilibrated columns were loaded with 15~20 ml of iodide solutions or iodate solutions, followed by flushing with artificial water. Effluents were collected by an automated fraction collector every 99 minutes and 59 seconds in the first 7 days, and manually collected every 12 hours for two month afterwards. The extended collection period allowed the possibility of detecting organo-iodine if present. Fractions collected were subjected to ^{125}I measurement by liquid scintillation and analyses of iodine species as described below.

6.3.3. Determination of retardation factor

The retardation factor, R_f , is a bulk property that describes the overall migration of the chemical species with respect to the water, dependent on factors that strongly affect the chemistry of soil, e.g., temperature, pH, redox potential, salinity, organic content, and

concentrations of other chemical species. It is defined as the ratio of the solution velocity to the velocity of a specific chemical (Bouwer, 1991), as given below.

$$R_f = V_{gw}/V_{sp}$$

where V_{gw} is the velocity of the water and V_{sp} is the velocity of the specific species. In addition, Bouwer (1991) also defined a relationship between retardation factor, R_f and distribution coefficient (K_d), as follows.

$$R_f = 1 + \psi K_d/n$$

where ψ is the dry bulk density and n is the porosity.

In column experiments, a breakthrough curve was obtained for each iodine species, and R_f was determined as the pore volume of the effluent in which the concentration of iodine species was 50% of the concentration of the loading solution.

6.3.4. Analyses of iodine species in fractions

Application of a recently developed method for iodide speciation determination (Zhang et al., in submission) enabled the investigation of iodide transport at ambient concentration. Briefly, the iodide concentrations were quantified using gas chromatography-mass spectrometry (GC-MS) after derivatization to 4-iodo-N,N-dimethylaniline. Iodate concentrations were quantified by measuring the difference of iodide concentrations in the solution before and after reduction by $\text{Na}_2\text{S}_2\text{O}_5$. Total iodine, including inorganic and organic iodine, was determined after conversion to iodate by the combustion at 900 °C and subsequent trapping. Organo-iodine was calculated as the difference between the total iodine and total inorganic iodine (iodide and iodate).

6.4. Results

6.4.1. Transport of iodide in surface soil of Savannah River Site

Figure 6.1 shows breakthrough curves for iodide at two concentration levels, 7.87 nM (ambient concentration) and 78.7 μM . For tritium or the low iodide concentration of 7.87 nM, breakthrough curves are plotted according to the relative concentration of tritium or ^{125}I in the effluents comparing to those in the loading solutions. Two breakthrough curves are plotted for the loading of iodide at a high concentration of 78.7 μM . One was based on the relative concentration of ^{125}I , the other is based on the relative concentration of iodide in the effluents. The agreement of the two breakthrough curves verified the reliability of the application of ^{125}I in the column experiments. It was evident that the transport of iodide in the soil columns greatly depended on the concentration of iodide that was applied. Iodide was significantly retarded in the soil column at ambient concentration (7.87 nM), resulting in a retardation factor greater than 4.3 which equals to $> 0.7 \text{ cm}^3/\text{g}$ in K_d value, assuming a porosity of 0.55 and a dry density of $2.5 \text{ g}/\text{cm}^3$ (Schwehr et al., 2009). However, the retardation factor for the transport of iodide was calculated as ~ 1.0 when the inflow concentration was 78.7 μM .

Variation of pH during the course of tritium and iodide transport (78.7 μM) was monitored and shown in Figure 6.2. The tendency for increase in pH was similar for the two species, increasing from 6.92 (freshwater) to 7.3 (effluents), which might be caused by the consumption of HCO_3^- when freshwater moved along the soil column. In addition, compared with tritium, a significant loss of ^{125}I during the transport of iodide at a

concentration of $78.7 \mu\text{M}$ was observed, i.e., the mass recovery of ^{125}I was 77.5%, while it was 90% for tritium.

