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An Investigation of Dissolved Organic Matter in a Shallow Coastal Bay Subject to *Aureococcus anophagefferens* Blooms

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AN INVESTIGATION OF DISSOLVED ORGANIC MATTER IN A SHALLOW COASTAL BAY SUBJECT TO *AUREOCOCCUS ANOPHAGEFFERENS* BLOOMS

by

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ABSTRACT

AN INVESTIGATION OF DISSOLVED ORGANIC MATTER IN A SHALLOW COASTAL BAY SUBJECT TO *AUREOCOCCUS ANOPHAGEFFERENS* BLOOMS

Jean-Paul Simjouw
Old Dominion University, 2004
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Aureococcus anophagefferens, the pelagophyte responsible for brown tide blooms, was identified in Chincoteague Bay in 1997 and has "bloomed" there since at least 1998. *Aureococcus anophagefferens* is capable of using dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) substrates to support growth, and this utilization is hypothesized to give the organism a competitive advantage relative to other phytoplankton when inorganic nutrient concentrations are low or depleted. Because previous studies suggest dissolved organic matter (DOM) is important in initiating and sustaining brown tide blooms, a field study of the variations in DOC concentration and DOM composition was performed at two sites in Chincoteague Bay, one where brown tide blooms had been reported and another where no *A. anophagefferens* blooms had been reported before. DOM collected before, during, and after brown tide events in 2002 and 2003 was characterized in terms of bulk DOC concentration and ultraviolet/visible light absorption. Stable isotope signatures and direct temperature-resolved mass spectrometry were performed on high-molecular-weight-DOM (HMW-DOM) isolated by ultrafiltration. Results from 2002 suggest that during the brown tide bloom, N-enriched HMW-DOM was released into the surface water and that this material was optically active and more aromatic. Comparison of results from 2002, a drought year, and 2003, a

wet year, show that spring DOM pools differed between the two years in DOC concentration and DOM composition; however, brown tide blooms developed in early summer of both years. During all the brown tide blooms monitored, the DOM pool shifted in composition, probably due to input of DOM by *Aureococcus anophagefferens*. In an attempt to expand the portion of DOM that can be molecularly characterized, the combination of ultrafiltration and C_{18} disk solid-phase extraction (SPE) for the isolation of DOM was also investigated. Using C_{18} SPE on LMW-DOM samples (ultrafiltration filtrate) increased the recovery of DOC from the total sample to about 70%, compared to the approximately 50% isolated within the ultrafiltration retentate alone.

This dissertation is dedicated to Jan Simjouw (1940-2000).

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I like to start by thanking my advisor Liz Minor for giving me the option to finish my Ph.D. career and for all the work she did and advice she gave along the way. I was fortunate she took a chance with me. I also like to thank my dissertation committee members Fred Dobbs, Margie Mulholland and Rodger Harvey. They helped me make this dissertation a presentable body of work.

For me, writing this dissertation was also a time of reflection on seven (!) years at Old Dominion University and the Norfolk area. When I arrived in Norfolk in August 1997 wearing my wooden shoes, tulips in my right hand and a suitcase in my left hand, I had no idea what I was getting myself into. Luckily, Scott Kline picked me up at the Norfolk International Airport and thanks to his organizational skills, which he exhibits to this day, I had an apartment to live in, a social security number, all my paperwork filled out, and a coffee maker (!) within two days. Scott has been a friend throughout my stay in Norfolk and I thank him for that.

Several people that came in the same year became friends stimulated by many drinking sessions and dart games in a bar. Hae-Cheol Kim, Andres Sepulveda (the Iced Tea King) and Isaac Schroeder joined me on many occasion in Batterson's at Colley Ave. (now known as Tanner's Creek) after a late class in physical oceanography. I know I was the only one of the group that didn't really know what was going on in that class. I think that the after class sessions increased my understanding in physical oceanography, so I like to thank them for that and their continued friendship to this day. Isaac was also a good person to talk to about that weird sport everybody in the rest of the world calls

football but in the USA is called soccer. We spent many hours watching (library time), and talking about "soccer" and we even played together in some intramural and pickup games. It was nice to have that outlet. My neighbor of the last two years, Oren Gruber, and Aron Stubbins, a post-doc in Ken Mopper's lab, were also good friends to talk footy and/or science with.

Another friend I made during my stay at ODU, who thought I was some weird dude from abroad at first (I thought she was annoying, so much for first impressions) is Shannon Meseck. She later agreed at the Silver Bells Chapel in Las Vegas (with 'Elvis' as a witness) to spend the rest of her life with me. Shannon was at one time the only reason I stuck it out at ODU and she was a tremendous support when my father became terminally ill.

This leads me to my biggest supporters for all my crazy ideas and decisions I made in my life so far and will make in the future to chase my dreams: my mother Corry Bruël-Simjouw and my father, Jan Simjouw. They showed by example how to stay positive, enjoy all the little things in life, and how to be a decent human being. My parents were always proud of the things I did and accomplished, and I know my father would be proud of me now. Dad, I miss you. My sister Natasja and her "boys", Django and Rafi, kept me entertained with funny postcards and beautiful drawings. I regularly have to update the fridge with new works of art. Here I also like to thank Shannon's family; especially Art, Carol, Aimee, and Granny for letting me become a part of their family including all the shish kebab duties.

In a random order I like to thank several people that helped me along the way or just made it nice to come to work: Pete Bernhardt, George Boneillo, Dana Oblak,

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Finally, I like to thank the fortune cookie fortune writers from Brooklyn, New York for keeping the morale high with phrases like: "Everybody around you is rooting for you. Don't give up!" and "Keep it simple. The more you say, the less people remember." along with "Love truth, but pardon error." And the Wednesday before I would see Shannon again: "There's a good chance of a romantic encounter soon." I never played their lucky numbers for the lotto, perhaps I should have.

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SECTION 1

GENERAL INTRODUCTION

1.1. *AUREOCOCCUS ANOPHAGEFFERENS*

Algal blooms that cause harm to the ecosystem, for instance by the production of toxins or by reducing the feeding capabilities of filter feeders, are grouped under the common descriptor harmful algal blooms (HABs). Even though these algal blooms can be natural phenomena, it appears that the number and frequency of HABs is increasing. This perception could be partly caused by the increasing number of studies, improved identification of the HAB species, and greater knowledge about these bloom events.

Aureococcus anophagefferens is a picoplankton (~2 μm diameter) that occurs in coastal bays along the eastern coast of the USA. An unusual bloom in Narragansett Bay, Rhode Island, during 1985 led to the identification and description of this organism (Sieburth et al. 1988). Its lack of obvious morphological features and small size were reasons the alga was not identified before the bloom event in Narragansett Bay, RI (Sieburth et al. 1988; Anderson et al. 1993). Due to the brown discoloration of the water when *Aureococcus anophagefferens* cell densities are high (e.g. 250,000 cells ml^{-1}), a bloom is also referred to as a brown tide bloom.

A brown tide bloom is harmful for coastal bay ecosystems because of the high cell density these blooms can reach. A first noted impact of a brown tide bloom event was in the feeding capabilities of the blue mussel *Mytilus edulis*. Tracey (1988) demonstrated reduced clearance rates of bloom algae independent of cell size and extra-

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The model journal for this dissertation was *Estuaries*.

cellular exudates. The study also showed that algal concentrations below $2.5x10^5$ cells ml^{-1} did not reduce the feeding of *Mytilus edulis*, suggesting a threshhold value before the bloom became harmful.

Even though *A. anophagefferens* does not appear to produce a toxin, the sheer density of the bloom alone has a significant impact by increasing the light attenuation, which has an effect on the submerged aquatic vegetation. Due to the high light requirement of eelgrass (*Zostera marina*) and because a brown tide bloom occurred during the growth season, *A. anophagefferens* was linked to the extensive loss of eelgrass beds on Long Island (Dennison et al. 1989). Indirect effects of the brown tide blooms, due to the reduced eelgrass beds, include decreased recruitment for shellfish and limiting nursery areas for fish in the coastal bay (Tettelbach and Wenczel 1993).

After initial *A. anophagefferens* blooms in Narragansett Bay, RI and in neighboring coastal waters and bays of Long Island and New Jersey, several subsequent blooms were reported for these areas. The development of an immunofluorescent technique (Anderson et al. 1989), one that uses a species-specific antibody attached to the outer cell wall to analyze samples for the presence of *A. anophagefferens*, increased the knowledge on areas that can possibly be impacted by brown tide. Surveys conducted in 1988 and 1990 for the presence of *A. anophagefferens* using the immunofluorescent technique indicated a population distribution centered around Long Island but also with more northern (New Hampshire) and southern bays and coastal waters (New Jersey) testing positive. The 1990 survey indicated the presence of *A. anophagefferens* was limited southward to the coastal bays of New Jersey, and that no positive sites were identified further south in Delaware, Maryland, and Virginia (Anderson et al. 1993).

Another detection and enumeration technique using quantitative polymerase chain reaction (qPCR) as reported in Popels et al. (2003) was also used to investigate the distribution of *A. anophagefferens* along the eastern United States. This study identified the presence of the alga from Delaware Bay south to northern Florida (Popels et al. 2003), significantly extending the distribution range reported in Anderson et al. (1993).

Because *A. anophagefferens* blooms significantly impact the ecosystem and fisheries, and because *A. anophagefferens* appears to be spreading along the east coast, it is important to investigate the factors that initiate and sustain these blooms. The fact that *A. anophagefferens* is present in certain coastal bays, but does not reach bloom densities, suggests that certain favorable conditions must be met to initiate a brown tide bloom.

A. anophagefferens bloom formation occurs mostly in May through July, indicating that surface water temperature might be a limiting factor in the growth of the organism. The highest growth rates of *A. anophagefferens* were measured at 20 and 25 °C in laboratory studies, but these studies also indicated that similar growth occurred at 5 °C, when given enough time for the organism to adapt (Cosper et al. 1990; Bricelj and Lonsdale 1997). Even though these studies do not fully explain the summer blooms, this physiological flexibility is important for the species to uphold the population in coastal bay ecosystem during the fall and winter months.

A similar relationship is exhibited between the salinity of the surface water and the growth of *A. anophagefferens*. Brown tide blooms occur in a relative broad salinity range of 18 to 32 psu (Cosper et al. 1990; Anderson et al. 1993; Glibert et al. 2001) while laboratory studies, however, indicate significant reduction in growth rates at salinities below 28 psu (Cosper et al. 1990). It thus appears *A. anophagefferens* is a saltwater

species that can adapt to lower salinities, possibly when other factors stimulate growth. In this context, it is important to note that lower salinities are obtained when freshwater input into coastal bays increases, simultaneous increasing the nutrient load to the system.

The input or presence of inorganic nutrients and the relationship with brown tide blooms has been a focus of study since the occurrence of the first *A. anophagefferens* bloom. Several studies have focused on inorganic nutrients and the impact on the initiation and maintenance of brown tide blooms, either by field measurements or with the use of laboratory cultures. Keller and Rice (1989) conducted mesocosm studies with Narragansett Bay water with *Aureococcus anophagefferens* present. These researchers found that this alga could grow, but not bloom, under low inorganic nutrient concentrations compared to other naturally present phytoplankton species. When they enriched the mesocosm water with inorganic macronutrients (NH_4^+, PO_4^{3-} and SiO_4), a full scale bloom failed to develop, but they did report other species, mainly diatoms, would appear after a short increase in brown tide density. Keller and Rice (1989) thus concluded that blooms of *Aureococcus anophagefferens* were not related to either a low or high nutrient level in Narragansett Bay water. Cosper et al. (1989a) reported similar observations from Long Island bays. These researchers could not find a relationship between the distribution of the brown tide bloom and the levels of macronutrients in the bay waters. They did, however, find a correlation between freshwater input pulses, rainfall runoff and possibly groundwater, with the initiation of brown tide blooms. Cosper et al. (1989a) speculated that these freshwater sources would increase the organic and/or nutrient concentrations. In addition, changes in inorganic nutrient levels (nitrate, nitrite, phosphate and ammonia) in Long Island bays during brown tide bloom years were not

different from pre-bloom years, also suggesting that the nutrient input alone was not a cause of brown tide blooms in these bays (Cosper et al. 1989b). Nutrient addition experiments, where both inorganic and organic nutrients were added to water samples taken prior to, during, and after a brown tide bloom in West Neck Bay (Long Island), indicated augmented growth of *Aureococcus anophagefferens* (Gobler and Sañudo-Wilhelmy 2001). In most samples, inorganic and organic N addition (nitrate and urea respectively) resulted in a similar response of the brown tide species but did not result in an increase in the relative abundance of *A. anophagefferens*. However, the addition of organic carbon in the form of glucose to the water samples did result in augmented growth and relative abundance of *A. anophagefferens* (Gobler and Sañudo-Wilhelmy 2001a). These results indicated that inorganic nutrients play a role in the growth and sustained presence of *A. anophagefferens* in coastal bays, but that organic nutrients could be instrumental in the development of brown tide blooms. Similar observations were made by Gobler et al. (2002) from nutrient addition experiments using water samples from Great South Bay (Long Island, NY) during a brown tide in the fall of 1999.

Thus besides inorganic nutrients, *A. anophagefferens* has also been shown to be able to utilize organic compounds. Dzurica et al. (1989) isolated and cultured *A. anophagefferens* from a summer bloom in 1986. Using ^{14}C labeled glutamic acid and urea as a sole nitrogen source, the researchers found uptake and incorporation of these compounds by *A. anophagefferens*. Glutamic acid also appeared to be a source of carbon for *A. anophagefferens* along with labeled glucose during uptake experiments. These experiments by Dzurica et al. (1989) indicated that *A. anophagefferens* might have a competitive edge over other phytoplankton due to the utilization of organic compounds

for their carbon and nitrogen needs.

Several other studies have looked at the use of organic compounds by *A. anophagefferens* as a N or C source. The uptake of dissolved organic nitrogen (DON) by *A. anophagefferens* from Shinnecock Bay (Long Island, NY) was investigated by Berg et al. (1997). In a study using ^{15}N labeled urea, amino acids from algal extract, glutamic acid, lysine, NH_4^+, and NO_3^-, Berg et al. (1997) observed the uptake of organic nitrogen by *A. anophagefferens* in significant proportions. They also measured a greater uptake capacity for urea than other compounds used in this study. From this result, the researchers suggested urea might be the preferred substrate because urea contains both nitrogen and carbon. Lomas et al. (2001) also measured a high specific uptake rate of urea by *A. anophagefferens*. They also found *A. anophagefferens* cultures grown on urea under low light conditions incorporated both the carbon and the nitrogen into particulate biomass. Lomas et al. (2001) concluded from these results that *A. anophagefferens* possessed the ability to acquire carbon for growth independent of photosynthesis. Mulholland et al. (2002) used dual-labeled (^{13}C and ^{15}N) amino acids glutamate and alanine as substrates to investigate the uptake of these organic compounds by *A. anophagefferens* during a bloom in Quantuck Bay (Long Island, NY) and in cultures. They concluded the amino acids were taken up and the amino acid uptake was an important source of nitrogen, and additionally supplied *A. anophagefferens* with a significant portion of the carbon required for growth supplementing photosynthetic carbon acquisition. This study again showed that *A. anophagefferens* can use organic compounds to enhance the uptake of nitrogen and carbon, an ability which could give the alga a competitive edge over other phytoplankton when inorganic nitrogen concentrations

in the water are low and when photosynthesis is not optimal, for instance due to self-shading or at night.

All of the studies discussed so far were conducted with field samples or in cultures with the natural bacteria assemblage present. It is possible that the results from these studies were influenced by the presence of the bacteria in the samples. Berg et al. (2002 and 2003) investigated the utilization of dissolved organic nitrogen (DON) substrates by *A. anophagefferens* in axenic (bacteria free) cultures. Comparisons of growth of *A. anophagefferens* on a wide variety of nitrogen sources in non-axenic and axenic conditions showed better growth of the axenic algal culture, suggesting no direct connection between *A. anophagefferens* and heterotrophic bacteria (Berg et al. 2002). Results from this study also indicated *A. anophagefferens* could hydrolyze and potentially utilize several organic nitrogen compounds like urea, small peptides and even chitin derivatives. To further investigate the possibility of the utilization of more complex organic nitrogen sources Berg et al. (2003) used high molecular weight DON (HMW-DON), > 1 kDa, as a sole nitrogen source *for A. anophagefferens*. This HMW-DON was collected from porewaters in West Neck Bay (Long Island, NY), concentrated using ultrafiltration, and then chemically characterized. Berg et al. (2003) reported that 25 to 36% of the nitrogen in the HMW-DON was available for growth of *A. anophagefferens*. This available fraction corresponded with the hydrolysable amino acid pool in the porewater HMW-DON pool. The data suggested that *A. anophagefferens* preferentially utilized the labile DON fraction, possibly introduced by the decline of a preceding phytoplankton bloom (Gobler et al. 2002) of the total HMW-DON pool. More refractory HMW-DOM collected from surface seawater (C:N ratio of 17 compared to 9 for the

porewater HMW-DOM) did not support growth of *A. anophagefferens* in culture studies (Berg et al. 2003).

It is now well established that *A. anophagefferens* is capable of utilizing organic compounds for growth. It also appears that not only low molecular weight, < 1 kDa, compounds can be utilized, but that *A. anophagefferens* can also utilize labile fractions of the HMW-DON pool. Even though Berg et al. (2003) did not measure the HMW-DOC utilization, it is possible that carbon was also utilized for growth based on the results from the study by Mulholland et al. (2002) using dual-labeled low molecular weight amino acids. Based on all the above results, DOM present in Chincoteague Bay or a change in the lability can cause and possibly sustain a bloom of *A. anophagefferens* in this system.

Several sources, such as *in situ* production/consumption (Gobler and Sañudo-Wilhelmy 2003), sediment porewater fluxes (Berg et al. 2003) or changes in groundwater flow (Gobler and Sañudo-Wilhelmy 2001b) could be likely candidates to change the overall DOM pool. Changes in the overall DOM pool due to phytoplankton blooms within a coastal bay system could also be responsible for sustaining the brown tide. More information on DOM characteristics, on a bulk and a molecular level, and the interactions of this DOM with an *A. anophagefferens* bloom event, is necessary to understand the development and impact of brown tide events in coastal bays.

In this thesis, the concentration and characteristics of DOM were studied at two sites in Chincoteague Bay on the Eastern Shore of Virginia and Maryland, USA (Fig. 1). At Public Landing, brown tide blooms had been reported previously, while at Greenbackville, no brown tide blooms have been reported. For this study I sampled both sites for *A. anophagefferens* abundance, chlorophyll abundance and DOM

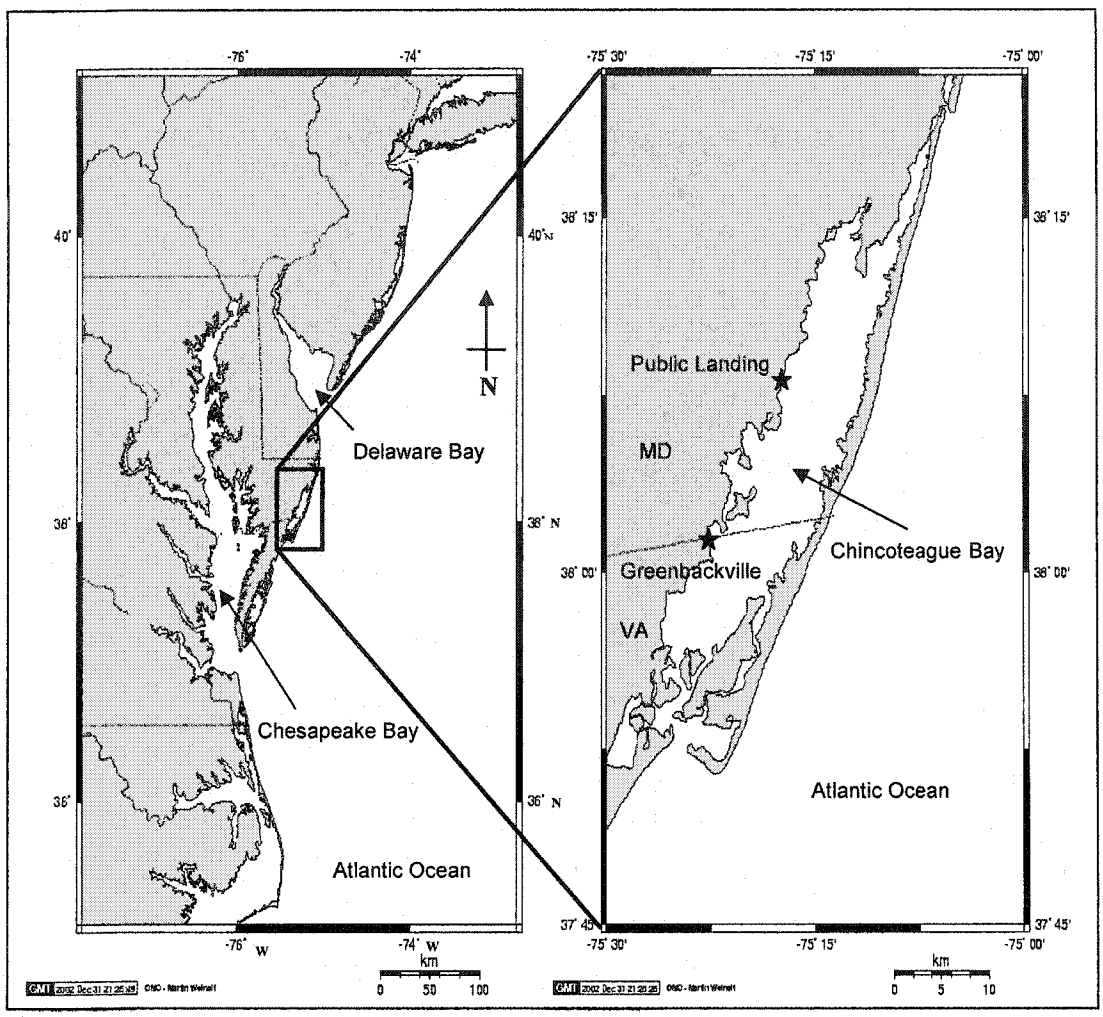

Fig. 1. Map of the sample sites in Chincoteague Bay used in this study. Greenbackville (GrBk) is on the Virginia-Maryland border. Public Landing (PL) is in Maryland, USA.

characterization on a bulk and molecular level. Sampling occurred on a monthly, biweekly, or weekly basis depending on bloom conditions of *A. anophagefferens*. Sampling at both sites allowed for comparison of the DOM pools present at the sites and the growth and bloom of *A. anophagefferens*.

1.2. DISSOLVED ORGANIC MATTER CHARACTERISTICS

Dissolved organic matter (DOM) comprises the largest amount of reduced organic carbon and is also the largest dynamic reservoir of reduced organic carbon in the ocean. The oceanic DOM pool (~10^{18} g carbon) is larger than the atmospheric CO_2 reservoir and is about the same magnitude as all of Earth's living vegetation (Hedges 1992). DOM is important in the carbon cycle, the scavenging and solubilization of trace contaminants, and biogeochemical cycles of other elements (Hedges 1992; Guo and Santschi 1997). In addition, DOM introduced in coastal regions can be a considerable food source for heterotrophic organisms present in the system (Lobbes et al. 2000). The cycling and interaction of dissolved organic matter with heterotrophic organisms is complex and is still open for debate. This lack of understanding is mostly due to the poor characterization of the DOM, with respect to its molecular structure, size distribution, and bioavailability. Direct analysis of DOM in seawater is preferred to avoid contamination and artifacts in the measurements, but is extremely challenging due to the low concentration of the DOM in a highly salty solution (Benner 2002) and the chemical heterogeneity of the DOM itself (Mannino and Harvey 2000).

Several techniques have been developed to concentrate DOM and possibly remove the salt matrix (Thurman 1985; Aiken 1985; Leenheer 1985). Ultrafiltration is a

technique that has been used most frequently to concentrate DOM from open ocean, estuarine, and coastal water samples and will be discussed further below.

1.2.1. Ultrafiltration

Ultrafiltration is a method by which macromolecules are separated according to molecular size by filtration through a membrane. Macromolecules with a smaller molecular size than the membrane pore size, defined as the molecular weight cut off, are removed along with the sample solution, which results in concentration of matter with a higher molecular size than the membrane pore size. For the stirred cell approach, which is used in this thesis, sample solutions are placed in a pressure cell with the membrane at the bottom. An inert gas is used to pressurize the sample cell to promote filtration of the sample solution. To avoid polarization of macromolecules and clogging of the membrane, a magnetic stirrer bar is suspended slightly above the membrane surface. Matter with a smaller molecular size than the membrane pore size is removed from the cell as filtrate. A schematic setup is shown in Fig. 2. A wide variety of membranes with different pore sizes can be used to isolate different size fractions of DOM.

Ultrafiltration techniques were initially developed to investigate the molecular weight distribution of DOM, mostly humic substances, in natural water samples to elucidate interactions within the different size classes (e.g. Wershaw and Aiken 1985; Swift 1985; Benner 1991). Interest in colloidal matter, operationally defined as matter between 0.2 μm and 1000 Da, in natural water and its role in biogeochemistry resulted in increased use of ultrafiltration as a technique to concentrate and isolate DOM from seawater. About 30-35% of oceanic surface water DOM can be repeatedly isolated

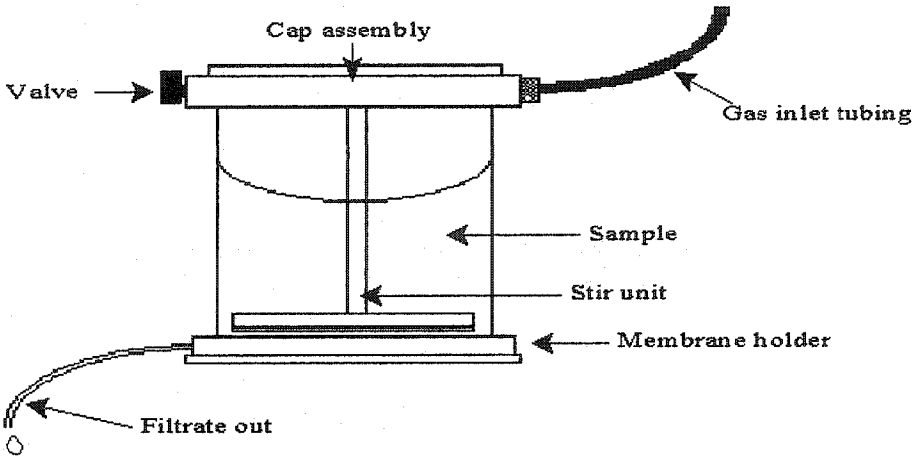

Fig. 2. Schematic of a stirred cell ultrafiltration setup.

using ultrafilters with a nominal pore size of 1000 Da (e.g. Carlson et al. 1985; Benner et al. 1992; McCarthy et al. 1996; Guo and Santschi 1997). Several characterizations have been done on the retentate of the ultrafiltration procedure using chemical techniques to evaluate DOM cycling and to determine sources and sinks of DOM (Benner 2002 and references therein). Profound advantages of the ultrafiltration technique are 1) the minimal sample handling because the separation is mostly based on the molecular size of the dissolved material, so there is no need for pH adjustments, and 2) the sample matrix of the DOM can be removed without removing the DOM sample from the system by additional desalting and concentration.

The increased interest in colloidal matter research also resulted in the development of other ultrafiltration approaches, such as cross-flow filtration (Buesseler et al. 1996). Cross-flow filtration is capable of handling larger sample volumes in less time compared to the stirred cell technique and is thus the preferred approach for studies in environments with low DOM concentrations.

A cut-off of 1000 Da is often used as a differentiation between high molecular weight (HMW) and low molecular weight (LMW) DOM in samples. LMW-DOM was originally considered as the readily available DOM fraction because this could be transported directly into bacterial cells (Münster and Chróst 1990). However, Amon and Benner (1994, 1996) showed that DOM decreases in bioavailabity with decreasing molecular size, and that in addition to the molecular size of the DOM, its nutritional value is also important for its bioavailability. In lakes, estuaries, and the coastal ocean, labile DOM is primarily thought to originate from autochthonous sources, like phytoplankton and submerged macrophytes, while more refractory DOM is from

allochthonous sources, and has experienced longer exposure time to microbial degradation (Lindell et al. 1995).

1.3. CHARACTERIZATION OF ULTRAFILTERED DOM

Using ultrafiltration, HMW-DOM is isolated from the sample for further analysis. In this study, the HMW-DOM fraction is characterized using mass spectrometry techniques, by determining stable isotope signatures and on a molecular level, by direct temperature-resolved mass spectrometry (DT-MS).

1.3.1. Stable Isotope-Monitoring Mass Spectrometry

To investigate the cycling of DOM, isotope studies (^{13}C and ^{15}N) have been conducted. Isotopic signatures, including $\delta^{13}C$, and $\delta^{15}N$, have been powerful tracers to establish the possible sources and sinks of DOM in the oceans (Druffel and Williams 1992). Even though isotope studies usually provide a lower degree of "specificity" with respect to biological sources compared to organic biomarkers, the isotopic signature can yield integrated information about the sources and cycling of the bulk DOM pool (Bauer 2002).

Stable isotope ratios are calculated in terms of

$$\delta\,{}^{X}Y = (R_{sample} / R_{standard} - 1) \times 1000 \qquad (1)$$

where ${}^{X}Y$ is either ^{13}C or ^{15}N and R is the ratio between ^{13}C to ^{12}C or ^{15}N to ^{14}N in sample or standard (PeeDee Belemnite for the carbon isotope signature and atmospheric N_2 for the nitrogen isotope signature).

The difference in both metabolism and inorganic nutrient sources in primary

productivity results in unique marine and terrestrial isotope signatures. The isotopic ratio of carbon in marine organic matter is brought about by the photosynthetic fixation of CO_2 or bicarbonate by phytoplankton. Fractionation of ^{12}C in preference to ^{13}C occurs at the enzymatic level, the carboxylation step by ribose 1,5-biphosphate carboxylase (RuBP), which causes reduced organic compounds to be depleted in ^{13}C. A similar fractionation is also seen for terrestrial C_3 plants. Other factors that can contribute to isotopic fractionation during inorganic carbon fixation include for instance temperature, CO_2 partial pressure, and phytoplankton community structure (Degens et al. 1968; Rau et al. 1991; Falkowski 1991). The $\delta^{13}C$ studies reveal a difference between terrestrial (-27 ‰) and marine (-20 ‰) sources. Guo et al. (1996) used $\delta^{13}C$ signature of different ultrafiltered DOM fractions to distinguish between the sources of the DOM and the mixing behavior of the DOM in the water column. Guo and Santschi (1997) measured the $\delta^{13}C$ signature of HMW DOM along the salinity gradient in both Galveston Bay and Chesapeake Bay. They observed a distinct terrestrial signature in the upper part of the bays, after which an increase in the $\delta^{13}C$ signature to a more marine source material was observed. They proposed removal of the terrestrial HMW DOM in the estuary as the reason for the heavier signature at the mouth of the bays.

The nitrogen isotope signature is mostly set by the inorganic sources of nitrogen used by primary producers. The ultimate nutrient source for terrestrial matter is atmospheric nitrogen, which is operationally defined as 0 ‰, and fractionation changes the signature significantly from this value. For most marine algae the main source of nitrogen is dissolved inorganic nitrogen in the form of ammonia or nitrate, with a range of 6 to 10 ‰, (Ostrom and Macko 1992). However the isotope signatures of the nutrients

could also be variable depending on the source, e.g. nitrate in groundwater influenced only by atmospheric deposition ranges between +2 to +8 ‰, while nitrate derived from human and animal waste ranges between +10 to +20 ‰ (McClelland et al. 1997). This variation makes it possible to identify the nitrogen source material and input sources within a system. For instance, Sigleo and Macko (2002) were able to use nitrogen isotope signatures of colloidal DOM to determine the source of the nutrients and trophic processes that changed the nitrogen isotope signature within estuarine systems.

The different geochemical pathways and turnover times of heterogeneous DOM can be obtained by isotopic ($\delta^{13}C$ and $\delta^{15}N$) and elemental (C/N) signatures on size fractionated DOM (Guo and Santschi 1997). Variations in the source of the DOM pool due to changes in inputs or in situ reactions can be measured using the isotopic signature of the DOM. For this study, stable isotope signatures of HMW-DOM fractions were determined to characterize the initial HMW-DOM pool and possible changes to this pool during the sampling period.

1.3.2. Direct Temperature-Resolved Mass Spectrometry

Direct temperature-resolved mass spectrometry (DT-MS) is a broadband technique that provides information on a wide range of chemical substances in marine samples and requires minimal sample manipulation (Eglinton et al. 1996). For analysis a sample is placed on a resistively heated probe. This probe is then inserted into the ionization chamber of the mass spectrometer. Desorption of volatile material and thermal dissociation of polymeric material (pyrolysis) occurs by a carefully programmed increase in temperature of the probe directly within the ionization chamber (Minor 1998).

A schematic of this process is shown in Fig. 3. Allowing the probe with the sample to increase in temperature at a preprogrammed rate gives more information about the sample during desorption of volatile and pyrolysis of biopolymeric material as compared to very rapid pyrolysis approaches such as Curie-point pyrolysis. The scans for each temperature region in DT-MS can then be summed to obtain composite mass spectra (Eglinton et al. 1996).

Different ionization approaches can be used in DT-MS and each has different advantages. Electron impact (EI) ionization conditions allow electrons formed in the ionization chamber to interact with the gas molecules formed by desorption or pyrolysis of the sample. The molecules lose an electron (or two) to become positively charged and subsequently undergo fragmentation.

$$ABC + e \rightarrow ABC^{+\cdot} + 2e \rightarrow A^{+} + BC^{\cdot} \quad (2)$$

$$\rightarrow A^{\cdot} + BC^{+} \quad (3)$$

If sufficiently excited, BC^{+} ions can further decompose resulting in an ion and a neutral species, e.g. B^{+} and C (McLafferty and Tureček 1993). The fragmentation pattern depends on the electron energy (Chapman 1993). Lower energy electron impact (EI) ionization, to 16eV from 70eV, has been used for characterization of lipids, polysaccharides, proteins, lignins, cutins, and commercial polymers (Boon et al. 1998) and for estuarine and marine POM studies (Klap 1997; Minor 1998).The lower energy EI reduces higher energy reactions that produce secondary products which are less representative of the original compound and is thus useful for structure elucidation (McLafferty and Tureček 1993). 16 eV EI is the ionization voltage used to obtain mass spectra of the HMW-DOM in this study.

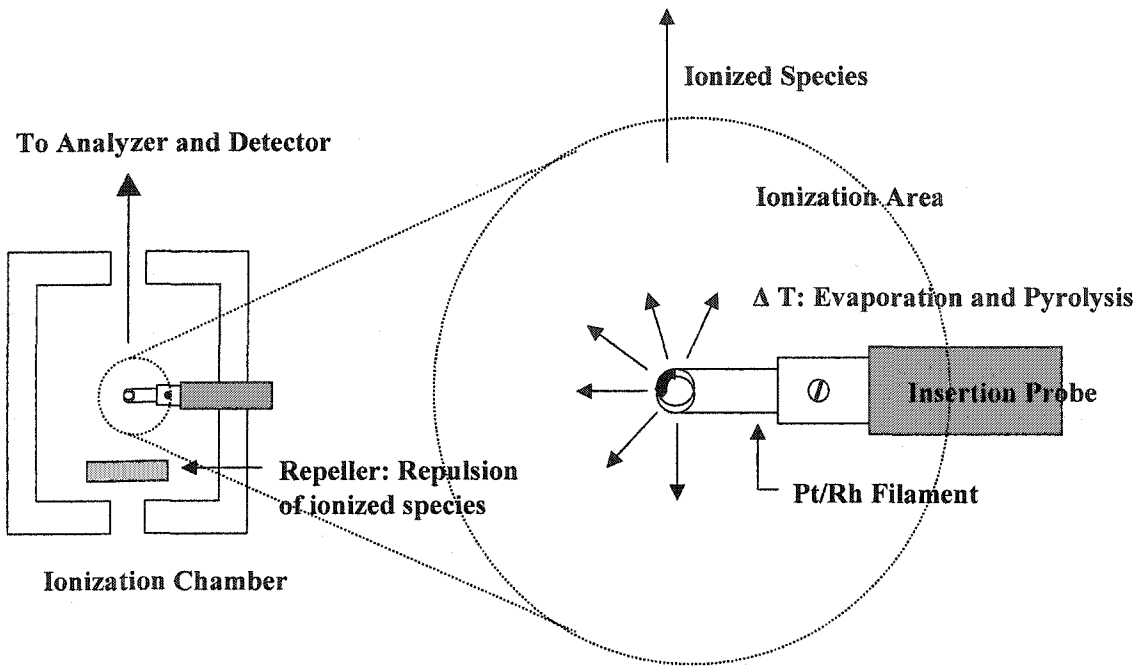

Fig. 3. Schematic of the DT-MS sample introduction. Based on a figure in Minor (1998).

Chemical ionization with ammonia as reactant gas is another technique to obtain specific mass spectra. Here ions characteristic of the sample are produced by ion molecule interactions. Chemical ionization is a widely used technique due to its ability to provide molecular weight information where EI fails to do so (Chapman 1993). This 'soft' ionization technique obtains much of its power from the fact that the characteristics of the spectra are dependent on the reactant gas used to ionize the sample. Using ammonia as a reactant gas provides more information about the (poly)saccharides in the sample (Boon et al. 1998).

Advantages of all these DT-MS techniques are rapid analysis time (2-5 min per sample), need for only a few micrograms of sample and that the data can be directly used for multivariate analysis (Minor 1998). A limitation of DT-MS, however, is that one needs to build up a library of compound classes for the DT-MS setup. This library can then be used to identify compounds and structure in the DOM samples. Data from DT-MS analyses of standard compounds as compiled in studies by Eglinton et al. (1996), Klap (1997), and Minor (1998) were used to identify compounds within the mass spectra of the HMW-DOM. Another limitation is that the low energy electron impact technique could obscure information on certain compound classes that could be important for identification of the DOM samples. Also another limitation in nominal-resolution DT-MS is that several ions can contribute to the same integer m/z value, which makes identification of particular compounds within a sample difficult.

1.4. MULTIVARIATE ANALYSIS

The mass spectra from the DT-MS analyses can be used for tentative

identification of possible compounds using mass spectra from standards run under the similar conditions. Because similar m/z values could indicate different pyrolysis products, it is often difficult to distinguish differences between complex samples within a large dataset. So, to elucidate molecular-level differences between HMW-DOM samples in this study, discriminant analysis was used on total mass spectra obtained by DT-MS.

Discriminant analysis was performed on the mass spectra by the program ChemoMetricks developed at FOM/AMOLF in Amsterdam. The program uses a two-stage principal component analysis approach as described in Hoogerbrugge et al. (1983).

In discriminant analysis the goal is to find the lowest number of linear combinations of the measured variables in a sample, the m/z values, that maximize the differences among the samples. A sample can then be described by its position along each discriminant function, the discriminant score. Now a two- or three-dimensional plot can visualize the differences among samples with the discriminant functions as the axes. This approach simplifies the whole dataset with 300 plus different m/z values into several discriminant functions that explain most of the variance among the samples and the respective discriminant scores for each sample. Another benefit of discriminant analysis is that the technique minimizes the variance among identical samples analyzed at least in triplicate, and maximizes the variance among the sample sets. Even though this procedure leads to an extra loss of variance, the differences among different samples are made more clear.

More in depth explanation of the discriminant analysis technique and the necessary calculations are provided by Davis (1986), Klap (1997), and Minor (1998).

1.5. DOM CHARACTERIZATION BY UV/VIS ABSORBANCE

DOM that is capable of absorbing visible (400 – 700 nm) or ultraviolet light (270 – 400 nm) is referred to as chromophoric DOM (CDOM). Using this definition, CDOM only represents a portion of the bulk DOM pool. However, since the CDOM fraction is determined in the sample and is not, like HMW-DOM, isolated from the sample for analysis, analyzing the CDOM component provides an additional window into DOM composition for comparison with HMW-DOM studies. Absorption spectra can be used to determine characteristics of the CDOM and perhaps the related bulk DOM pool and possible changes in this pool due to input sources or removal processes. Absorption spectra are broad, unstructured and typically show a logarithmic decrease in absorption value with increasing wavelength. A typical spectrum for samples collected in this study is shown in Fig. 4A. Several approaches using the UV/Vis absorbance spectra have been used to infer DOM characteristics, and the parameters used in this study will be discussed.

Typical spectra can be fitted by the following equation:

$$a_\lambda = a_{\lambda 0}\, e^{S(\lambda 0-\lambda)} \qquad (4)$$

where a_λ is the absorption coefficient at a certain wavelength, λ, $a_{\lambda 0}$ is the absorption coefficient at a reference wavelength, λ_0, and S is the exponential slope coefficient that indicates how the absorption coefficient decreases with respect to wavelength. The absorption coefficient is obtained from the absorbance value A, also called the optical density, at wavelength λ from the following relation,

$$a_\lambda = 2.303 A_\lambda / L \qquad (5)$$

where L is the cuvette path length in meters.

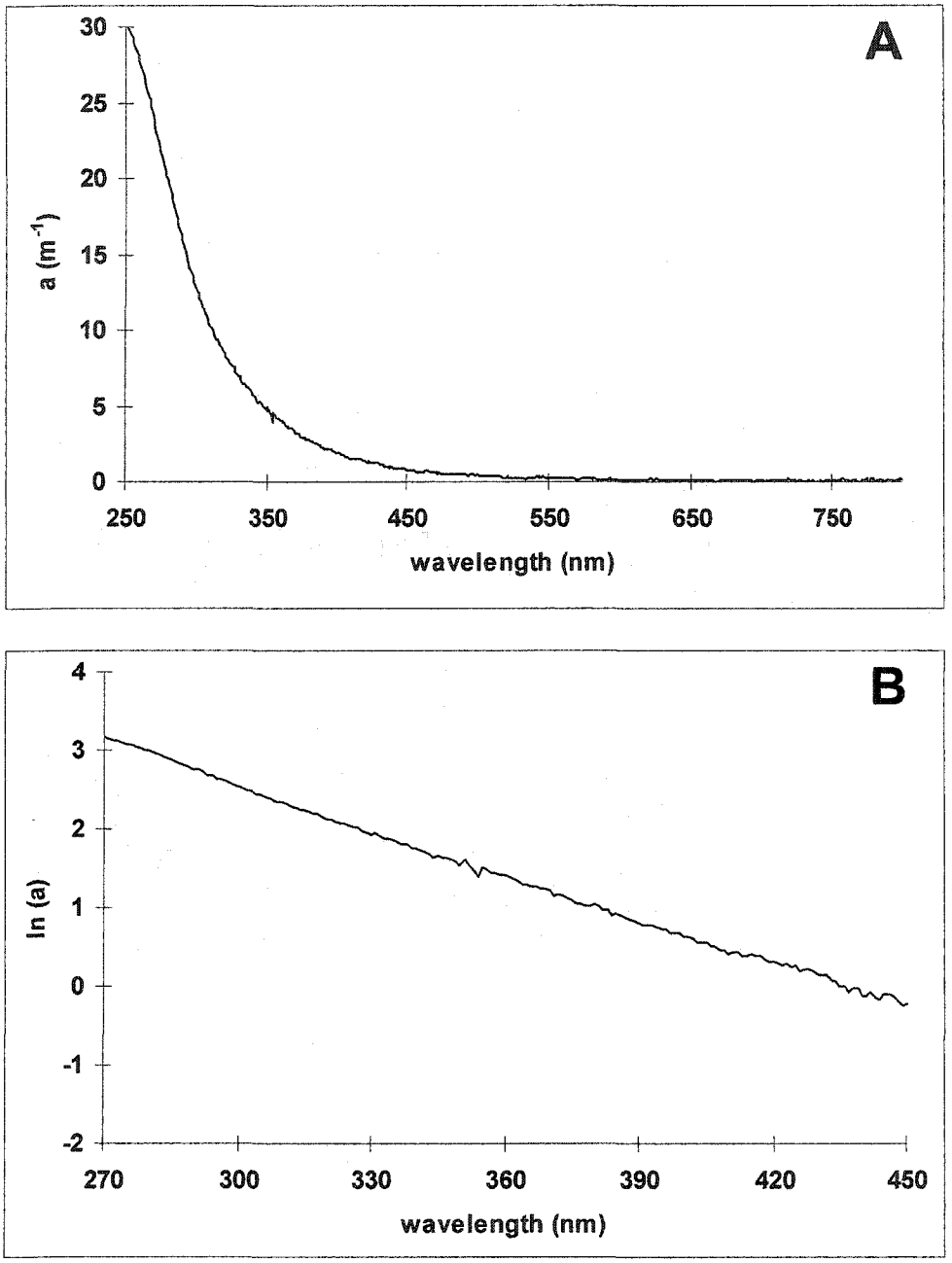

Fig. 4. UV/Vis absorption spectra of CDOM. (A) An example of an UV/Vis absorption spectra. (B) An example of a log-normalized absorption spectra for the range 270-450 nm as used in this study.