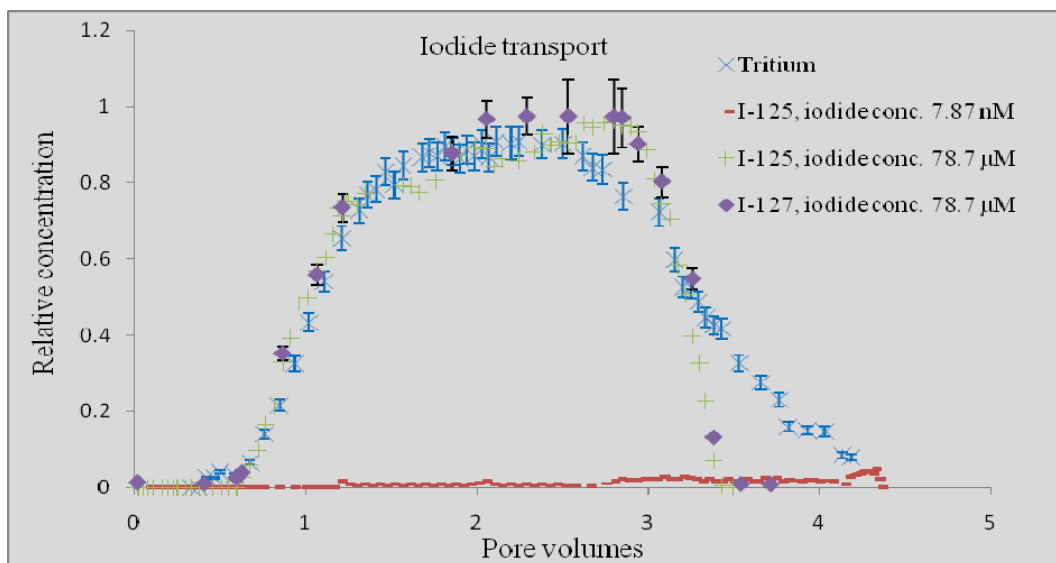


Fig. 6.1 Breakthrough curves of tritium and iodide in SRS surface soil column

6.4.2. Transport of iodate in surface soil of Savannah River Site

Figure 6.3 is a series of breakthrough curves of tritium and iodate at two concentration levels, 78.7 nM (ambient concentration) and $78.7 \mu\text{M}$. Interestingly, in the column experiment with an inflow concentration of iodate at $78.7 \mu\text{M}$, iodate was completely reduced to iodide after travelling through the surface soil. No iodate could be detected in the effluents. After the relative concentration of iodide in the effluents compared to initial concentrations of iodate in the loading solution was plotted versus pore volumes, a retardation factor of 1.1 was obtained, which is similar to that in the iodide transport experiment at the same concentration. For the low concentration of iodate (78.7 nM),

iodate or interconversion of iodate to iodide have not been observed in the effluents at the given loading volume (2 pore volumes), but longer term samples (one month) are still being run.

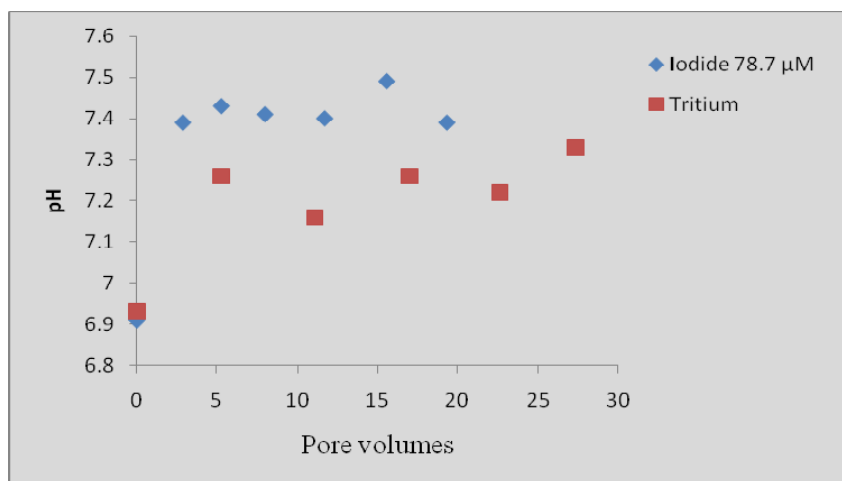


Fig. 6.2 Variation of pH in the course of transport of tritium and iodide (78.7 μM)