The S value, also referred to as the spectral slope of a sample, can thus be calculated for each UV/Vis spectrum by determining the slope of the linear fit through log transformed absorption coefficient data. The result of such a transformation is shown in Fig. 4B for the same sample as in Fig. 4A. The approach as used in this study is now believed to bias the slope downward by enhancing the relative weights of low absorption values at long wavelengths (see Blough and Del Vecchio 2002, for an in depth discussion). However, because in this study samples are compared with each other and not directly with samples from other studies, the observed changes in the S value of the samples will be real (Blough and Del Vecchio 2002).

The CDOM in a sample can thus be described by the S value calculated from the UV/Vis absorption spectrum. The S value provides information about the "nature" of the CDOM in the sample and varies with the source of the CDOM with S values as low as ~ 0.01 nm^{-1} for terrestrial humic acids and as high as ~ 0.03 nm^{-1} for oligotrophic seawaters (see Blough and Del Vecchio 2002, for a summary of CDOM sources and corresponding S values). Changes in the S value for samples from a specific site can occur as a result of several different processes, e.g. input from other sources, bacterial degradation, and photodegradation (Stedmon et al. 2000). Based on properties of humic substances, the S value increases with decreasing molecular weight and decreasing aromatic content. Changes of the S value of samples from a time series can thus give information on the changes in the CDOM pool with respect to possible processes that can occur at the sampling site.

Another parameter to describe the CDOM pool is the specific absorption coefficient or specific UV absorbance value. This parameter is obtained by normalizing

the absorption coefficient at λ (m^{-1}) to the organic carbon concentration (in mg org C l^{-1}). In this study the preferred wavelength for the determination of aromatic structures, 254 nm, is used to calculate this specific UV absorbance value (referred to as SUVA in this study). SUVA is shown to increase with increasing aromatic content (Chin et al. 1994) due to the presence of more light absorbing aromatic structures or an increase in possible charge transfer interactions in the CDOM structures (Blough and Del Vecchio 2002). Changes in the SUVA value of samples from a site will thus indicate changes in the aromatic content of the CDOM pool during a sample period and along with the S value measurements could indicate possible sources of the DOM that is responsible for these changes.

1.6. OUTLINE OF THIS DISSERTATION

This dissertation summarizes results from two years (2002 and 2003) of intense sampling in Chincoteague Bay before, during and after *Aureococcus anophagefferens* bloom events. The techniques described above were used to characterize bulk and isolated HMW DOM in the samples.

Section 2 summarizes results from samples collected in 2002. This section is focused on the characterization of the DOM pool in the surface water prior to and up to the maximum bloom density of *Aureococcus anophagefferens* at the bloom site (Public Landing) and the non-bloom site (Greenbackville), with an emphasis on the utilization and input of DOM during bloom conditions by *Aureococcus anophagefferens*.

In Section 3, the results from the DOM characterization of samples collected in 2002, a drought year and 2003, which had above-average precipitation, are compared. In

contrast to 2002, *Aureococcus anophagefferens* bloomed at both sampling sites in 2003. The results from these two years provide insights into the DOM type that could possibly be utilized by *Aureococcus anophagefferens* and the changes in the DOM pool brought about by brown tide blooms.

Molecular-level characterization by DT-MS and stable isotope signature was only possible on the high molecular weight fraction of the DOM pool in Sections 2 and 3. To extend DOM studies to other fractions, Section 4 describes the comparison of the ultrafiltration technique, as used in Sections 2 and 3, for the concentration and isolation of DOM with a C_{18} disk solid phase extraction method. This section compares the two isolation techniques based on the recovery of DOM and the molecular-level characteristics of the isolated DOM. The C_{18} disk solid phase extraction method is investigated as a way to isolate DOM from the low molecular weight DOM pool to supplement HMW-DOM isolation by ultrafiltration. As future work, knowledge of molecular-level characteristics of the LMW-DOM pool could give us more insight in the type of DOM possibly utilized and the potential input of DOM by *Aureococcus anophagefferens*.

Section 5 discusses the results and conclusions of Sections 2, 3, and 4. Also, possible new areas of study are identified in this section to further our knowledge on the interaction between the DOM pool and *Aureococcus anophagefferens* in Chincoteague Bay.

SECTION 2

CHANGES IN DISSOLVED ORGANIC MATTER CHARACTERISTICS IN CHINCOTEAGUE BAY DURING A BLOOM OF THE PELAGOPHYTE *AUREOCOCCUS ANOPHAGEFFERENS*

2.1. INTRODUCTION

Blooms (> 200,000 cells ml^{-1}) of the pelagophyte, Aureococcus anophagefferens, have been observed in coastal bays along the northeastern United States since 1985 (Sieburth et al. 1988; Bricelj and Lonsdale 1997; Glibert et al. 2001). This small (~2 μm) alga is responsible for "brown tides." Brown tide blooms have had significant ecological impacts on eelgrass (Zostera marina), by limiting light penetration, and on blue mussels (Mytilus edulis), by reducing their feeding rates. Brown tide blooms also indirectly affect fish and shellfish due to the resulting decline in available spawning and nursing areas (Tracey 1988; Bricelj and Lonsdale 1997). Understanding how A. anophagefferens blooms are initiated and sustained, and the effects of blooms on the ecosystem, is important for future predictions and controls of impacted regions.

Several environmental factors have been linked to the occurrence of *A. anophagefferens* blooms, including water temperatures between 20.0 and 28.0 °C, salinities between 24.4 and 31.5 psu, low inorganic nutrient concentrations, availability of iron, and reduced flushing rates of the coastal areas (Keller and Rice 1989; Glibert et al. 2001; Gobler and Sañudo-Wilhemy 2001; Vieira and Chant 1993; Nixon et al. 1994). The presence of dissolved organic nutrients could also be important for *A. anophagefferens*. Growth and uptake experiments using cultures of *A. anophagefferens*

have demonstrated that this species is capable of taking up both dissolved organic nitrogen (DON) (Dzurica et al. 1989; Berg et al. 1997; Lomas et al. 2001; Mulholland et al. 2002), including natural high-molecular weight DON from sediment pore waters (Berg et al. 2003) and dissolved organic carbon (DOC) (Dzurica et al. 1989; Lomas et al. 2001; Mulholland et al. 2002). The ability to take up DON and DOC is hypothesized to give *A. anophagefferens* a competitive advantage relative to other phytoplankton under organic nutrient enriched conditions and thus to promote the initiation of brown tide blooms. However, it is unclear what fraction of the dissolved organic matter (DOM) pool is preferred by *A. anophagefferens* and whether DOM serves as a carbon or nitrogen source, or both. Further, brown tide blooms can affect the DOM pool both by uptake and release of DOM, and the impact of a bloom on the quality and quantity of the DOM has not been extensively studied.

To ascertain how the composition of the DOM pool affects and is affected by growth, bloom, and decay of *A. anophagefferens*, we studied DOM characteristics prior to, during, and after a brown tide bloom in 2002 in Chincoteague Bay. We compare these results with those from a site in the same Bay that did not experience a brown tide bloom. Because DOM analyses are methodologically difficult, we analyzed both bulk and HMW-DOM (> 1000 Da) pools with a variety of techniques and compared these results with brown tide cell density and chlorophyll *a* concentrations. The resulting data provides insights regarding DOM dynamics under bloom and non-bloom conditions in Chincoteague Bay. To our knowledge this is the first study that extensively analyzes characteristics of water-column DOM in a coastal bay and their relationship with *A. anophagefferens.*

2.2. MATERIALS AND METHODS

2.2.1. Site Selection

Chincoteague Bay is a mid-Atlantic coastal bay along Maryland and Virginia's Eastern Shore (Fig. 1). *A. anophagefferens* was first identified in this bay in 1997 and the bay has been monitored for brown tide since 1998 (Glibert et al. 2001; www.dnr.state.md.us/coastalbays). Chincoteague Bay is roughly 72 kilometers in length and 16 kilometers in width. It is bordered on the west by a divide, which separates it from the Pocomoke River watershed, on the north by a divide that defines the Assawoman Bay watershed, on the east by a barrier island that borders the Atlantic Ocean, and on the south by salt marshes. The bay basin is flat with a maximum elevation of 15 meters, contains large areas of salt marsh, and is interlaced with numerous small creeks, guts, and drainage ditches (Cerco et al. 1978). The coastal region is devoid of topographic relief and, although numerous small streams drain directly into Chincoteague Bay, because of the small watershed area, the freshwater input is small compared to tidal exchange (Dillow et al. 2002; www.epa.gov/owow/estuaries). The bay system is connected to the Atlantic Ocean through several inlets at the south end around Chincoteague Island. The residence time of the water in the system is estimated to be > 60 hrs (Brumbaugh 1996), but has never been measured for the sampling sites used in this study. Dye experiments by Vaccaro and Jacobson (1976) around Chincoteague Island inlet during ebb tide showed that none of the dye was moved out of the bay to the ocean. The dye, instead, was lost at the site by dispersion through the water in the bay.

The sites sampled in this study are Greenbackville, VA and Public Landing, MD (Fig. 1). The Greenbackville site (GrBk) is close to the Virginia-Maryland state line and

as of spring 2002, no *A. anophagefferens* blooms had been reported there. The Public Landing site (PL) in Maryland, which is approximately 18 kilometers North of the Greenbackville site, has had reported brown tide blooms since 1999 (www.dnr.state.md.us/coastalbays).

2.2.2. Sample Collection

Surface water samples were collected between March and November, 2002, at the two sites in Chincoteague Bay. Immediately before sample collection, environmental parameters were measured using a Hydrolab Water Quality Multiprobe equipped with sensors for temperature, salinity, dissolved oxygen, and irradiance. Sample collection was conducted on a monthly basis from May-April and July-November and three times a month during May and June. At both sites surface water samples were collected from a dock using a stainless steel bucket, and were transferred immediately into acid rinsed and combusted (450 °C, overnight) 4 l amber bottles. The samples were transported in a cooler to a lab for further processing within 1 hour. In the lab ~ 1 l of each sample was pumped (via peristaltic pump) through a 0.2 μm surfactant-free cellulose acetate Sartorius in-line filter cartridge to remove suspended particles and bacteria. Sample tubing was rinsed before each sample filtration with deionized (DI) water and an aliquot of the sample; the filter was rinsed with about 50 ml sample prior to sample collection. DOC measurements made on samples before and after inline filtration have shown that the method results in no addition of DOC to the filtered sample (Simjouw and Minor, unpublished results). Aliquots (6 ml) of < 0.2 μm water were taken for DOC concentration and ultraviolet/visible (UV/Vis) absorbance measurements. Remaining

sample was stored in the dark at 4°C. The samples for DOC and UV/Vis measurements were stored in acid cleaned and muffled borosilicate clear glass vials with teflon lined caps. To remove inorganic carbon and minimize bacterial activity, 50 µl of 6 N HCl was added to the DOC samples. DOC samples were stored frozen until further processing.

2.2.3. DOC and UV/Vis Measurements

DOC concentrations were measured by high temperature combustion using a Shimadzu TOC-5000 as described in Burdige and Homstead (1994).

In addition to DOC analysis, UV/Vis absorbance spectra were measured on the 0.2 µm filtrate from surface water samples. UV/Vis samples were stored in the dark at 4 °C and processed immediately upon return in the lab. UV/Vis absorbance was measured from 190 to 800 nm using a Varian Cary 3 Bio with DI water as a blank. The absorbance values from 700 to 800 nm were averaged and used for background correction. The absorption coefficient a, at wavelength λ, is calculated using $a_{(\lambda)} = 2.303 * A_{(\lambda)} l^{-1}$ where $A_{(\lambda)}$ is the absorbance at wavelength λ and l is the cell path length in meters.

The absorption coefficient spectrum is fitted by an exponential decay curve and transformed to a linear relationship by plotting the natural log of the absorption coefficient against wavelength. The spectral slope, S, is the slope of this transformed relationship from 270 to 450 nm using a linear least square fitting routine. The spectral slope has been used in many studies to infer the "nature" of organic matter in all types of water bodies and ranges from 0.02-0.03 nm^{-1} for oligotrophic seawater to 0.01 nm^{-1} for terrestrial humic acids (Blough and Del Vecchio 2002 and others therein). A lower spectral slope could indicate higher molecular weight and/or more aromatic DOM.

All reported spectral slopes in this study have a regression coefficient of at least 0.98.

The DOM that can be characterized using UV/Vis absorbance, however, is limited to the UV-B, UV-A, and visible light-absorbing portion of the DOM pool. This portion of the total DOM pool is referred to as chromophoric DOM, CDOM (Blough and Del Vecchio 2002).

Specific ultraviolet absorbance (SUVA), the absorption coefficient at 254 nm divided by the DOC concentration in mg l^{-1}, is referred to as an indicator for changes in aromaticity (Chin et al. 1994; Blough and Del Vecchio 2002) and photochemical reactivity, i.e. the potential to form LMW-substrates from DOM due to photochemical reactions (Mopper and Kieber 2000 and 2002; Stubbins 2001). An increase in SUVA could be attributed to more photochemically reactive and more aromatic DOM.

2.2.4. Ultrafiltration

Ultrafiltration was performed to obtain high molecular weight DOM (HMW-DOM), > 1000 Dalton, for further analysis. An Amicon 8400 stirred cell with a 1000 Dalton (Da) regenerated cellulose membrane was used to concentrate 600 ml samples by a factor of 20. DOC concentrations from the whole sample, retentate, and filtrate, were used to monitor the efficiency of the ultrafiltration procedure and to calculate the HMW-DOC concentration of the sample as described in Benner (1991) and Klap (1997). The total DOC recovery of the ultrafiltration (98.4 +/- 8.0 %) indicated no addition or loss of sample from the filter membrane. Following ultrafiltration, retentates were desalted within the stirred cell by repeatedly rinsing with DI water. Blank runs using DI water showed that the desalting procedure did not contribute significantly to the sample DOC

and mass spectrometry measurements.

Desalted, ultrafiltered dissolved organic matter (HMW-DOM) samples were frozen and then freeze-dried using a Heto FD4. Freeze-dried samples were stored at –20 °C until further analysis.

2.2.5. Elemental Composition and Stable Isotope Measurements

To determine possible sources and infer differences in the HMW-DOM from both sites in Chincoteague Bay, the C:N ratio and the C and N isotopic signatures of the HMW-DOM samples were measured using a PDZ-Europa ANCA-GSL with a 20-20 Stable Isotope Analyzer. Samples and standards were weighed into tin capsules and squeezed shut using a cleaned pincet. Samples were measured in duplicate while standards were measured in triplicate. DL-asparagine (Eastman Organic Chemicals) was used as the standard and reference compound to correct for instrument drift. Stable isotope ratios are reported as $\delta^{13}C$ and $\delta^{15}N$ using equations and standards as described by Hayes et al (1978) and Mariotti (1983).

2.2.6. Direct Temperature-Resolved Mass Spectrometry (DT-MS)

A broad overview of the chemical composition of the HMW-DOM samples was obtained by measuring each sample in triplicate using direct temperature-resolved mass spectrometry (DT-MS). DT-MS was chosen because it yields information on a wide range of chemical substances in marine samples and because minimal sample manipulation is required (Eglinton et al. 1996). For DT-MS analysis, tens to hundreds of micrograms of HMW-DOM was redissolved in 20 µl DI water and a 1-2 µl aliquot was

dried onto a Pt/Rh (90/10) probe (0.125 mm diameter wire). This probe was then inserted into the ionization chamber of a VG AutospecQ magnetic sector mass spectrometer and resistively heated from 0 to 1.1 Amps over two minutes. A programmed increase in temperature of the sample probe promoted desorption of volatile material and pyrolysis, thermal dissociation of polymeric material (Boon 1992; Eglinton et al. 1996; Minor 1998). The resulting volatilized components were ionized using 16 eV electron impact (EI^+) ionization. Instrument settings were as follows: acceleration voltage 6.0 kV, direct inlet, mass range 41 – 795, scan rate 1.16 seconds with a 0.5 second delay, resolution 1000. The scans for each temperature region were summed to obtain composite mass spectra (Boon 1992; Eglinton et al. 1996).

2.2.7. Statistical Analysis

The DT-MS method generates complex mass spectra that can be interpreted against or by comparing standard compound runs (Eglinton et al. 1996; Minor 1998; Minor and Eglinton 1999). We performed discriminant analysis on the dataset to ascertain trends among the time series samples of HMW-DOM from the two sample sites. The mass spectra of samples from both sites were exported to a multivariate statistics program, ChemoMetricks (FOM-AMOLF Institute, the Netherlands). ChemoMetricks was used to perform a type of discriminant analysis on the dataset using a two-stage principle component analysis technique (see Hoogerbrugge et al. 1983 and Minor and Eglinton 1999 for more information). Discriminant analysis was used here to maximize differences between samples while minimizing differences in replicate samples. The ratio of variance between samples to the variance within replicate samples

(B/W) was used to indicate the significance of the discriminant function. The B/W is reported along with the total variance of the dataset explained by the discriminant function (%var).

Scores for the two major discriminant functions were used to construct a two-dimensional score plot for the samples from both sites and to understand relative similarities among samples. Plotting discriminant scores against measured parameters was used to identify possible correlations between the parameter and changes in HMW-DOM composition.

Using the loadings of a discriminant function for the m/z values multiplied with the respective standard deviation we were able to reconstruct difference spectra. The difference spectra show enrichments or depletions of the m/z values along the discriminant function axis (Minor 1998; Minor and Eglinton 1999). This indicates what components of the HMW-DOM are responsible for the separation in the discriminant analysis plot.

2.3. RESULTS

2.3.1. Environmental Parameters

A bloom of *A. anophagefferens* (> 200,000 cells ml^{-1}) was found at the Public Landing, MD site from May 30 to June 12, 2002. The water temperature during onset and bloom conditions (May 2 through June 12, 2002) ranged from 17.7 to 27.5 °C (Table 1), which is the same temperature range other researchers found to be ideal for *A. anophagefferens* (Cosper et al. 1990; Gobler et al. 2002). However, the surface water temperature at the Greenbackville site, where there was no bloom, also fell within the

TABLE 1. Summary of environmental parameters (temperature and salinity) and surrogate biomass measurements (chlorophyll *a* and brown tide cell counts) from both sites. The standard deviation of the value is given in parenthesis. In the shaded area are the parameters during the brown tide period (defined as > 10^4 cells ml^{-1}).

	Date	T (°C)	S	Chl a ($\mu g\ l^{-1}$)	BT counts (10^5 cells ml^{-1})
Public Landing					
PL2	03/01/02	6.15	33.2	1.2 (0.24)	0.0002 (.0001)
PL3	04/05/02	13.6	30.5	1.8 (0.38)	0.0001
PL4	05/02/02	17.7	29.9	5.2 (0.19)	0.152 (0.02)
PL5	05/15/02	18.9	30.6	2.4 (0.16)	0.004
PL6	05/30/02	27.5	30.5	10.2 (1.06)	3.96 (0.16)
PL7	06/06/02	25	33.0	14.4 (1.52)	5.88 (0.51)
PL8	06/12/02	24.8	32.6	19.5 (1.46)	12.1 (2.11)
PL9	06/26/02	29.8	32.2	18.4 (1.68)	0.011 (0.001)
PL10	07/24/02	28.1	35.2	16.7 (1.00)	0.001 (0.0002)
PL11	08/21/02	28.0	35.2	22.4 (4.46)	0.002 (0.0008)
PL12	09/13/02	23.9	36.0	11.2 (0.85)	0
PL13	10/21/02	15.7	34.0	3.02 (0.26)	0.0001 (0.0001)
Greenbackville					
GrBk2	03/01/02	6.49	33.3	0.8 (0.04)	0.00027
GrBk3	04/05/02	12.9	30.4	1.2 (0.05)	0
GrBk4	05/02/02	17.5	30.8	8.7 (0.42)	0
GrBk5	05/15/02	17.5	31.7	1.7 (0.23)	0.00018
GrBk6	05/30/02	25.7	32.0	3.5 (1.49)	0.00093
GrBk7	06/06/02	23.3	34.0	7.4 (0.75)	0.00096
GrBk8	06/12/02	24.3	33.7	5.1 (2.4)	0.00075
GrBk9	06/26/02	29.1	34.1	10.1 (1.71)	0.00013
GrBk10	07/24/02	27.6	35.3	14.2 (0.61)	0.00026
GrBk11	08/21/02	27.7	35.3	11.8 (1.4)	0
GrBk12	09/13/02	23.3	37.0	6.7 (0.60)	0
GrBk13	10/21/02	15.4	35.9	2.3 (0.09)	0

same range.