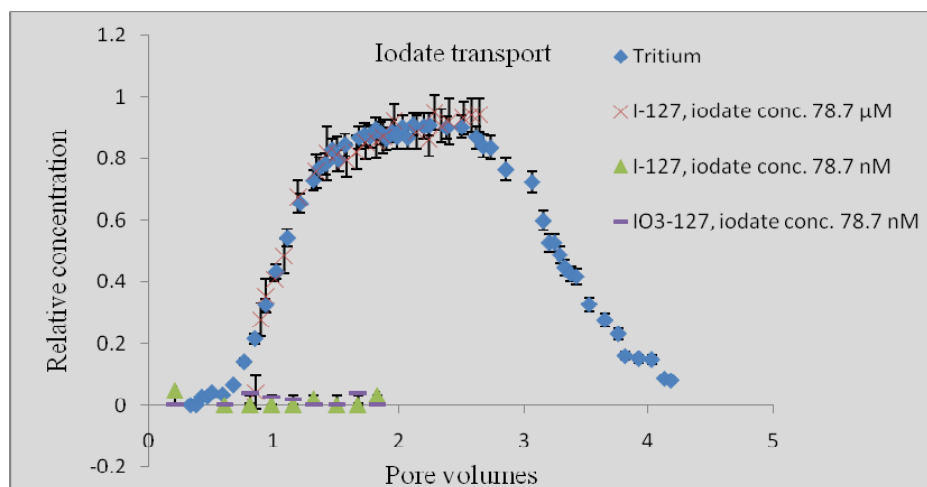


Fig. 6.3 Breakthrough curves of tritium and iodate in SRS surface soil column

6.5. Discussion

6.5.1. *Physicochemical properties affecting sorption and transport of iodide*

In the column experiments, tritium exhibited ideal breakthrough with symmetrical behavior and negligible tailing. The extent of iodide sorption onto soils has been reported to be extremely limited (Kaplan et al., 2000; Hu et al., 2005). However, significant iodide sorption has been observed in SRS subsurface samples (Hu et al., 2005; Schwehr et al., 2009), which was found to possess positively charged surfaces and thus facilitated the ion interaction between iodide and the sorbent. While reversible interactions are important for iodide retardation, our results suggest that some irreversible processes, perhaps iodination of organic matter, have occurred during the transport of iodide due to the 22% loss of iodide. In order to verify the presence of organo-iodine that was formed during the course of travelling of iodide, the column was continued to be flushed by artificial freshwater for an extended period of 1 month and will be running for one more month. Schwehr et al., (2009) observed the formation of organo-iodine by inoculating sediments with groundwater with an ambient concentration of iodide. The kinetics of iodination of thyroxine and protein are well-established in the literature (Huber et al., 1989; Pommier et al., 2005). In addition, bacterial enzymes, such as oxidases and peroxidases, were found to be involved in the halogenation of natural organic matter (Amachi et al., 2005a, 2005b). Even though the formation of organo-iodine has not been observed in the column effluents during the given transport time (i.e., one month), the fact that the surface soil was non-sterile and possessed a high content of organic carbon (108 mg/g dry soil) suggests interaction of iodide with organic matter, mediated by

bacterial enzymes. Moreover, in this study, retardation of iodide depends significantly on the concentration of iodide, i.e., iodide at ambient concentration was retarded to a greater degree in the surface soil column than at elevated concentration. The retardation of iodide implied interaction of iodide with the soil matrix, which might be through electrostatic adsorption to mineral surfaces in the soil, anion exchange with the positively charged soil surface, or irreversible covalent binding with organic matter. However, the fact that iodide apparently travelled along with the water when the iodide concentration was $78.7 \mu\text{M}$ suggests a limited sorption capacity of the soil, which must be between $78.7 \mu\text{M}$ and 7.87 nM .

In order to estimate the potential role of natural organic matter on iodide transformation, an approximation calculation was carried out as below. Given that 10 g of soil with a 50% porosity was packed in the column, there was 540 mg organic carbon (OC) in the soil column and 151 mg aromatic C as aromatic carbon (22-35% OC in typical soils, Schwehr et al., 2009 and references therein). Considering that one aromatic ring possesses one reactive site for iodide binding, there would be 2 mmol equivalent reactive sites for iodide to bind. However, iodide loading was only about 1.2 μmol (15 ml, $78.7 \mu\text{M}$), and only 0.25 μmol of I (22% loss of iodide) was found in a potentially organic form. This is much lower than the estimated 2 mmol equivalent reactive sites. Therefore reactive sites in organic matter of soil must be limited but abundant enough to interact with iodide at ambient concentrations.