Salinity differed by about 1 to 2 units between the two sites during the spring and summer, with the lower values measured at the Public Landing site (Table 1). The lower salinity at Public Landing could indicate a freshwater input though we did not explicitly explore possible freshwater sources to either Public Landing or Greenbackville for this study. The salinity range during May and June at Public Landing was 29.9 to 33.0 psu, and is consistent with ranges reported in some studies (Cosper et al. 1990), but is higher than values reported by others (Gobler et al. 2002).

2.3.2. Chlorophyll *a* and *Aureococcus anophagefferens* Measurements

The chlorophyll *a* (chl *a*) and brown tide data clearly showed several events between April and November, 2002 (Table 1). First, chl *a* increased at the beginning of May 2002, where the Greenbackville site had almost twice the amount of chl *a* as Public Landing. In late-May, there was a simultaneous increase in chl *a* and *A. anophagefferens* cell numbers at Public Landing with cell numbers reaching a maximum of 1.2×10^6 cells ml^{-1} during the middle of June. Using a chl *a* content of 0.035 pg/cell (Mulholland et al., 2002; Gobler et al, 2002; Gobler and Sañudo-Wilhelmy, 2001), *A. anophagefferens* was responsible for all the chl *a* during the simultaneous increase in chl *a* and *A. anophagefferens* cell numbers. The calculated chl *a* concentration actually exceeded the measured concentration at the height of the bloom, which may indicate a decrease in the chl *a* content of the *A. anophagefferens* cells due to self-shading. Chl *a* decreased slightly just after the brown tide bloom at Public Landing and then increased again later in the summer (Table 1). This second increase was not associated with an increase in

TABLE 2. Summary of dissolved organic carbon concentration of both the bulk and high molecular weight organic matter pool, and C:N ratio and stable isotope measurements of the high molecular weight dissolved organic matter pool. The standard deviation of the value is given in parenthesis. When no standard deviation is given only one measurement was taken. n.m. is not measured because of ssample size constraints. The HMW-DON concentration is calculated using the HMW-DOC concentration and the C:N ratio as given in the table. In the shaded area are the parameters during the brown tide period (> 10^4 cells ml^{-1}).

Sampling site	Date	DOC (μM)	HMW-DOC (μM)	% HMW-DOC	C:N ratio HMW-DOM	$\partial^{13}C$ HMW-DOM (‰)	$\partial^{15}N$ HMW-DOM (‰)	HMW-DON (μM)
Public Landing								
PL2	03/01/02	366 (1.9)	71 (0.2)	19.5	10.4 (1.54)	-21.25 (0.62)	-0.87 (2.79)	7
PL3	04/05/02	360 (7.3)	109 (0.1)	30.2	13.0 (0.22)	-20.74 (0.22)	2.39 (0.67)	8
PL4	05/02/02	381 (3.6)	147 (1.3)	38.6	14.9 (0.34)	-21.57 (0.03)	3.99 (0.69)	10
PL5	05/15/02	369 (5.1)	131 (0.3)	35.5	n.m.	n.m.	n.m	
PL6	05/30/02	429 (3.1)	210 (1.8)	49.0	13.1	-22.07	4.30	16
PL7	06/06/02	424 (8.6)	146 (0.7)	34.5	12.7	-20.98	3.87	11
PL8	06/12/02	491 (1.2)	210 (0.2)	42.8	n.m.	n.m.	n.m	
PL9	06/26/02	592 (3.0)	257 (0.7)	43.4	15.5 (0.04)	-21.74 (0.09)	4.74 (0.15)	17
PL10	07/24/02	508 (2.0)	273 (0.5)	53.7	14.9 (0.58)	-21.63 (0.08)	4.76 (0.93)	18
PL11	08/21/02	678 (1.4)	405 (0.3)	59.7	18.0 (0.45)	-22.23 (0.32)	4.86 (0.02)	22
PL12	09/13/02	499 (0.8)	286 (0.6)	57.3	15.5 (0.65)	-21.75 (0.07)	3.75 (0.67)	18
PL13	10/21/02	443 (3.4)	227 (0.9)	51.1	14.8 (0.10)	-21.21 (0.02)	3.40 (0.47)	15

TABLE 2. Continued.

Sampling site	Date	DOC (μM)	HMW-DOC (μM)	% HMW-DOC	C:N ratio HMW-DOM	$\partial^{13}C$ HMW-DOM (‰)	$\partial^{15}N$ HMW-DOM (‰)	HMW-DON (μM)
Greenbackville								
GrBk2	03/01/02	215 (0.9)	75 (2.8)	35.1	10.7 (0.98)	-20.71 (0.34)	-1.61 (2.56)	7
GrBk3	04/05/02	277 (1.5)	126 (0.8)	45.6	13.8 (0.44)	-20.49 (0.04)	4.36 (0.73)	9
GrBk4	05/02/02	270 (3.2)	101 (1.0)	37.4	13.3 (0.35)	-20.33 (0.04)	4.16 (1.42)	8
GrBk5	05/15/02	245 (1.1)	123 (2.9)	50.3	13.0	-20.21	5.58	9
GrBk6	05/30/02	276 (1.0)	112 (0.6)	40.6	11.1 (0.50)	-21.26 (0.03)	3.03 (1.58)	10
GrBk7	06/06/02	258 (2.8)	119 (0.8)	46.0	12.3 (0.22)	-20.23 (0.06)	3.76 (0.36)	10
GrBk8	06/12/02	229 (1.2)	117 (0.3)	51.1	11.6	-20.32	4.19	10
GrBk9	06/26/02	297 (0.3)	147 (0.6)	49.5	12.9 (0.35)	-19.94 (0.12)	4.61 (0.65)	11
GrBk10	07/24/02	303 (1.8)	147 (0.9)	48.5	12.5 (0.08)	-19.95 (0.09)	5.19 (1.00)	12
GrBk11	08/21/02	281 (1.5)	151 (0.6)	54.0	12.5 (0.05)	-19.75 (0.14)	5.29 (0.33)	12
GrBk12	09/13/02	458 (2.7)	268 (4.3)	58.6	13.8 (0.27)	-19.71 (0.00)	4.33 (0.03)	20
GrBk13	10/21/02	445 (3.0)	230 (0.4)	51.6	n.m.	n.m.	n.m.	

A. anophagefferens cell numbers and was probably due to growth of other phytoplankton that succeeded the brown tide bloom. At Greenbackville, chl *a* reached a maximum in July but *A. anophagefferens* was never abundant.

2.3.3. DOC Measurements

The surface water DOC concentrations were consistently higher at Public Landing than at Greenbackville from May-August (Table 2). When *A. anophagefferens* cells were abundant, the DOC concentration increased; however, the DOC maximum, 678 μM, coincided with the non-brown-tide-associated chlorophyll *a* maximum in August at Public Landing (Fig. 5A). The DOC concentrations at the Greenbackville site were about 150-200 μM lower than those observed at Public Landing until September, when the concentrations at both sites were 440 – 500 μM (Table 2 and Fig. 5B).

The HMW-DOC concentration at the Public Landing site (Table 2 and Fig. 6A) increased throughout the spring and summer with a maximum on August 21, 2002 again coinciding with the August non-brown tide chlorophyll *a* maximum. At the Greenbackville site, HMW-DOC increased only slightly through the year, with most of this increase in September and October (Table 2 and Fig. 6B). Interestingly, the HMW-DOC fraction of the total DOC increased from spring values of about 20% for the Public Landing site and 35% for the Greenbackville site, to a high of around 60% for both sites in August and September (Table 2).

2.3.4. Absorbance Characteristics of DOM

At the Public Landing site, there was a clear decrease in the spectral slope (S)

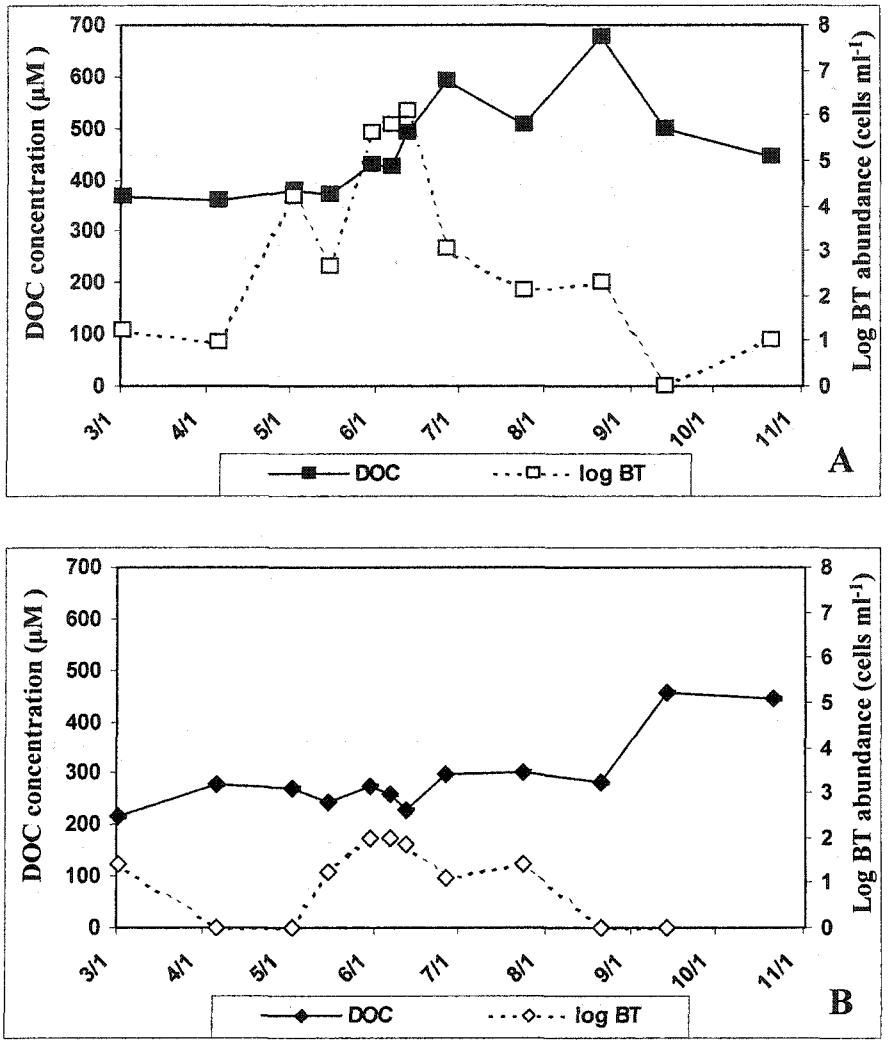

Fig. 5. Dissolved organic carbon concentrations in µM for (A) Public Landing and (B) Greenbackville between March and November 2002 with the corresponding log brown tide cell numbers (cells ml^{-1}) on the secondary axis.

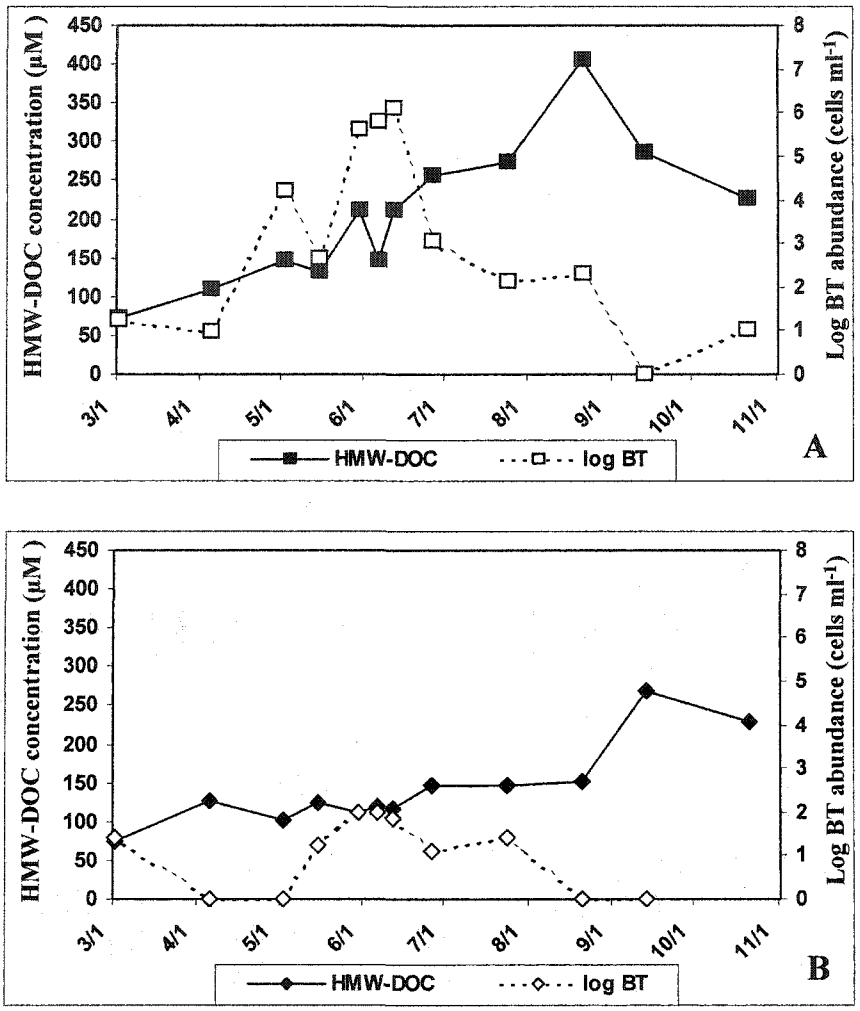

Fig. 6. High molecular weight dissolved organic carbon concentrations in µM for (A) Public Landing and (B) Greenbackville between March and November 2002 with the corresponding log brown tide cell numbers (cells ml^{-1}) on the secondary axis.

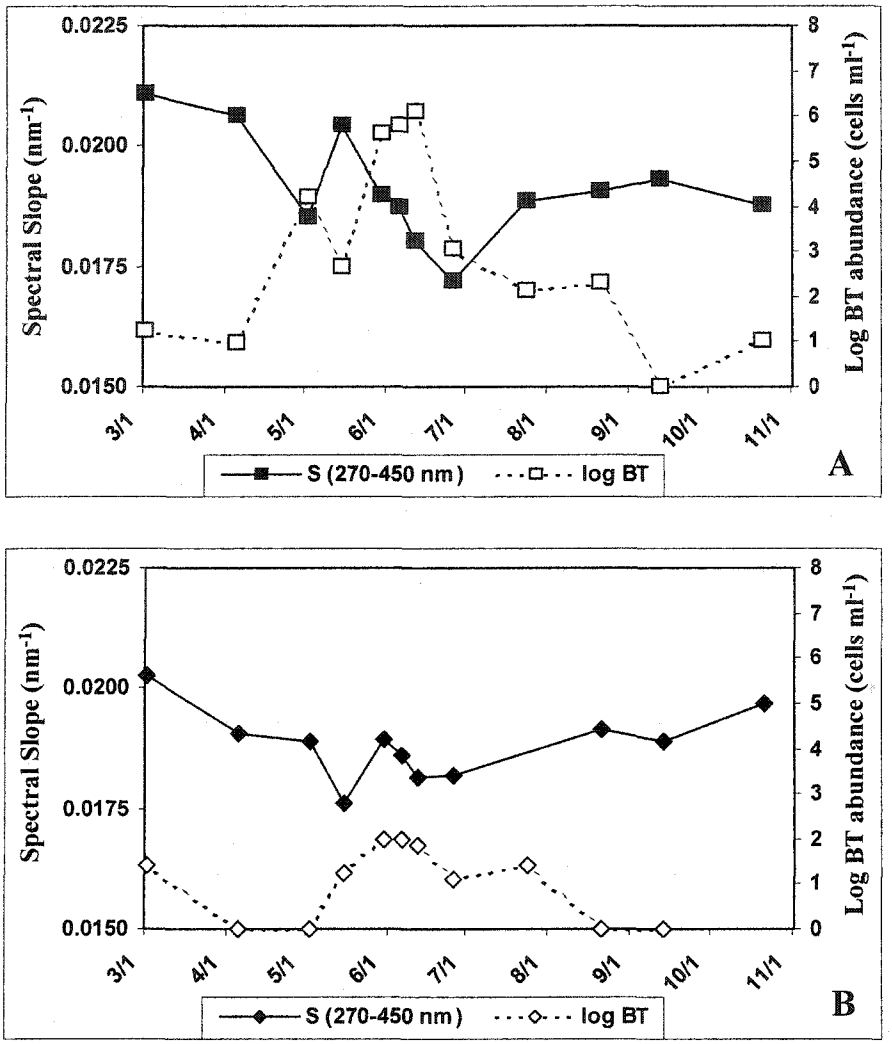

Fig. 7. Spectral slope in nm^{-1} for (A) Public Landing and (B) Greenbackville between March and November 2002 and the corresponding log brown tide cell number (cells ml^{-1}).

leading up to and during the bloom (Fig. 7A). The spectral slope decreased from 0.021 nm^{-1} on May 2nd to 0.017 nm^{-1} on June 26th, just after peak *A. anophagefferens* cell density. At Greenbackville, S was less variable but decreased from 0.020 to 0.018 nm^{-1} during May and June (Fig. 7B). These spectral slope values are within the range reported for coastal and estuarine waters (between 0.015-0.021 nm^{-1}; Blough and Del Vecchio 2002, and references therein). The S values did not correlate with the chlorophyll *a* concentrations at either site (data not shown), but did have a significant correlation (R^2 = 0.86) with the log brown tide cell counts at Public Landing (Fig. 8).

SUVA values increased at both sites during and immediately after the brown tide bloom at Public Landing, increasing from around 4.8 to 6.5 l mg^{-1} m^{-1} (Fig. 9A and 9B). After the maximum the SUVA value decreased to between 5.5 and 6.0 l mg^{-1} m^{-1} at both sites.

2.3.5. C:N, $\delta^{13}C$ and $\delta^{15}N$ of HMW-DOM

The C:N ratio of HMW-DOM from the Public Landing and Greenbackville sites increased in early spring and then decreased in late May/early June, during which the brown tide bloom occurred at Public Landing (Table 2). During and after the brown tide period the C:N ratio was higher at the Public Landing site relative to the Greenbackville site (Table 2). Based on the measured C:N ratio and the HMW-DOC we calculated the HMW-DON for the samples (Table 2). Both sampling sites had similar HMW-DON concentrations before the brown tide period, and both showed an overall increase in HMW-DON in late spring and summer. At Public Landing, HMW-DON generally increased until late August when chl *a* reached a maximum. The overall HMW-DON

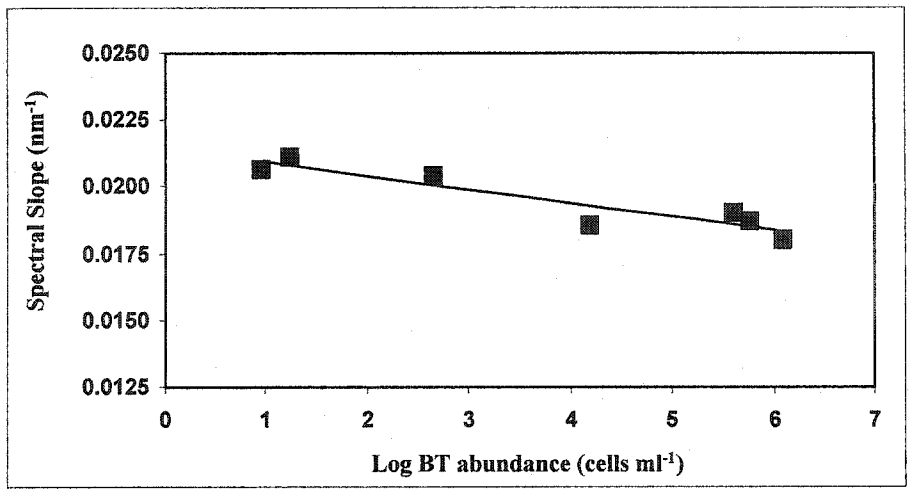

Fig. 8. The spectral slope (nm^{-1}) versus log brown tide cell number (cells ml^{-1}) for Public Landing samples prior to and during the period when brown tide cell number was $> 10^4$ cells ml^{-1}. The R^2 for the regression line is 0.86.