6.5.2. Sorption and transport of iodate in the SRS surface soil

Iodate was reported to commonly exhibit more retardation behavior than iodide (Sheppard and Thibault, 1992; Yoshida et al., 1998). The difference in I^- and IO_3^- sorptive behavior was presumably due to the harder base nature of iodate, as compared to I^- , which would favor the hard-hard interactions with the mineral surfaces (Kaplan et al., 2000). However, in this study, iodate was completely reduced to iodide when flowing through the soil column, and thus, the subsequent transport behavior was controlled by the high mobility of iodide. The geochemical basis for the conversion of iodate to iodide will be discussed separately below.

6.5.3. Conversion of iodate to iodide

The reduction of IO_3^- to I^- could be abiotic or biotic processes. Hu et al., (2005) compared reduction capacity for different minerals. Illite, montmorillonite and kaolinite were reported to show an appreciable capacity for IO_3^- reduction. In addition, a recent study of Fox et al., (2010) indicated the complete reduction of IO_3^- occurred in the soils which contained an abundance of reductants, such as iron (II). In this study, rapid and complete reduction to I^- was also observed. The presence of iron and data about the mineralogy of the surface soil has yet to be reported. However, the high content of natural organic carbon might be an important substratum for IO_3^- reduction, as iodate is still present in groundwater with much lower organic carbon concentration (Schwehr et al., 2009; Zhang et al., in submission). The mechanism was further investigated in Steinberg et al. (2008b) study, who postulated an intermediate, I_2 or HIO that would be produced during the reaction of iodate with natural organic matter. The intermediate

species would then subsequently be incorporated into the organic matter or further reduced to I^- . As discussed in the section above, limited reactive sites for the iodination of organic matter in the soil is present in adequate concentration for IO_3^- reduction to dominate the IO_3^- transport behavior and be mediated by organic matter. In addition, microbes might contribute to IO_3^- reduction as well as the iodination of organic matter since the surface soil used in the experiments wasn't air-dried and sterilized. Therefore, control experiments where microbes will be killed by gamma irradiation will be conducted to estimate the role of microbes in the transport of iodine species.

6.6. Conclusions

Using a newly developed analytical technique, ambient concentrations of iodine species were examined for their transport behavior in the surface soil from the Savannah River Site, in contrast to artificially high concentrations typically used for soil column experiments. It was shown here that the mobility of iodine species greatly depends on the iodine concentration that was applied. At ambient concentrations, iodide and iodate were significantly retarded during the column experiment. Little or no retardation was observed in the case of high input concentrations of iodide (78.7 μ M). This probably was caused by the limited sorptive capacity of the surface soil. In addition, iodination of natural organic matter during the transport of iodide, which might be facilitated by enzymes (e.g., oxidases and peroxidases) was likely responsible for the loss of iodide during transport. The mechanism of complete reduction of iodate to iodide has yet to be

explored. Most likely, it was related to high content of natural organic carbon in the surface soil.

6.7. Future work

In order to validate the role of natural organic matter in the transport of iodine species, a soil with a low content of natural organic matter from the Savannah River Site should be prepared to compare with the soil which was used in this study. In addition, if iodinated natural organic matter formed during transport of iodine species through the columns, these organo-iodine species would not be eluted from the soils, even when running for one-month. Thus, soils in the column should be subjected to extraction and iodinated natural organic matter should be separated and characterized by FTIR and NMR (if possible).

The transport of iodine species should also be investigated by comparing fresh soils with corresponding sterile soils, in order to evaluate the role of bacteria in the iodination of natural organic matter.

CHAPTER VII

SUMMARY AND CONCLUSIONS

A series of laboratory and field investigation were carried out to elucidate the importance of natural organic matter (NOM), i.e., trace element scavenging (e.g., ^{234}Th) by exopolymeric substances (EPS), formation of biofilms, as well as interactions with ^{129}I .

In order to chemically characterize strongly Th (IV)-binding exopolymeric substances (EPS) from a single organism, cross flow ultrafiltration, along with some necessary improvements, followed by a three-step cartridge soaking and stirred-cell diafiltration was developed for isolating EPS from phytoplankton cultures, especial in seawater media. EPS isolated from a marine diatom, *Amphora sp.* was then subjected to semi-quantitative (e.g., carbohydrate, proteins) and quantitative analysis (e.g., neutral sugars, acidic sugars, sulfate) after further purification and separation by anion exchange acid. More than 90% of the isolated EPS was found to be composed of two different acidic polysaccharides, f1 and f2. Unpurified EPS, fractions f1 and f2 showed peaks at isoelectric points (pH_{IEP}) of about pH 3 during isoelectric focusing after labeling with ^{234}Th (IV), indicating that Th (IV) binding by EPS was dominated by the acidic polysaccharides in f1 and f2. The strong binding of ^{234}Th (IV) to these acidic polysaccharide-rich EPS compounds enables us to locate and closely look at the agents who are responsible for binding of ^{234}Th (IV), which is relevant for a better understanding of the oceanographic applications of $\text{POC}/^{234}\text{Th}$ ratios to particle and organic carbon dynamics in marine systems.