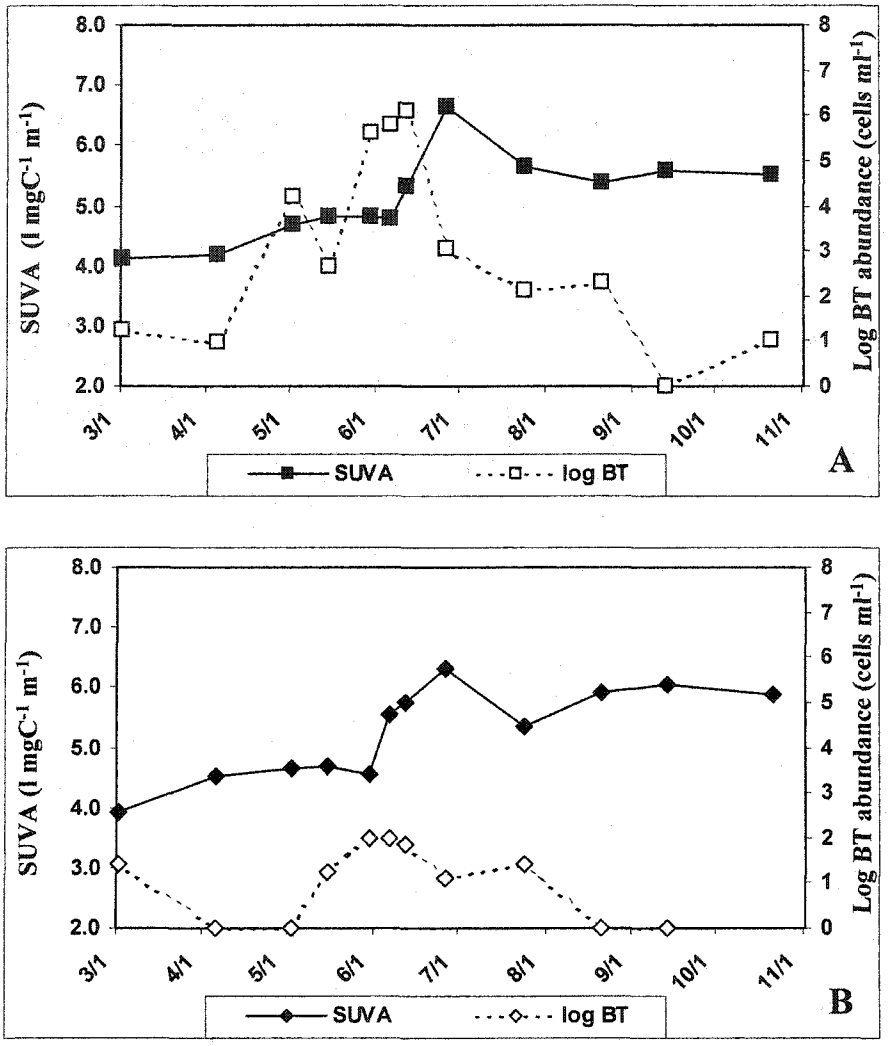

Fig. 9. Specific UV absorbance (SUVA) in l mgC^{-1} m^{-1} for (A) Public Landing, MD and (B) Greenbackville, VA with corresponding log brown tide cell number (cells ml^{-1}) on the secondary axis.

concentration increased slightly at Greenbackville until August-September after which there was a large (8 μM) increase in concentration.

Throughout the year the HMW-DOM was on average more ^{13}C depleted at the Public Landing site than at Greenbackville (Table 2). During the brown tide bloom the $\delta^{13}C$ of the HMW-DOM was more variable, but was consistently lighter than the Greenbackville samples. After the brown tide period (from July to September), the HMW-DOM samples were less variable at both sites, with Public Landing samples about 2‰ lighter than the Greenbackville HMW-DOM samples. The $\delta^{15}N$ at both sites increased after March and then remained at + 4.2‰ ± 0.8 for most of the year (Table 2).

Based on a property-property plot of the $\delta^{13}C$ and $\delta^{15}N$ (Fig. 10), the HMW-DOM from the two sites is distinct, mostly due to the $\delta^{13}C$ signatures of the HMW-DOM.

2.3.6. DT-MS and Discriminant Analysis

DT-MS and discriminant analysis were conducted on samples from both sites collected between March and mid-June, 2002 (see Table 1).

Discriminant function 1 (DF1) appears to separate Public Landing and Greenbackville samples with most Public Landing samples having positive DF1 scores and most Greenbackville samples having negative DF1 scores. Using the DF1 scores, no significant correlations could be found with the measured environmental parameters or DOM characteristics for either site (data not shown). PL6 to PL8, brown tide bloom samples (cell densities > 395,000 cells ml^{-1}; Table 1) and PL4 (*A. anophagefferens* cell density > 15,000 cells ml^{-1}; Table 1) cluster together in the space defined by DF1 and DF2. This implies that HMW-DOM from samples with high brown tide cell numbers

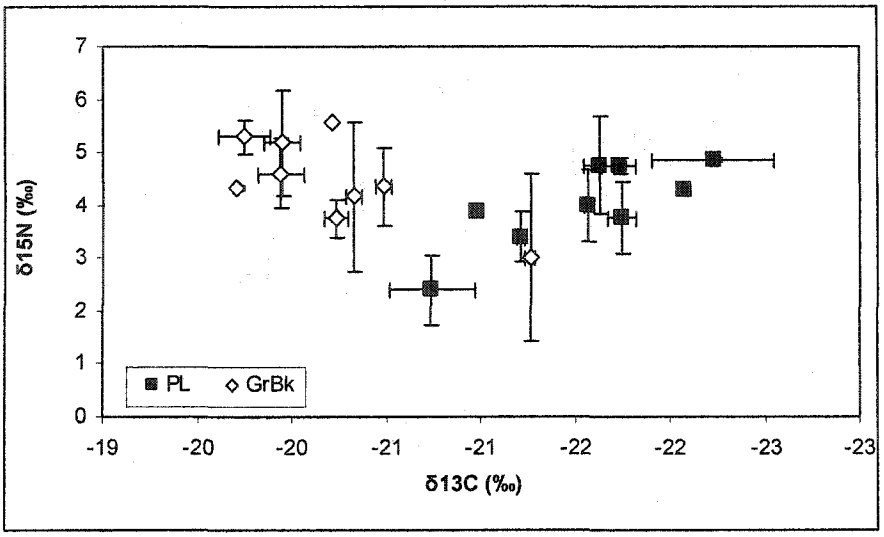

Fig. 10. $\delta^{15}N$ (‰) vs. $\delta^{13}C$ (‰) of high molecular weight dissolved matter from surface water of Public Landing, MD and Greenbackville, VA. Negative $\delta^{15}N$ values are not included in the figure.

were similar at the molecular level.

There appears to be separation of the Public Landing HMW-DOM along DF2. The scores of DF2 for all Public Landing samples from Fig. 11 had significant correlations (95% level of significance as calculated using Davis, 1986) with measured characteristics of the DOM pool (HMW-DOC and $\delta^{15}N$ of the HMW-DOM) as well as the log brown tide (BT) cell numbers (Fig. 12). For all three comparisons the trend was similar, i.e. most negative DF2 scores for the highest cell abundance, HMW-DOC concentration, or $\delta^{15}N$ value (Fig. 12A, 12B, and 12C respectively). The negative DF2 scores corresponded with PL samples from the brown tide bloom (cell count > 10^4 cells ml^{-1}) while the positive values were from the non-bloom PL samples. Discriminant analysis on only the Public Landing samples (rather than samples from both sites) results in a similar separation now shifted from DF2 to DF1 with an increase in %var and B/W parameters to 13.9 % and 19.9% respectively (data not shown). This indicates that the grouping of the Public Landing samples as shown in Fig. 11 does not occur due to the influence of the Greenbackville samples included in that data set. Also, the DF1 scores for the Public Landing samples remain correlated (95% level of significance) with HMW-DOC, $\delta^{15}N$ of the HMW-DOM, and the log BT cell numbers (data not shown). To further characterize the differences in molecular structure we constructed a difference spectrum from the loadings for DF2 from Fig. 11 (Fig. 13). The most striking DT-MS difference peaks are associated with benzene and phenol, m/z 78 and 92 respectively. They indicate that HMW-DOM associated with high brown tide abundance had more aromatic structures than the HMW-DOM when brown tide was not abundant. This is consistent with the shift in spectral slope seen for the same samples (Fig. 7A).

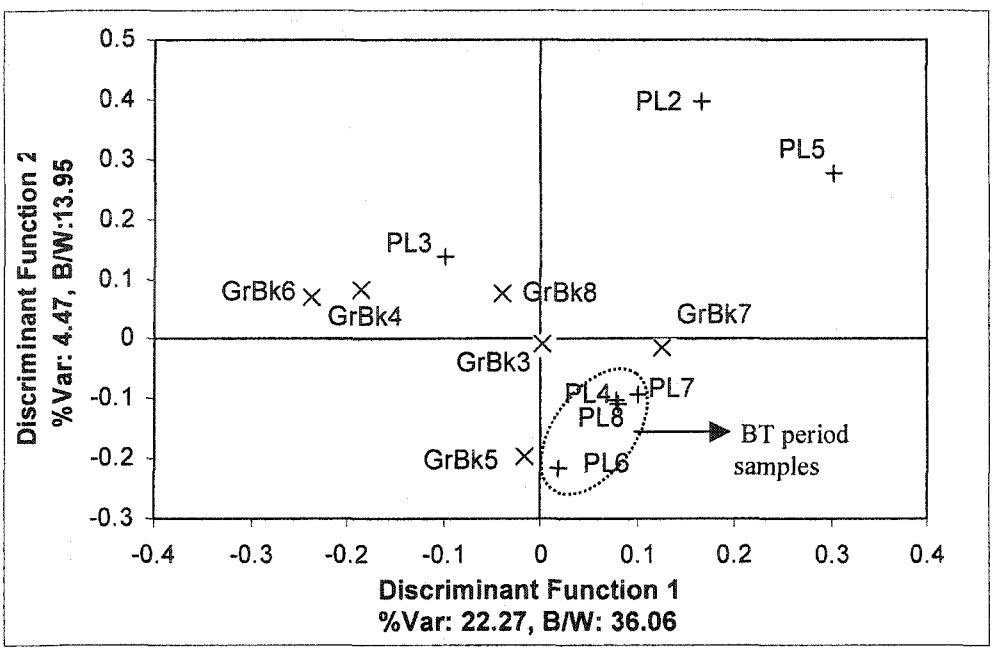

Fig. 11. Discriminant analysis score plot showing the average scores for high molecular weight dissolved organic matter samples from both Public Landing (PL) and Greenbackville (GrBk) prior to and during the bloom of *A. anophagefferens* (samples 2-8) analyzed by direct temperature resolved mass spectrometry. Discriminant function 1 has a between to within ratio of 36.06 and % variance of 22.27%; discriminant function 2 has a between to within ratio of 13.95 and % variance of 4.47 %. Note: all samples were run in triplicate.

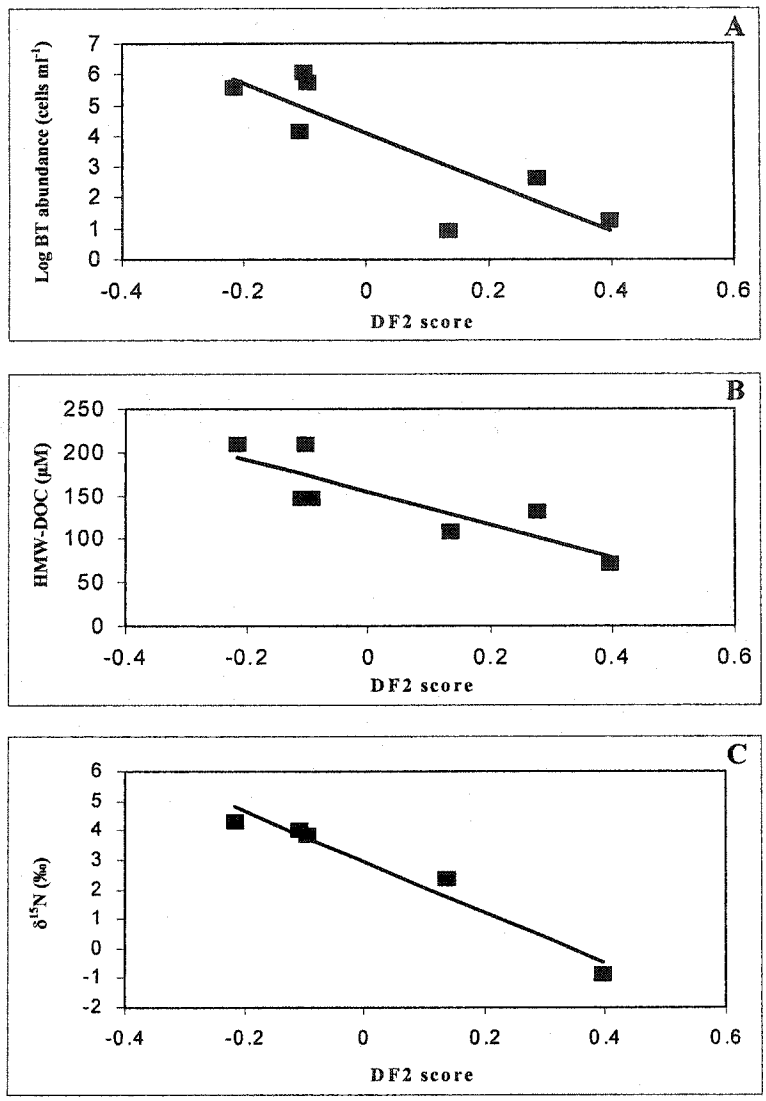

Fig. 12. Correlations of discriminant function 2 scores for Public Landing high molecular weight dissolved organic matter with external variables. Discriminant function 2 scores vs. log. brown tide number (cells ml^{-1}) with r = -0.85, (B) Discriminant function 2 scores vs. high molecular weight dissolved organic carbon concentration (µM) with r = -0.85, (C) Discriminant function 2 scores vs. $\delta^{15}N$ of the high molecular weight dissolved organic matter (‰) with r = -0.99.

The exact nature of the aromatic compounds is not yet known. Separate DT-MS analyses of HMW-DOM samples from after the brown tide bloom period (samples 9 to 13) indicate that the aromatic structures persist; m/z 78 and 92 are still present. However, due to shifts in DT-MS instrument parameters (primarily a change in sensitivity), the post-bloom data could not be included in the discriminant analysis. Therefore we cannot directly compare the relative abundance of both m/z 78 and 92 during and after the bloom period.

2.4. DISCUSSION

2.4.1. DOC Dynamics

The DOC concentrations at both sites (Table 2) correspond to the range of DOC values found in a previous study of brown tide, where summer DOC ranges of 154-442 μM and 213-888 μM were reported for Shinnecock Bay, Long Island, NY and the Maryland coastal bays respectively (Lomas et al. 2001). The high DOC concentrations at Public Landing are also consistent with concentrations (mean DOC of 426 ± 66 μM) measured in Great South Bay, New York, during a fall bloom of A. anophagefferens in 1999 (Gobler et al. 2002). However, Lomas et al. (2001) found no significant difference between the DOC concentrations when brown tide was abundant (> 100,000 cells ml^{-1}) and when it was not. In our study (2002), DOC concentrations before, during, and immediately after the brown tide period were 90 to 400 μM higher at Public Landing, the brown tide bloom site, than at Greenbackville, the non-bloom site. This difference in DOC concentration could indicate that DOC concentration is an important variable in brown tide development and maintenance. However, at the same sites in 2003 (Simjouw

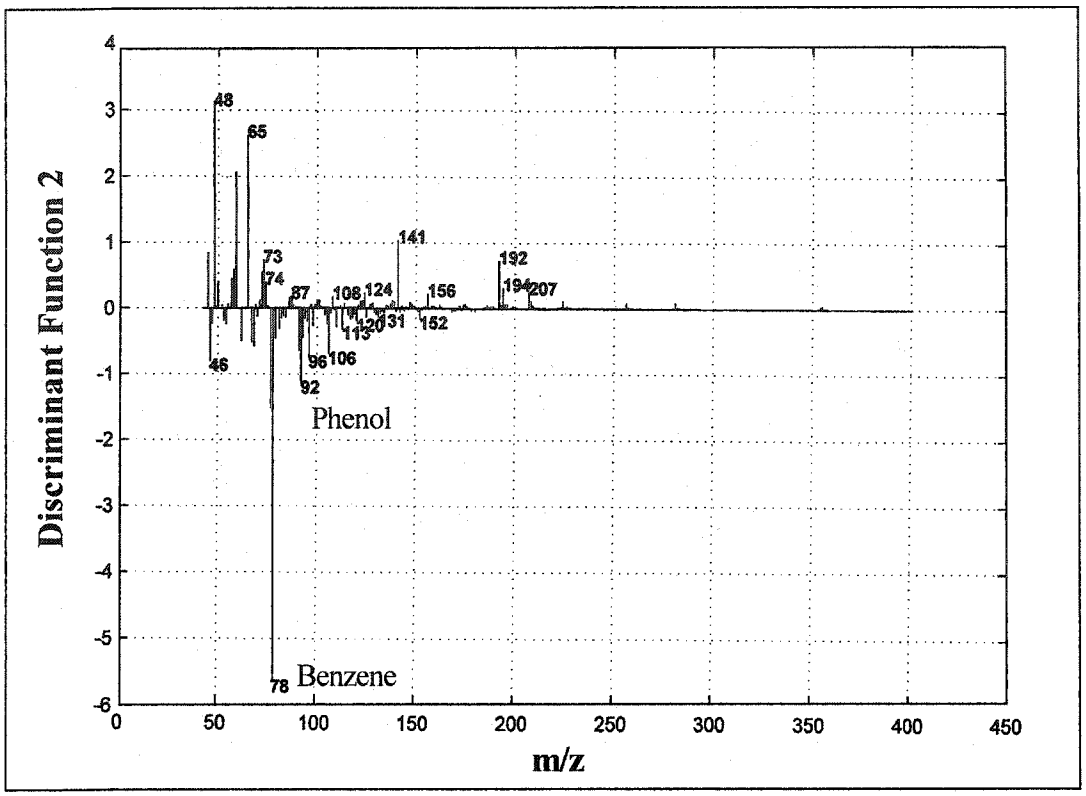

Fig. 13. Difference spectrum reconstructed from along discriminant function 2 loadings. The discriminant analysis of HMW-DOM samples from Public Landing, MD and Greenbackville, VA prior to and during the brown tide period (> 10^4 cells ml^{-1}) is the same as shown in Fig. 11.

et al. in prep.) DOC concentrations were similar to those at Greenbackville in Spring-Summer 2002, yet a significant brown tide developed.

The increase in DOC concentrations at Public Landing during the 2002 summer could have been due to inputs from an external DOC source or from the *A. anophagefferens* bloom. A significant correlation ($r = 0.98$) was found between the brown tide cell number (cells ml^{-1}) and DOC concentration (μM) for the samples prior to and during the brown tide bloom (samples PL2-PL8), and is similar for chl *a* (mg l^{-1}) and DOC concentration ($r = 0.97$) for the same period. These correlations, combined with the chl *a* calculations earlier, further support our assumption that *A. anophagefferens* is the most abundant phytoplankton during the brown tide period. After the brown tide bloom, the change in DOC concentration only exhibited a significant correlation with chl *a* ($r = 0.90$). We speculate that the increase in DOC concentration during late-spring to mid-summer is due to the presence of *A. anophagefferens*, and that other phytoplankton are responsible for the late-summer changes in DOC concentration.

It is known that *A. anophagefferens* produces polysaccharide-like exocellular material (Sieburth et al. 1988). The molecular size of this material is unknown but has been determined not to interfere with filter feeding capabilities of the blue mussel *Mytilus edulis* (Tracey 1988). In addition to the release of exocellular material, an increase in DOC concentration immediately after the brown tide bloom might result from release of material by viral cell lysis or bacterial degradation of the cellular material (Gobler et al. 1997; Gastrich et al. 1998; Garry et al. 1998). We calculated that our increase of about 100 μM DOC corresponds to a release of 1 pg DOC $cell^{-1}$ l^{-1}. This value is comparable to release rates calculated by Gobler et al. (2002) after viral cell lysis of *A. anophagefferens*

(about 0.4 pg DOC $cell^{-1}$ l^{-1}). Actual release rates of DOC may have been higher or lower if there were additional increases in *A. anophagefferens* cell abundance after our measurements on 12 June or if released POC was utilized and subsequently released as DOC by grazers and/or bacteria in the water column.

Results from ultrafiltration studies show an increase from March through August 2002 in the HMW-DOC and the percentage HMW-DOC of the total DOC pool at the Public Landing site. This indicates that the DOM added to the surface water contained a large proportion of high molecular weight compounds. This is consistent with the idea that the additional DOM is derived from cells. The percentage of HMW-DOC of the total DOC pool (34.5 – 49.0%) was higher than the 20-30% measured at stations in the Pacific Ocean, Atlantic Ocean, and the Gulf of Mexico (Benner et al. 1997). This higher percentage at Public Landing, a coastal bay site, is most likely due to higher input sources, either from coastal run-off or *in situ* phytoplankton growth. Supporting this is the fact that the Public Landing % HMW-DOC is comparable to values observed by Mannino and Harvey (2000) on a transect down the Delaware River (31-61%). It is also important to point out that the % HMW-DOC at Public Landing from May through October is within the same range as the % HMW-DOC at the Greenbackville site even though the total DOC pool at Greenbackville did not exhibit the same increase as at Public Landing.

2.4.2. C:N and Stable Isotope Signature of the HMW-DOM

Both the C:N ratio and the stable isotope signatures suggest that the HMW-DOM from the two sites was different. The average C:N ratio of the HMW-DOM from Public

Landing, 14.3 ± 2.1, and Greenbackville samples, 12.5 ± 1.0, fall between average surface ocean values of 15-18 (Benner 2002), and reported ratios for transects from the Potomac River and the Chesapeake Bay, ranging from 10 to 12 (Sigleo and Macko 2002). The higher C:N ratio at the Public Landing site relative to Greenbackville could be due to more terrestrial contributions to the HMW-DOM or due to diagenesis of the HMW-DOM in the surface water at this site.