EPS of biofilm not only serve as a matrix for biofilm growth by holding cells together, but also provide sorption sites for inorganic and organic solutes such as nutrients and trace elements. The enrichment of inorganic and organic nutrients facilitates cell growth in biofilms. In this study, biofilms were grown on four substrata, brick, glass, plastic and wood, at two heavily contaminated Houston, TX, bayous, White Oak Bayou and Buffalo Bayou. After four weeks of growth, biofilms were collected and extracted for analysis of EPS. Proteins were the most abundant components in EPS, followed by carbohydrates. However, the chemical composition of carbohydrates or proteins, i.e., monosaccharides and amino acids, respectively, varied with environmental conditions and substrata applied, which suggests that the formation of biofilms on different substrate is regulated by specific properties of microorganism cells, environmental conditions and nature of substratum. No correlation between relative hydrophobicity of substratum and development of biofilm was found in this study.

Unlike ^{234}Th , the interaction of natural organic matter with ^{129}I are of covalent nature, i.e., through electrophilic substitution of aromatic carbon, which probably is mediated by bacterial activities. In aquatic environments, iodine mainly exists as iodide, iodate and organic iodine. The high mobility of iodine in aquatic systems has led to ^{129}I contamination problems at sites where nuclear fuel has been reprocessed, such as the Savannah River Site. In order to assess the distribution of ^{129}I and stable ^{127}I in environmental systems, a sensitive and rapid GC-MS method was developed. This method distinguishes itself by providing 10-50 fold higher sensitivity than HPLC or IC without compromising accuracy. In addition, this method provides a much simpler, more

convenient or cheaper way to analyze ^{129}I species when compared to currently available techniques such as AMS or neutron activation methods. As such, it is a valuable and complementary method to AMS for the analysis of environmental samples that are highly contaminated with ^{129}I , such as groundwater near nuclear reprocessing facilities. However, the detection limit of our method (0.08 nM or 2 pCi ^{129}I /L) does not permit the determination of ^{129}I in more pristine, natural samples. Finally, the primary innovation of this method is that it permits full speciation at ambient concentrations of ^{127}I and ^{129}I , thereby permitting greater mechanistic understanding of the terrestrial biogeochemical fate and transport of radioiodine.

At the F-area of the Savannah River Sites, the ratio of $^{129}\text{I} / ^{127}\text{I}$ was found to be extremely high, up to 0.03, compared to $10^{-6} \sim 10^{-10}$ in natural waters. Furthermore, the relative constant ratios of $^{129}\text{I} / ^{127}\text{I}$ for each iodine species (iodide, iodate and organo-iodine) implied fully equilibrated behavior in the groundwater. In addition, iodine species in the groundwater of the polluted F-area consisted of 48.8% of iodide, 27.3% iodate and 23.9% organo-iodine. In contrast, iodate was the primary iodine component in the water from Galveston Bay due to the presence of sufficient oxygen in the surface water. Each of these iodine species sorbs differently to sediment and therefore moves at different speeds through the environment. Results from column experiments using a surface soil from the F-area demonstrate that the mobility of iodine species greatly depends on the iodine concentration that is used. At ambient concentrations (7.87 nM-78.7 nM), iodide and iodate were significantly retarded, while at artificially high concentrations of 0.1 mM, iodide traveled along with the water with a retardation factor

of ~ 1 , which might be due to the limited sorptive capacity for anions of the surface soil. Microbial enzymes (e.g., haloperoxidases) could be responsible for the binding of iodide by iodination of natural organic carbon, especially the aromatic C, which caused the loss of 22% iodide during the course of column transport. At high input concentrations of iodate (78.7 μM), iodate was found to be completely reduced to iodide and subsequently followed the transport behavior of iodide. The marked reduction of iodate concentrations was probably caused by reactions with natural organic matter that was facilitated by microbial activities. Alternatively, it could have been caused by reactions with inorganic reductants (e.g., Fe^{2+}) in sediments and pore water.

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