During the brown tide bloom at Public Landing, the HMW-DOM pool seemed to be very dynamic. The C:N ratio decreased during the brown tide period (PL6 and PL7). This decrease in C:N could have been due to an increase in N content of the HMW-DOM being produced during this time since the total DOC pool and the HMW-DOC pool sizes also increased (Table 2). Based on the change in HMW-DOC and HMW-DON concentration among any two samples, the C:N ratio of the removed or added HMW-DOM can be calculated. The decrease in C:N ratio at May 30th (PL6) could have been caused by the net addition of HMW-DOM with a C:N ratio of 10.3 into the surface water. However, to maintain the lower C:N ratio on June 6th (PL7) a net loss of HMW-DOM with C:N ratio of 13.9 was necessary. Both samples indicate a net increase of N-enriched HMW-DOM in the surface water during the brown tide. The net increase in N-enriched HMW-DOM in the surface water during this period, and thus the possible release of this material by *A. anophagefferens* around May 30th, could be due to adaptation of the organism to the changing environment, e.g. a shift in available nutrients and/or shading due to the increase in cell numbers during this period. The exact mechanism cannot be clarified with this data set. The increase in the C:N ratio combined with the increase in HMW-DOC between June 6th and June 26th could have been caused by an net addition of

HMW-DOM with a C:N ratio of 21.6. This could indicate release of C-enriched HMW-DOM such as the polysaccharide-like exocellular material *A. anophagefferens* is known to produce (Sieburth et al. 1988) or lysis of *Aureococcus anophagefferens* cells coupled with reworking of this material by bacteria as the bloom crashes. In contrast to the dynamic Public Landing site (where the HMW-DOM C/N ratios vary from 10.4 to 18.0), the HMW-DOM C:N ratio at the Greenbackville site was more stable, ranging from 10.7 to 13.8.

The $\delta^{13}C$ and $\delta^{15}N$ data show that the HMW-DOM from Public Landing and Greenbackville differed mainly in $\delta^{13}C$ values; this suggests a difference in carbon source material at the two sites. The $\delta^{13}C$ values from both sites were within the range for marine origin HMW-DOM (-20 to -25 ‰). The $\delta^{15}N$ values for both stations, average 4.2 ± 0.79 ‰, were slightly higher than values from river and coastal end-members, ~3 ‰ (as compiled in Guo and Santschi 1997), and about 2-3 ‰ lower than oceanic surface water (Benner et al. 1997).

The combination of a higher C:N and lower $\delta^{13}C$ for the HMW-DOM at Public Landing could point to a more terrestrial origin, while the lower C:N and higher $\delta^{13}C$ suggests a more marine source of the HMW-DOM at the Greenbackville site. However, as both sites have $\delta^{13}C$ values within the marine range, the difference between the two sites could also be due to differences in autochthonous source material and diagenesis patterns (e.g. contribution of more organic matter from organisms higher up the food chain or the relative proportion of seagrass organic matter at the Greenbackville site). During our sampling period, the carbon and nitrogen isotope signatures for the HMW-DOM did not correlate with the increase in salinity at Public Landing. Such a correlation

would be expected from a two-member mixing model where the terrestrial isotopic signature water of the Chincoteague Bay would be diluted with the isotopic signature of seawater (Guo et al. 1996; Sigleo and Macko 2002). Even though the data set is incomplete, the stable isotope data coupled with the spectroscopy and mass spectrometry data suggests that HMW-DOM was formed *in situ* at Public Landing during the brown tide bloom.

2.4.3. Molecular Level Changes in HMW-DOM

Discriminant analysis of the DT-MS spectra from HMW-DOM (Figs. 8 and 10) separates the Public Landing samples along DF2 (B/W is 13.96 and %var is 4.47%). The spread of the samples from both sites in DF1, DF2 space indicates that the HMW-DOM pool was more varied (see, for example, PL2, 3, and 5 in Fig. 11) when there were fewer brown tide cells. The close grouping of samples collected when *A. anophagefferens* abundance was > 10^4 brown tide cells ml^{-1} (PL4, 6, 7, and 8) suggests that brown tide affects the HMW-DOM pool composition in the surface water.

DF2 scores for the Public Landing samples have a significant correlation with the concentration of HMW-DOC, $\delta^{15}N$ of the HMW-DOM, and the log BT abundance (Fig. 12). This indicates that the compositional change in HMW-DOM during the sampling season is related to the increase in HMW-DOC, increase in $\delta^{15}N$ of the HMW-DOM, and the occurrence of the brown tide bloom.

The DF2 difference spectrum implies that the samples from the brown tide period have a higher aromatic content than non-bloom samples, also indicating a relation between the HMW-DOM and the presence of *A. anophagefferens*.

2.4.4. UV/Vis Absorbance of Bulk DOM

Spectral slope and SUVA profiles, as calculated from UV/Vis analysis, support the DOC and HMW-DOM data by indicating an increase in molecular weight and/or aromaticity of the chromophoric DOM observed at the Public Landing site. The increased percentage of HMW-DOC as determined by ultrafiltration, and the increase in benzene and phenol DT-MS peaks are both consistent with this UV/Vis trend.

The spectral slopes measured in this study, between 0.021 and 0.017, are similar to the range of reported values by Blough and Del Vecchio (2002) and Rochelle-Newall and Fisher (2002) for surface water samples. The spectral slope decreased at Public Landing with increasing *A. anophagefferens* abundance (Fig. 7A), thus indicating that brown tide may be a source of HMW and/or aromatic DOM. The smaller decrease in spectral slope values at the Greenbackville site suggest that other processes affecting DOM characteristics also occur in the surface water. It is likely that the measured decrease of the spectral slope at Public Landing is due to a combination of such processes and input source. Preliminary results from sampling in 2003, where both the Public Landing and the Greenbackville sites experienced a brown tide bloom, show spectral slopes at both sites converging to a common value of roughly 0.0175 at the height of the bloom, the result of an increase in spectral slope at Public Landing and a decrease in spectral slope at Greenbackville (Simjouw et al. in prep.). This indicates that during brown tide blooms DOM of similar optical properties is present.

In 2002, the shift in DOM composition at Public Landing coincided with an increase in DOC and HMW-DOC concentration, which began during the brown tide bloom and was extremely pronounced just after the bloom. The post-bloom concurrent

increase in SUVA indicates that photochemically reactive material was released.

The increase in SUVA at the Greenbackville site during the summer (Fig. 8B) was not associated with significant changes in the total DOC concentration. At the Greenbackville site, *A. anophagefferens* cells were never abundant (<100 cells ml^{-1}) and DOC and HMW-DOC concentrations did not increase nearly as much as at Public Landing. The increase of the SUVA value at Greenbackville could indicate another process that produced or added photochemically reactive material within the total DOC pool. Because SUVA is a DOC-normalized value, the added total DOC at the Public Landing site must have consisted of more photochemically reactive compounds to have obtained an increase in SUVA, even if a similar process to that at Greenbackville is occurring.

In situ production of chromophoric DOM (CDOM) by phytoplankton is an active research area and results have been contradicting (Blough and Del Vecchio 2002). The results presented in this study suggest that *A. anophagefferens* could be responsible for *in situ* production of CDOM in Chincoteague Bay. The SUVA values decreased about 50% two weeks after the height of the bloom and remained higher than the pre-bloom value, which could indicate a significant input of relative refractory CDOM into the system due to the brown tide bloom. However, the CDOM could also be produced by other phytoplankton or by microbial reworking of the DOM after the brown tide bloom.

2.5. CONCLUSIONS

While this study does not result in the identification of a direct link between DOM characteristics and the initiation of an *A. anophagefferens* bloom, it does allow us to

observe changes in DOM characteristics in the surface water due to the brown tide bloom. Our results indicate that *A. anophagefferens* can change the overall DOM pool. During the bloom, DOM that is more aromatic, has a higher molecular weight, and is possibly enriched in N, is introduced into the surface water. How this input effects the ecosystem is not known. The released DOM could be beneficial to the presence and growth of *A. anophagefferens* the next year or to other organisms later during the summer of the same year. It is also interesting to note that a phytoplankton source appears to be creating chromophoric DOM in the Chincoteague Bay, a shallow coastal bay.

In order to elucidate the relationship between DOM and the onset of brown tide blooms (if one exists) several approaches need to be undertaken. We need to be able to sample both high molecular weight and a significant portion of low molecular weight compounds for enrichment studies using axenic cultures of *A. anophagefferens* and for molecular-level identification using mass spectrometry and NMR. The molecular-level analyses can be used to identify representative test molecules for uptake studies using axenic *A. anophagefferens* cultures; this work should monitor both C and N uptake. To provide constraints on DOM sources, isolated high molecular weight and low molecular weight field DOM samples should be analyzed for their stable carbon and nitrogen isotope signatures. Field monitoring should encompass several sites and should occur over several bloom periods. This monitoring could clarify both potential DOM uptake and release by *A. anophagefferens*. The biological and chemical field studies should be done in conjunction with a concerted physical oceanography effort to understand the flushing rates and circulation patterns in the shallow bays where brown tide blooms occur. Such approaches would be labor intensive but would lead to an understanding of

the relationships between *A. anophagefferens* and DOM components in shallow bays prone to brown tide blooms.

SECTION 3

DISSOLVED ORGANIC MATTER CONCENTRATION AND CHARACTERISTICS DURING *AUREOCOCCUS ANOPHAGEFFERENS* BLOOMS IN 2002 AND 2003: A COMPARISION

3.1. INTRODUCTION

Brown tides blooms caused by the pelagophyte, *Aureococcus anophagefferens,* have occurred in increasing numbers of coastal bays along the Eastern USA since 1985 (Cosper et al. 1990; Anderson 1993; Bricelj and Lonsdale 1997; Glibert et al. 2001) and the presence of *A. anophagefferens* has been identified in waters as far south as northern Florida (Popels et al. 2003). *A. anophagefferens* can form intense localized blooms where they dominate (> 90%) the phytoplankton community in these coastal bays (Bricelj and Lonsdale 1997; Gobler and Sañudo-Wilhelmy 2001; Simjouw et al. in press).

Several environmental factors such as temperature, salinity, and low flushing rates of the coastal bay (Keller and Rice 1989; Vieira and Chant 1993; Nixon et al. 1994), low inorganic nutrient concentrations, high dissolved organic matter (DOM) concentrations, and the availability of iron (Glibert et al. 2001; Gobler and Sañudo-Wilhemy 2001a and b; Simjouw et al. in press) have been linked to the occurrence of brown tides. *A. anophagefferens* has been shown to be capable of taking up organic nitrogen compounds (Lomas et al. 1996; Berg et al. 1997; Berg et al. 2002) and organic carbon to supplement autotrophic carbon uptake (Dzurica et al. 1989). The ability to take up organic compounds is hypothesized to be a factor in the initiation of a brown tide, helping *A. anophagefferens* to out-compete other phytoplankton species and to continue blooming

when light limitation due to self-shading limits the autotrophic carbon uptake. Studies measuring uptake of organic compounds have mainly focused on low molecular weight compounds such as glucose, urea and amino acids, but have also included high molecular weight DOM such as algal extract, humic acids, and chitin derivatives. All the above compounds were utilized at least to some degree, indicating that *A. anophagefferens* is capable of using compounds of varied chemical structures as a foodsource.

Aureococcus anophagefferens was first identified in Chincoteague Bay in 1997 and has been monitored since 1998 (Glibert et al. 2001; www.dnr.state.md.us/ coastalbays). A 2002 field study in Chincoteague Bay indicated that the DOM pool changed during and after an *A. anophagefferens* bloom, even though a direct relationship between DOM composition and bloom initiation and development could not be established (Simjouw et al. in press). During the brown tide bloom in 2002, there was intense recycling of the high molecular weight (HMW) DOM, indicated by rapidly changing C:N ratios. The result was a net increase of N-enriched HMW-DOM. The total DOC concentration increased significantly during the bloom and immediately after the bloom. These results were contradictory to findings by Gobler and Sañudo-Wilhemy (2003), who reported an increase in C-enriched HMW-DOM during a brown tide bloom in a small Long Island embayment during the bloom and a rapid decrease in the organic pools when the bloom collapsed. Bulk DOM absorbance characteristics and molecular level characterization of the HMW-DOM during the 2002 Chincoteague Bay bloom indicated a change in DOM composition to a more optically active and more aromatic nature (Simjouw et al. in press).

The 2002 Chincoteague Bay study occurred during a drought year in which

rainfall and streamflow into the bay was about 50% of the average (http://cdo.ncdc.noaa.gov/ancsum/ACS; http://nwis.waterdata.usgs.gov/md/ nwis/discharge). The same suite of measurements was continued in 2003, a year of extensive rainfall in the region. Despite the difference in rainfall, streamflow, and associated nutrient and organic matter inputs, a brown tide bloom again developed in Chincoteague Bay. In this paper we compare DOC concentrations and DOM characteristics before, during, and after brown tide events during 2002 and 2003.

3.2. MATERIALS AND METHODS

3.2.1. Site Selection

We sampled two sites, Public Landing and Greenbackville, in Chincoteague Bay on the Eastern Shore of Maryland and Virginia (Fig. 1). The Public Landing site (PL), which is approximately 18 kilometers north of the Greenbackville site, has had reported brown tide blooms since 1999 (www.dnr.state.md.us/coastalbays). The presence of *Aureococcus anophagefferens* has been reported for the Greenbackville site but not in bloom concentrations (Simjouw et al. in press). During our sampling brown tide blooms were observed at Public Landing in 2002 and 2003 and at Greenbackville in 2003.

3.2.2. Sample Collection

Sampling was performed as described for year 2002 in Simjouw et al. (in press). In 2003, sterile-filtered (<0.2 μm) surface water samples were collected on a monthly basis in March, April, July and August and on a weekly basis during May and June. From these samples aliquots for dissolved organic carbon (DOC), ultraviolet/visible

(UV/Vis) light absorbance measurements, and ultrafiltration were taken. DOC samples were stored frozen and UV/Vis and ultrafiltration samples were stored at 4 °C until processing.

Ultrafiltration was performed using an Amicon 8400 stirred cell with a 1000 Dalton (Da) regenerated cellulose membrane to obtain high molecular weight DOM (HMW-DOM). 600 ml samples were concentrated by a factor of 20. Mass balance and recoveries were monitored by DOC analysis on whole sample, retentate, and filtrate as described in Benner (1991) and Klap (1997). The total DOC recovery of the ultrafiltration (98.4 +/- 8.0 %) indicated no addition or loss of sample from the filter membrane. Desalting of the HMW-DOM fraction occurred within the stirred cell by repeated addition of DI water to a total volume of 1500 ml. The desalting approach did not contribute significantly to the sample DOC and mass spectrometry measurements as shown by blank runs using DI water. The desalted HMW-DOM samples were freeze-dried and stored at 4 °C.

3.2.3. Sample Analysis

DOC concentrations were measured by high temperature combustion using a Shimadzu TOC-5000 as described in Burdige and Homstead (1994). UV/Vis absorbance was measured from 190 to 800 nm using a Varian Cary 3 Bio with DI water as a blank. From the measured UV/Vis absorbance the spectral slope, the absolute value of the slope (S) of the natural log of the absorption coefficient from 270 to 450 nm, and the specific ultraviolet absorbance (SUVA), the absorption coefficient at 254 nm divided by the DOC concentration in mg l^{-1}, were determined.

The stable carbon isotopic signature of the HMW-DOM samples was measured using a PDZ-Europa ANCA-GSL with a 20-20 Stable Isotope Analyzer. Samples were measured in duplicate when possible, and DL-asparagine (Eastman Organic Chemicals), used as the standard and reference compound, was measured in triplicate. Stable isotope ratios are reported as $\delta^{13}C$ using equations and standards as described by Hayes et al. (1978) and Mariotti (1983).

HMW-DOM was also analyzed by direct temperature-resolved mass spectrometry (DT-MS) using 16eV electron impact (EI^+) ionization as in Simjouw et al. in press, Minor 1998, and Eglinton et al. 1996. In this mode, DT-MS analysis gives a broad overview of the chemical composition of the HMW-DOM. The results can be interpreted by comparing the spectra to earlier DT-MS runs of standard compounds as summarized in Minor (1998) and Klap (1997).

To determine trends in the molecular level characteristics of the HMW-DOM samples, principal component and discriminant analysis was performed upon DT-MS data using the program ChemoMetricks (FOM-AMOLF Institute, the Netherlands). The discriminant analysis approach used here is a two-stage principal component analysis technique (see Hoogerbrugge et al. 1983 and Minor and Eglinton 1999 for more information). For discriminant analysis, we selected three HMW-DOM samples from each sampling site each year, one from the time of the highest chl *a* concentration before the brown tide bloom, one from the time of the brown tide bloom maximum, and one from the time of the post-brown tide chl *a* maximum. This allowed us to investigate the differences in the HMW-DOM of the sample set without biasing the multivariate analysis with a dataset consisting primarily of brown tide bloom samples. In the score plot, we

also included HMW-DOM from a nutrient replete, with nitrate as the N source, *A. anophagefferens* culture isolated by ultrafiltration. The culture's HMW-DOM was included as a test set in the discriminant analysis, which means that it was not included in the determination of the two dimensional score plot, but was plotted in the space defined by the DT-MS characteristics of the field samples. To address possible instrument variability, the dataset included replicates (n=3) of four samples (PL3, GrBk3, PL8, and GrBk8) that were analyzed by DT-MS in both 2002 and 2003. Principal component analysis indicated that the replicates from both years have very similar scores for Principal Component 1 and 2 (data not shown), a result indicating that molecular level differences due to instrument variation are negligible compared to the differences in the HMW-DOM from both 2002 and 2003.

3.3. RESULTS

3.3.1. Temperature and Salinity Measurements

In both 2002 and 2003, the surface water temperature pattern from the Greenbackville and the Public Landing sites were very similar, although the surface water temperature at both sites was generally slightly lower for most of the sampling period in 2003 than in 2002 (Fig. 14A, Table 3). In both years the temperature profiles show an increasing trend from 5 to 6 °C in March to a maximum temperature of about 29°C in July.

The salinity profiles for the Public Landing and Greenbackville sites (Table 3, Fig. 14B) show significantly higher salinity values in 2002 relative to 2003. The salinity values for Public Landing and Greenbackville ranged from 30.4 psu in early May 2002 to

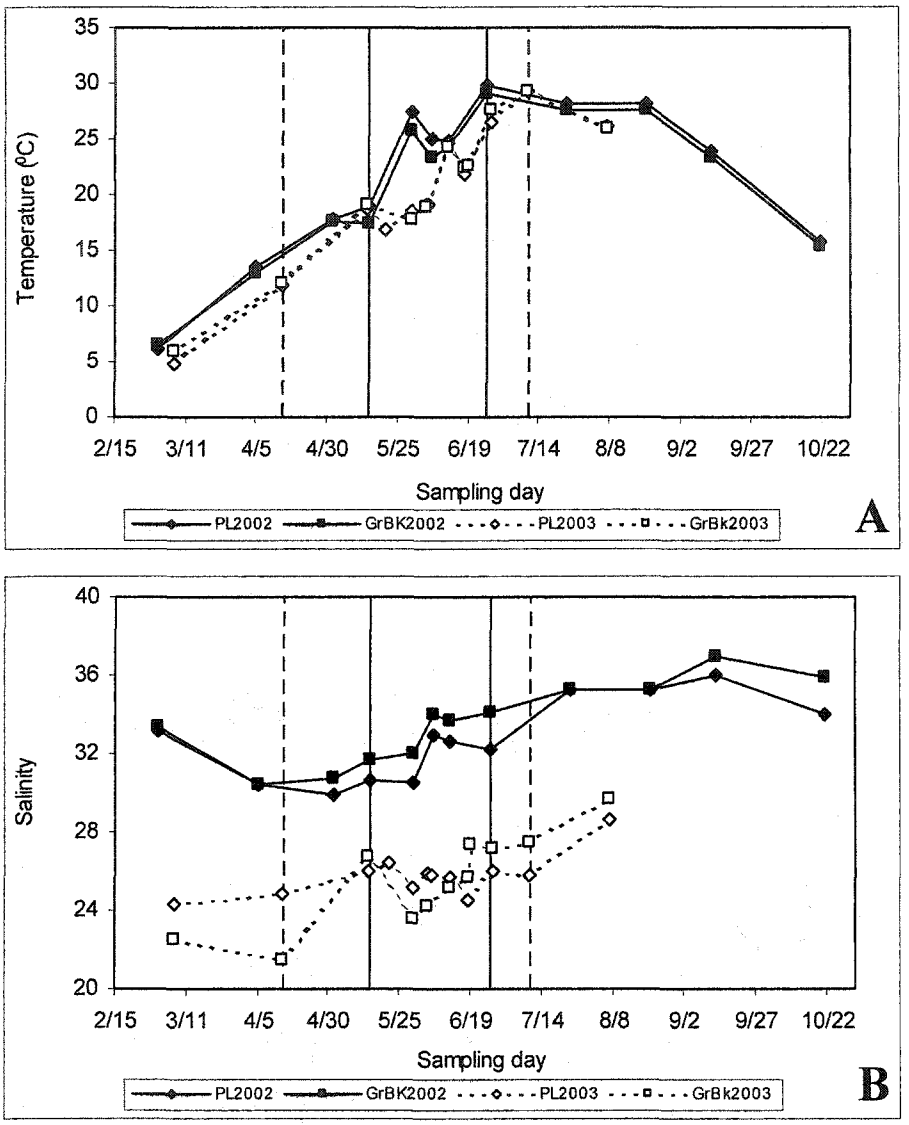

Fig. 14. Temperature (A) and Salinity (B) of surface water samples from Public Landing and Greenbackville in 2002 and 2003. The vertical solid lines and the interrupted lines indicate the brown tide bloom periods (> 10^4 cells ml^{-1}) in 2002 (Public Landing only) and 2003 respectively.

TABLE 3. Summary of environmental parameters (temperature and salinity) and surrogate biomass measurements (chlorophyll *a* and brown tide cell counts) from the Public Landing and Greenbackville site from both sampling years. The standard deviation of the value is given in parentheses. In the shaded area are the parameters during the brown tide period (defined as > 10^4 cells ml^{-1}).

Sample Site Public Landing	Date	T (°C)	Salinity (psu)	Chl *a* ($\mu g\ l^{-1}$)	BT counts (10^5 cells ml^{-1})
2002					
PL2	03/01/02	6.15	33.2	1.2 (0.24)	0.0002 (.0001)
PL3	04/05/02	13.6	30.5	1.8 (0.38)	0.0001
PL4	05/02/02	17.7	29.9	5.2 (0.19)	0.152 (0.02)
PL5	05/15/02	18.9	30.6	2.4 (0.16)	0.004
PL6	05/30/02	27.5	30.5	10.2 (1.06)	3.96 (0.16)
PL7	06/06/02	25	33.0	14.4 (1.52)	5.88 (0.51)
PL8	06/12/02	24.8	32.6	19.5 (1.46)	12.1 (2.11)
PL9	06/26/02	29.8	32.2	18.4 (1.68)	0.011 (0.001)
PL10	07/24/02	28.1	35.2	16.7 (1.00)	0.001 (0.0002)
PL11	08/21/02	28.0	35.2	22.4 (4.46)	0.002 (0.0008)
PL12	09/13/02	23.9	36.0	11.2 (0.85)	0
PL13	10/21/02	15.7	34.0	3.02 (0.26)	0.0001 (0.0001)
2003					
PL14	03/07/03	4.89	24.3	5.10 (0.07)	0
PL15	04/14/03	11.8	24.9	4.4 (0.27)	0.007 (0.001)
PL16	05/14/03	18.8	26.0	0.8 (0.47)	1.59 (0.169)
PL17	05/21/03	16.9	26.5	7.2 (0.71)	1.68 (0.087)
PL18	05/30/03	18.4	25.2	6.9 (1.29)	2.50 (0.313)
PL19	06/04/03	19.1	25.9	9.7 (0.68)	4.91 (0.535)
PL20	06/05/03	19.1	25.8	8.8 (0.26)	-
PL21	06/12/03	24.6	25.7	4.5 (0.24)	0.062 (0.015)
PL22	06/18/03	21.9	24.5	21.8 (0.53)	3.51 (0.563)
PL23	06/19/03	-	-	-	-
PL24	06/27/03	26.46	25.99	11.19 (0.45)	0.889 (0.161)
PL25	07/10/03	29.31	25.76	8.04 (0.20)	0.005 (0.001)
PL26	08/07/03	26.08	28.61	12.49 (1.28)	0

TABLE 3. Continued.

Sample Site Greenbackville	Date	T (°C)	Salinity (psu)	Chl *a* (μg l^{-1})	BT counts (10^5 cells ml^{-1})
2002					
GrBk2	03/01/02	6.49	33.3	0.8 (0.04)	0.00027
GrBk3	04/05/02	12.9	30.4	1.2 (0.05)	0
GrBk4	05/02/02	17.5	30.8	8.7 (0.42)	0
GrBk5	05/15/02	17.5	31.7	1.7 (0.23)	0.00018
GrBk6	05/30/02	25.7	32.0	3.5 (1.49)	0.00093
GrBk7	06/06/02	23.3	34.0	7.4 (0.75)	0.00096
GrBk8	06/12/02	24.3	33.7	5.1 (2.4)	0.00075
GrBk9	06/26/02	29.1	34.1	10.1 (1.71)	0.00013
GrBk10	07/24/02	27.6	35.3	14.2 (0.61)	0.00026
GrBk11	08/21/02	27.7	35.3	11.8 (1.4)	0
GrBk12	09/13/02	23.3	37.0	6.7 (0.60)	0
GrBk13	10/21/02	15.4	35.9	2.3 (0.09)	0
2003					
GrBk14	03/07/03	5.97	22.5	5.1 (0.02)	0
GrBk15	04/14/03	12.0	21.5	6.9 (0.43)	0
GrBk16	05/14/03	19.0	26.3	0.6 (0.03)	0.002 (0.0001)
GrBk17	05/21/03	-	-	-	-
GrBk18	05/30/03	17.9	23.6	8.3 (0.56)	2.90 (0.334)
GrBk19	06/04/03	18.9	24.2	10.9 (0.35)	6.77 (0.399)
GrBk20	06/05/03	-	-	-	-
GrBk21	06/12/03	24.3	25.1	14.9 (1.99)	7.24 (0.512)
GrBk22	06/18/03	22.4	25.7	9.5 (0.27)	3.58 (0.426)
GrBk23	06/19/03	22.6	27.3	13.6 (3.10)	-
GrBk24	06/27/03	27.5	27.1	13.7 (1.12)	1.11 (0.059)
GrBk25	07/10/03	29.3	27.5	15.6 (0.16)	0.007 (0.001)
GrBk26	08/07/03	25.9	29.7	11.2 (0.90)	0

35.3 psu on July 24, 2002 as compared to 21.5 to 29.7 psu for April 14 to August 7, 2003. Public Landing, the brown tide bloom site, had a slightly lower salinity during the 2002 brown tide period compared to the Greenbackville site, where no bloom occurred in 2002. The salinity in 2003 was comparable at both sites during the bloom period but lower than in 2002.

3.3.2. Chlorophyll *a* and *Aureococcus anophagefferens* Density Measurements

The chlorophyll *a* data showed several different phases of phytoplankton growth at both sites during both sampling years (Fig. 15A). First, a small spring bloom, from March to early May, occurred at both sites in both years. This small phytoplankton bloom occurred earlier in 2003, in mid-March, than in 2002 when the first maximum was in early May. In both years the chl *a* concentration at Greenbackville was higher during this period than at Public Landing. Second, in mid May of both years, both sampling experienced a decrease in chl *a* concentration to very low levels just before the brown tide period.

After May 15, the chl *a* concentration increased considerably, simultaneous with the rapid increase in *A. anophagefferens* cell abundance (Table 3; Fig. 15B) at Public Landing in 2002 and at both sampling sites in 2003. The chl *a* increase and the brown tide cell density peaked in mid-June at 19.5 mg l^{-1} in 2002 and at 9.7 mg l^{-1} in 2003 at Public Landing and at 14.9 mg l^{-1} at Greenbackville in 2003. The chl *a* concentrations at Greenbackville in 2002, when no brown tide bloom was measured, also increased but at a slower rate than at bloom sites.

When the brown tide bloom was diminishing at the sampling sites in mid to late

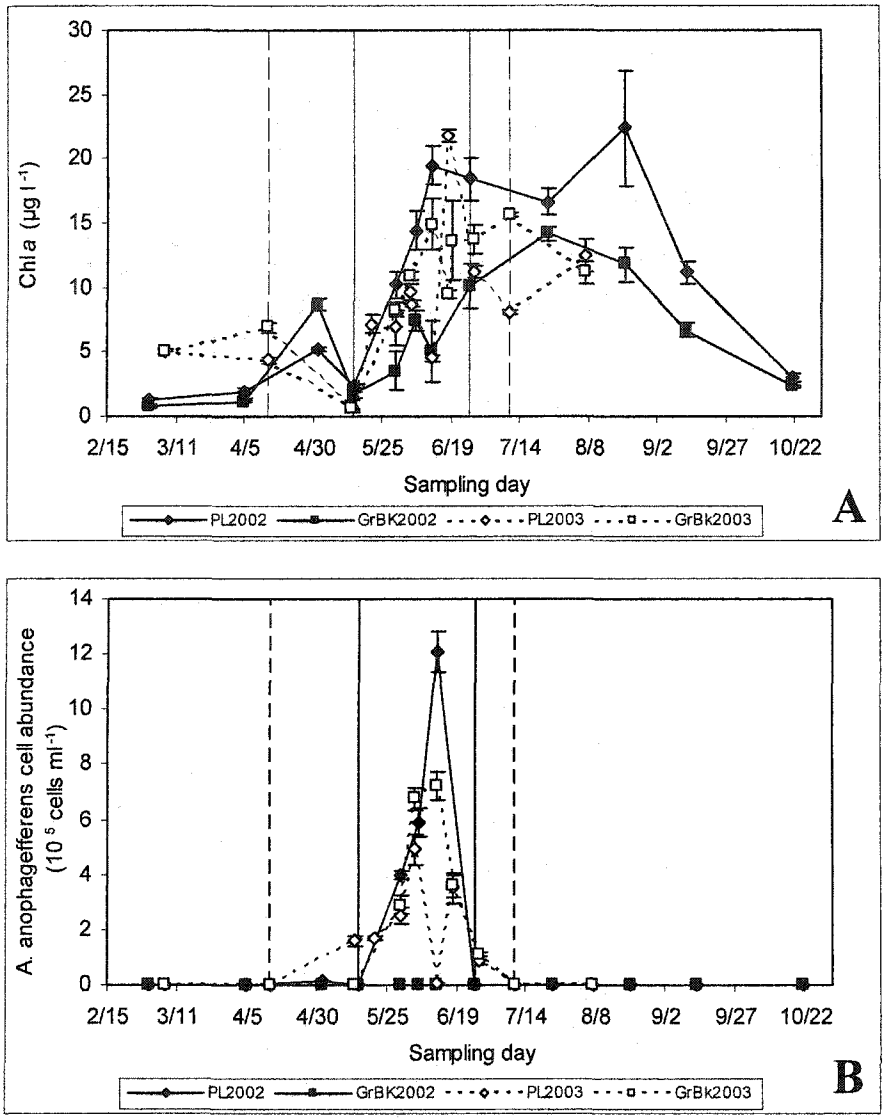

Fig. 15. Chl *a* concentration (A) and *A. anophagefferens* cell abundance (B) of surface water samples from Public Landing and Greenbackville in 2002 and 2003. Vertical lines as in Fig. 14.

June (Fig. 15B), the chl *a* concentration at these brown tide bloom sites also decreased (Table 3; Fig.15A). At Public Landing in 2002, the chl *a* decreased only slightly before a new non-brown tide bloom occurred in late August. In 2003 similar post-brown tide chl *a* increases took place at both sampling sites but earlier than in 2002. At Greenbackville in 2003 this chl *a* peak occurred right after the brown tide bloom, while at Public Landing in 2003, the chl *a* increase was simultaneous with another increase in brown tide cell density in mid June, followed by a crash and a second, non-brown tide, chl *a* increase in early August.

Assuming a chl *a* content of 0.035 ± 0.003 pg cell^{-1}, the value found for *A. anophagefferens* in nutrient replete cultures (Gobler et al. 2002; Gobler and Sañudo-Wilhelmy 2001), one can estimate the contribution of this alga to field measurements of chl *a* concentration (Fig. 16). For the Greenbackville site in 2003, the spring to fall sampling period could be broken down into three stages, a pre-brown tide bloom with a chl *a* maximum and a decrease, the brown tide bloom itself, and a following non brown tide chl *a* maximum (Fig. 16C). For the Public Landing site in 2003 we see a similar pattern for the first two stages, pre brown tide bloom and brown tide bloom, but the third stage included a simultaneous increase in brown tide chl *a* and chl *a* due to other phytoplankton in mid June (Fig. 16B) when about 55% of the total chl *a* was due to the presence of *A. anophagefferens*. For Public Landing in 2002 the chl *a* and brown tide density measurements showed three successive bloom stages similar to those at Greenbackville in 2003 (Fig. 16A). The *A. anophagefferens* cell abundance at Greenbackville in 2002 was not responsible for the chl *a* concentration profile during the sample period. All three bloom events (Fig. 16) indicate that brown tide dominated chl *a*

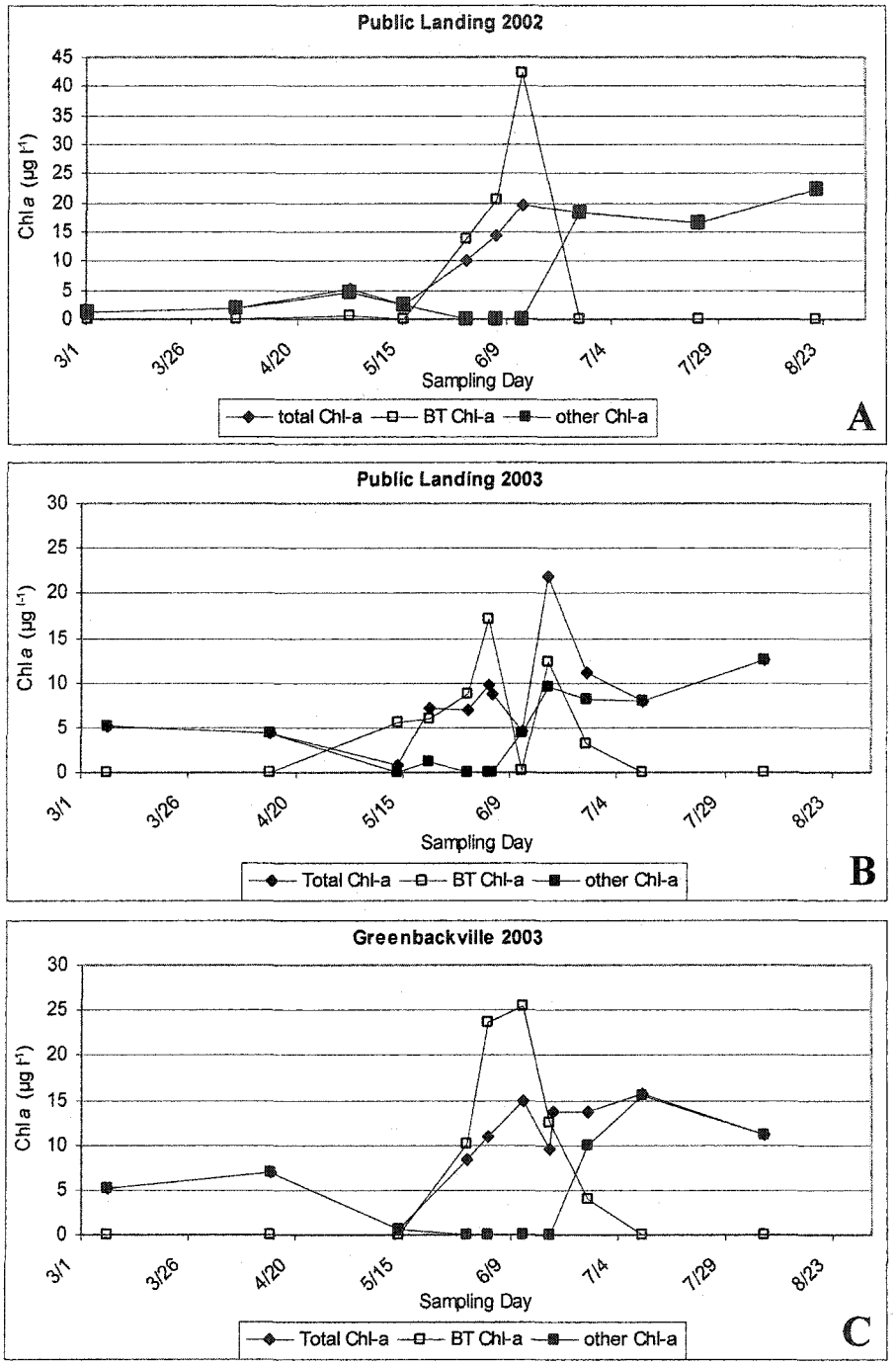

Fig. 16. Total chl *a* concentration, chl *a* concentration due to the presence of brown tide based on a chl *a* content of 0.035 pg cell^{-1}, and chl *a* concentration due to phytoplankton other than *Aureococcus anophagefferens* in surface water samples from Public Landing in 2002 (A), Public Landing in 2003 (B), and Greenbackville in 2003 (C).

concentrations during blooms while before and after the brown tide bloom other phytoplankton controlled the chl *a* concentration.

3.3.3. UV/Vis Absorbance Characteristics

Both the spectral slope (S) and the specific UV absorbance (SUVA) values showed contrasting trends during the two sampling years (Fig. 17A; Table 4). In 2003, the S values ranged from 0.0151 to 0.0182 nm^{-1} for Public Landing and between 0.0164 and 0.0192 nm^{-1} for Greenbackville. In 2002, they varied between 0.0172 and 0.0211 nm^{-1} for Public Landing and between 0.0176 and 0.0203 nm^{-1} for Greenbackville. Surface water samples taken in March and April exhibited lower S values for both sampling sites in 2003 compared to 2002, indicating the presence of more aromatic DOM in 2003. The S value decreased at Public Landing in 2002 and at Greenbackville in 2003 during the brown tide blooms, but increased during the brown tide bloom at Public Landing in 2003. Even though the S values at sites where blooms occurred differed by sites and at the beginning of the sampling periods, they converged during the maximum brown tide bloom to a range of 0.0174 and 0.0180 nm^{-1} (Table 4; Fig. 17A). It thus appears that during the peak of a brown tide bloom, DOM with similar absorbance characteristics was present in the surface water at the bloom sites. However, no bloom occurred at Greenbackville in 2002, yet it also exhibited a similar S value (0.0181 nm^{-1}). The SUVA values for both sites in 2003 were higher prior to and during the brown tide bloom than the SUVA values for both sites in 2002. Unlike the spectral slope data, the SUVA values of samples from 2002 and 2003 did not converge at the peak of the brown tide blooms, but instead reached similar values after the brown tide bloom.

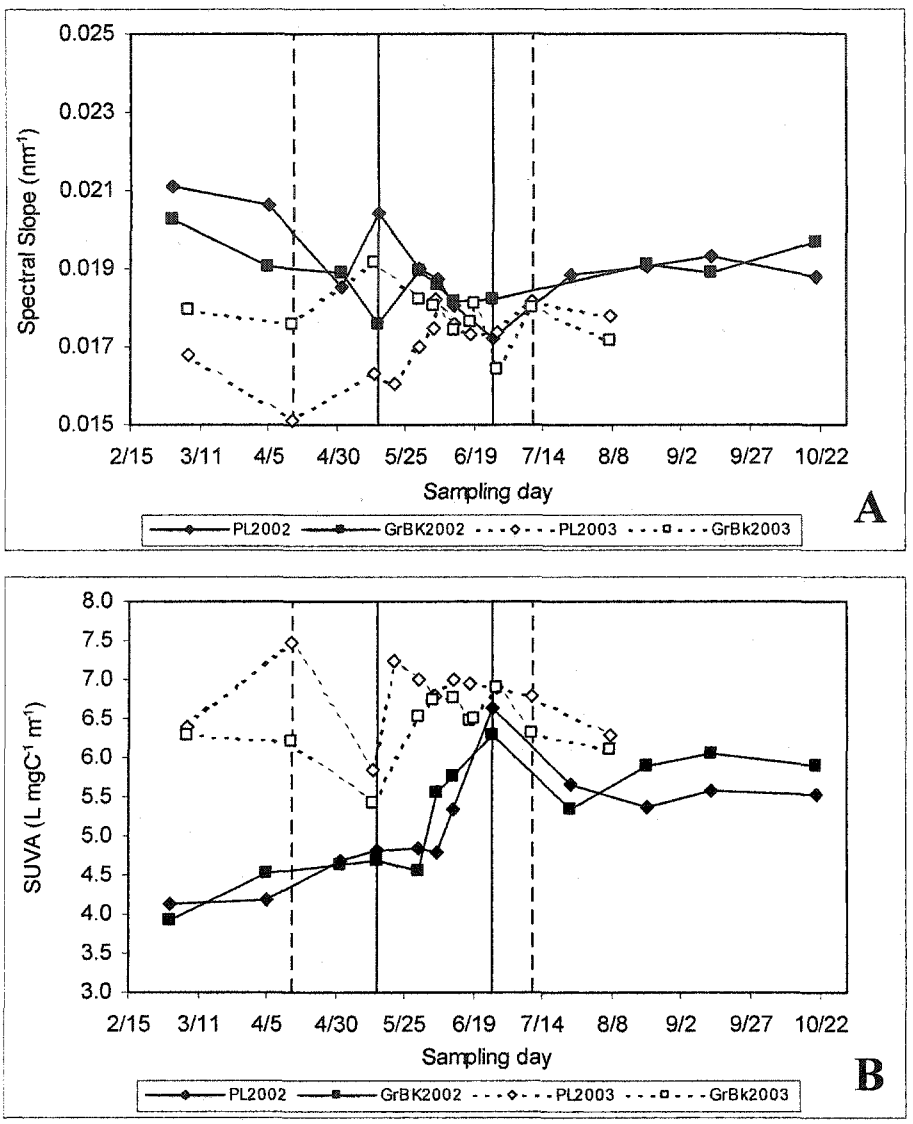

Fig. 17. The spectral slope (A) and the specific UV absorbance (B) calculated from UV/Vis absorbance measurements of surface water samples from Public Landing and Greenbackville in 2002 and 2003. Vertical lines as in Fig. 14.

TABLE 4. Summary of bulk ultraviolet/visible light absorbance parameters, dissolved organic carbon concentration of both the bulk and high molecular weight organic matter pool, and stable carbon isotope measurements of the high molecular weight dissolved organic matter pool for the Public Landing and Greenbackville from both sampling years. The standard deviation of the value is given in parentheses. When no standard deviation is given only one measurement was taken. n.m. is not measured because of sample size constraints and n.s. is not sampled. In the shaded area are the parameters during the brown tide period (> 10^4 cells ml^{-1}).

Sample Site Public Landing	Date	Spectral Slope (nm^{-1})	SUVA (L mg C^{-1} m^{-1})	DOC (µM)	HMW-DOC (µM)	% HMW-DOC	$\partial^{13}C$ HMW-DOM (‰)
2002							
PL2	03/01/02	0.0211	4.12	366 (1.9)	71 (0.2)	19.5	-21.25 (0.62)
PL3	04/05/02	0.0206	4.19	360 (7.3)	109 (0.1)	30.2	-20.74 (0.22)
PL4	05/02/02	0.0185	4.67	381 (3.6)	147 (1.3)	38.6	-21.57 (0.03)
PL5	05/15/02	0.0204	4.81	369 (5.1)	131 (0.3)	35.5	n.m.
PL6	05/30/02	0.0190	4.83	429 (3.1)	210 (1.8)	49.0	-22.07
PL7	06/06/02	0.0187	4.79	424 (8.6)	146 (0.7)	34.5	-20.98
PL8	06/12/02	0.0180	5.33	491 (1.2)	210 (0.2)	42.8	n.m.
PL9	06/26/02	0.0172	6.63	592 (3.0)	257 (0.7)	43.4	-21.74 (0.09)
PL10	07/24/02	0.0189	5.66	508 (2.0)	273 (0.5)	53.7	-21.63 (0.08)
PL11	08/21/02	0.0191	5.37	678 (1.4)	405 (0.3)	59.7	-22.23 (0.32)
PL12	09/13/02	0.0193	5.57	499 (0.8)	286 (0.6)	57.3	-21.75 (0.07)
PL13	10/21/02	0.0188	5.52	443 (3.4)	227 (0.9)	51.1	-21.21 (0.02)
2003							
PL14	03/07/03	0.0168	6.40	276 (1.28)	163 (1.5)	59.1	-23.06 (0.62)
PL15	04/14/03	0.0151	7.48	268 (9.24)	166 (2.7)	62.1	-24.87
PL16	05/14/03	0.0163	5.84	260 (1.35)	135 (0.3)	52.1	-23.62
PL17	05/21/03	0.0161	7.23	295 (2.97)	160 (2.7)	54.1	-24.11
PL18	05/30/03	0.0170	7.01	313 (3.97)	184 (0.3)	58.8	-23.81
PL19	06/04/03	0.0175	6.79	294 (1.27)	167 (1.2)	56.8	-23.43 (0.17)
PL20	06/05/03	0.0182	6.79	287 (4.85)	148 (0.4)	51.4	-22.92
PL21	06/12/03	0.0176	7.00	286 (6.71)	163 (1.5)	57.1	-23.42 (0.59)
PL22	06/18/03	0.0173	6.96	382 (11.54)	206 (1.4)	54.1	-22.99 (0.49)
PL23	06/19/03	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
PL24	06/27/03	0.0173	6.89	353 (2.46)	183 (0.3)	51.9	-23.76
PL25	07/10/03	0.0181	6.78	397 (2.86)	242 (0.6)	60.9	-23.33
PL26	08/07/03	0.0178	6.28	376 (1.24)	210 (1.4)	55.8	-21.41 (0.38)

TABLE 4. Continued.

Sample Site Greenbackville	Date	Spectral Slope (nm^{-1})	SUVA (L mg C^{-1} m^{-1})	DOC (µM)	HMW-DOC (µM)	% HMW-DOC	$\partial^{13}C$ HMW-DOM (‰)
2002							
GrBk2	03/01/02	0.0203	3.92	215 (0.9)	75 (2.8)	35.1	-20.71 (0.34)
GrBk3	04/05/02	0.0191	4.52	277 (1.5)	126 (0.8)	45.6	-20.49 (0.04)
GrBk4	05/02/02	0.0189	4.64	270 (3.2)	101 (1.0)	37.4	-20.33 (0.04)
GrBk5	05/15/02	0.0176	4.68	245 (1.1)	123 (2.9)	50.3	-20.21
GrBk6	05/30/02	0.0189	4.54	276 (1.0)	112 (0.6)	40.6	-21.26 (0.03)
GrBk7	06/06/02	0.0186	5.55	258 (2.8)	119 (0.8)	46.0	-20.23 (0.06)
GrBk8	06/12/02	0.0181	5.76	229 (1.2)	117 (0.3)	51.1	-20.32
GrBk9	06/26/02	0.0182	6.3	297 (0.3)	147 (0.6)	49.5	-19.94 (0.12)
GrBk10	07/24/02	n.m.	5.35	303 (1.8)	147 (0.9)	48.5	-19.95 (0.09)
GrBk11	08/21/02	0.0191	5.91	281 (1.5)	151 (0.6)	54.0	-19.75 (0.14)
GrBk12	09/13/02	0.0189	6.05	458 (2.7)	268 (4.3)	58.6	-19.71 (0.00)
GrBk13	10/21/02	0.0197	5.89	445 (3.0)	230 (0.4)	51.6	n.m.
2003							
GrBk14	03/07/03	0.0180	6.28	344 (2.04)	202 (1.6)	58.5	-22.66 (0.11)
GrBk15	04/14/03	0.0176	6.21	310 (4.43)	177 (2.5)	57.0	-22.05 (0.01)
GrBk16	05/14/03	0.0192	5.41	242 (2.23)	129 (0.2)	53.4	-20.73 (0.51)
GrBk17	05/21/03	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
GrBk18	05/30/03	0.0182	6.53	350 (2.68)	201 (1.5)	57.3	-23.02 (0.26)
GrBk19	06/04/03	0.0180	6.73	315 (2.51)	186 (1.4)	58.8	-22.39 (0.21)
GrBk20	06/05/03	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
GrBk21	06/12/03	0.0174	6.75	295 (4.97)	176 (0.7)	59.7	-23.62 (0.73)
GrBk22	06/18/03	0.0176	6.46	336 (3.86)	186 (1.0)	55.4	n.m.
GrBk23	06/19/03	0.0181	6.51	298 (0.35)	165 (0.1)	55.6	-23.23 (0.25)
GrBk24	06/27/03	0.0164	6.91	330 (1.06)	186 (0.7)	56.4	-21.70 (0.27)
GrBk25	07/10/03	0.0180	6.32	338 (11.07)	211 (1.0)	62.4	-20.75
GrBk26	08/07/03	0.0172	6.10	264 (0.53)	135 (0.8)	51.2	-19.69 (0.53)

The significant increase in SUVA value at Public Landing and Greenbackville after the brown tide period in 2002 was not reiterated in 2003. In both 2002 and 2003, SUVA values at Greenbackville showed similar overall trends to the Public Landing dataset. This result, coupled with the shift in timing of convergence, indicates processes governing the SUVA value could be separate from processes that change the spectral slope of the surface water.

3.3.4. DOC Measurements

In March 2003, the DOC concentration of the surface water at Greenbackville (344 μM) was significantly higher than at Public Landing (276 μM) and was comparable to the DOC concentration at Public Landing in 2002 (366 μM ; Table 4; Fig. 18A). By mid-May, 2003, the DOC concentration had decreased at Greenbackville and at Public Landing and was similar to the DOC concentration at Greenbackville in 2002. During the brown tide bloom at Public Landing and Greenbackville in 2003, the DOC concentration increased slightly at both sampling sites, but the drastic increase in DOC concentration at Public Landing in 2002 during and immediately after the brown tide bloom did not occur at either sampling site in 2003.

The profiles for the 2003 HMW-DOC concentrations show the same general features as the 2003 DOC profiles for Public Landing and Greenbackville (Table 4; Fig. 18B). The HMW-DOC concentration for the March and April 2003 samples were higher than the corresponding samples taken in 2002, possibly due to an increased terrigenous DOM input in 2003 that might have introduced HMW-DOC into the surface water. Even so, all samples from both 2002 and 2003 exhibit a similar HMW-DOC concentration

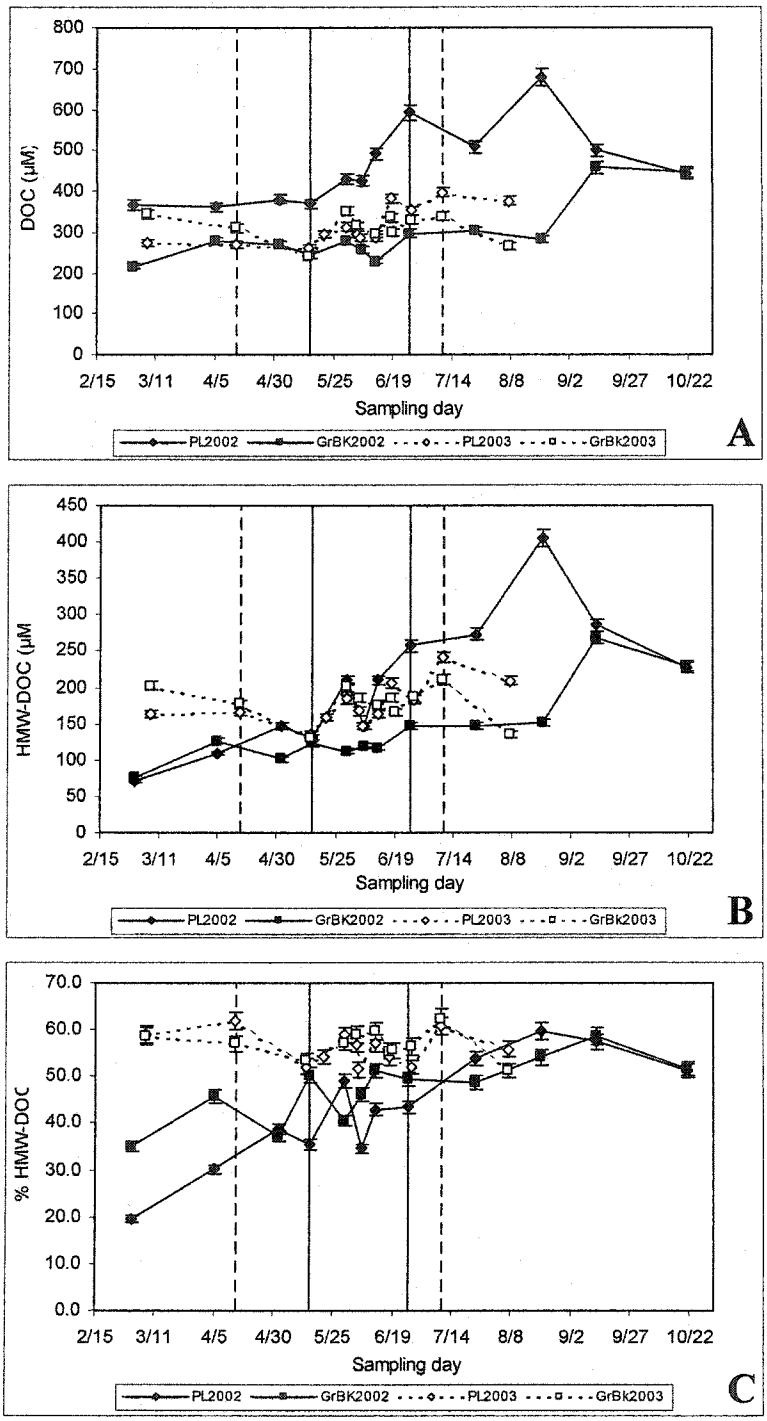

Fig. 18. The dissolved organic carbon concentration (A), the high-molecular-weight dissolved organic carbon concentration (B), and the % high-molecular-weight dissolved organic carbon of the dissolved organic carbon pool (C) of surface water samples from Public Landing and Greenbackville in 2002 and 2003. Vertical lines as in Fig. 14.

around mid May, right before the start of the brown tide bloom. The HMW-DOC concentrations at Public Landing in 2002 and at both sampling sites in 2003 increased during and after the brown tide bloom until mid July. The HMW-DOC concentration of the 2002 Greenbackville samples, where no bloom occurred, increased only slightly until August. The bigger increase in HMW-DOC after the brown tide bloom at Public Landing in 2002 compared to the HMW-DOC increase at both sampling sites in 2003 was associated with a higher brown tide cell density.

Initial %HMW-DOC values in 2003 were higher, about 59% for both sampling sites, than in 2002 (19.5% for Greenbackville and 35.1% for Public Landing, Table 4; Fig. 18C). In 2002, however, the %HMW-DOM increased at both sampling sites to a maximum of 59.7% in August for Public Landing and 58.6% in September at Greenbackville. The samples from 2003 varied much less over time, between 51 to 62 %HMW-DOC. Between mid May and mid June, the brown tide bloom period in 2002 and 2003, the %HMW-DOC values were higher for the 2003 sampling sites.

3.3.5. $\delta^{13}C$ of the HMW-DOM

The HMW-DOM from both sites had lower early spring $\delta^{13}C$ values in 2003 than in 2002 (Table 4; Fig. 19). The $\delta^{13}C$ values for the HMW-DOM collected from both sampling sites in March 2003 was about -23 ‰, while the HMW-DOM in March 2002 was more enriched and exhibited $\delta^{13}C$ values of around –21 ‰. There were very different trends in the $\delta^{13}C$ values in 2003 relative to 2002. In 2002, the HMW-DOM $\delta^{13}C$ values from Public Landing decreased overall in $\delta^{13}C$ values during the sampling period and the first half of the brown tide bloom, while the $\delta^{13}C$ values for the 2002 samples from

Greenbackville, where no bloom occurred, slightly increased to more enriched HMW-DOM. The $\delta^{13}C$ values for the HMW-DOM indicated a more dynamic system at both sampling sites in 2003. The $\delta^{13}C$ values for Greenbackville in 2003 initially increased to –20.73 ‰, but then decreased to –23.62 ‰ during the maximum brown tide bloom. The $\delta^{13}C$ values of the HMW-DOM from Public Landing increased during the brown tide bloom and at the maximum brown tide cell abundance exhibited similar $\delta^{13}C$ values to those of the 2003 Greenbackville maximum bloom samples. When the brown tide bloom diminished at both sampling sites in 2003, the $\delta^{13}C$ values increased again at both sites, reaching August values of –19.7 ‰ at Greenbackville and –21.4 ‰ at Public Landing. This increase occurred during the time of the third chl *a* maximum at the two sites. During the 2003 brown tide blooms, the $\delta^{13}C$ values for HMW-DOM were more depleted than during the brown tide bloom at Public Landing in 2002.

3.3.6. DT-MS Results

Discriminant analysis of selected DT-MS data shows a clear separation of most HMW-DOM samples based upon sampling year (Fig. 20). This separation, primarily along discriminant function 1 (DF1), explains 26.5% of the variance with a B/W of 50.4. Samples collected prior to and during the maximum brown tide bloom in 2002 plot with a relatively similar positive DF1 score, while the 2003 bloom samples plot along the negative DF1 score axis. The post brown tide bloom samples from 2002 plot away from the rest of the 2002 data set and indeed separately from the rest of the samples in both DF1 and DF2 (which explains 17.6% of the variance with B/W of 33.7). The AA UDOM samples, which were run as a test rather than a training set, plots near

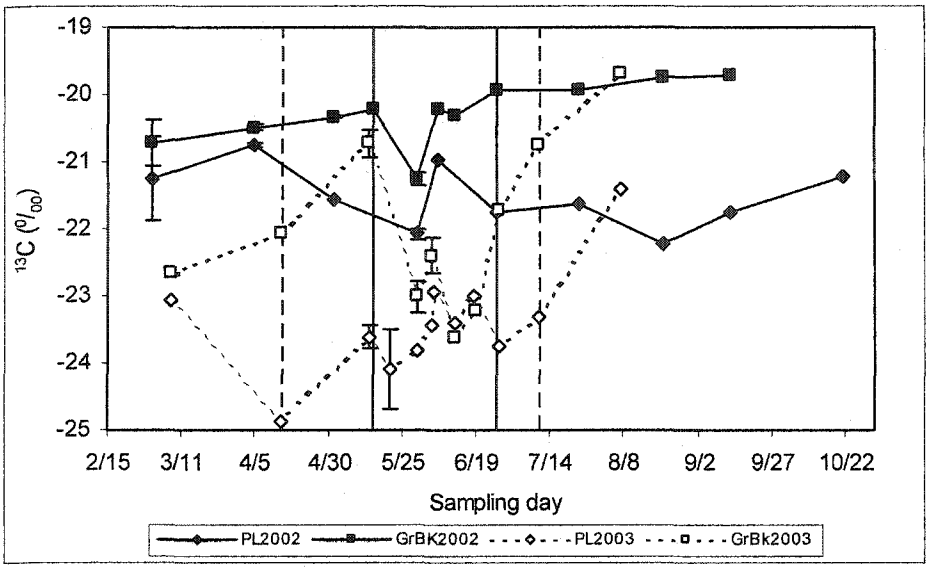

Fig. 19. Stable carbon isotope values of high-molecular-weight dissolved organic matter isolated from surface water samples from Public Landing and Greenbackville in 2002 and 2003. Error bars represent the standard deviation from Table 4. Vertical lines as in Fig. 14.

the PL post brown tide bloom sample from 2002 (PL11). The maximum and post brown tide bloom samples from both sampling years appear to shift along DF1 to smaller score values and to similar DF1 scores than the *A. anophagefferens* culture HMW-DOM DF1 score (Fig. 20).The difference spectrum along DF1 (Fig. 21A) revealed the separation between the 2002 and 2003 samples is due in large part to the enriched presence of acetamide (m/z 59), benzene (m/z 78), phenol (m/z 92), and aromatic protein pyrolysis products from tyrosine (m/z 94, 108, and 120), phenylalanine (m/z 91, 92, and 106), and tryptophan (m/z 117 and 131) in the positive DF1 scores where the 2002 HMW-DOM samples plot. The separation was also due to the enriched presence of pentose sugars (m/z 85 and 114),furfurals (m/z 96 and 110), and some potential lignin pyrolysis products (m/z 124, 138, and 151) for the 2003 dataset and the 2002 post brown tide bloom samples with negative DF1 scores. Along DF2, the difference spectrum indicates potential enrichment of sterols (m/z 368, 380, and 286), fatty acids (m/z 228, 256, 278, and 242), and several lignin-like pyrolysis products (m/z 210, 180, 167, 164, 137, 124 for syringyl and guaiacyl units of lignin) for the 2003 HMW-DOM samples and the Greenbackville 2002 post-brown tide period sample (Fig. 21B). To further investigate differences between HMW-DOM samples, the position of the m/z values (the loadings) were superimposed onto the score plot to yield information on which compounds (actually the enrichment of these compounds in the sample) are most likely responsible for the separation of the samples from both years. The *A. anophagefferens* culture HMW-DOM sample and the post brown tide bloom sample from Public Landing in 2002 separated from the rest of the HMW-DOM samples predominantly due to enrichment in furfural compounds (m/z 96 and 110). The separation between the rest of the samples from both

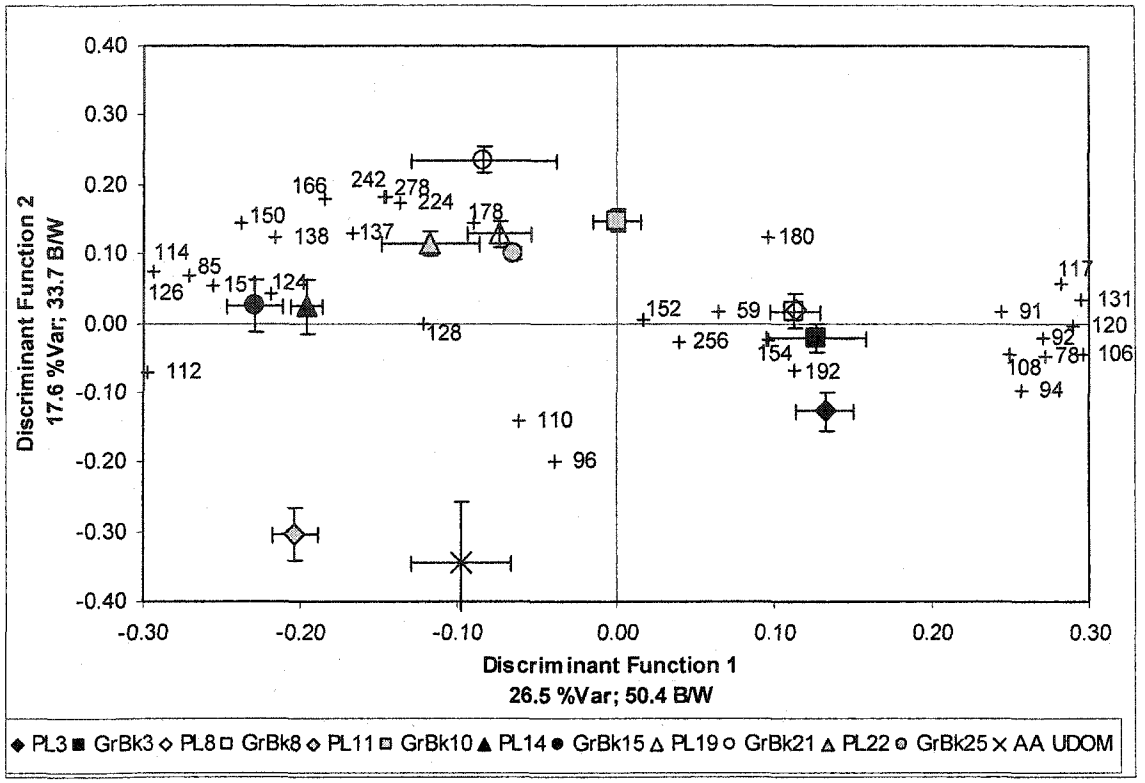

Fig. 20. Discriminant analysis score plot showing the average scores for high-molecular-weight dissolved organic matter samples from Public Landing (PL) and Greenbackville (GrBk) pre-brown tide bloom (filled black symbols), maximum brown tide bloom (open symbols), and post-brown tide bloom (filled gray symbols) in 2002 (♦ and ■ symbols) and 2003 (▲ and ● symbols). Samples were analyzed by direct temperature resolved mass spectrometry. See Tables 1 and 2 for further information concerning individual samples. AA UDOM represents high molecular weight dissolved organic matter isolated from an *Aureococcus anophagefferens* culture. The AA UDOM sample was run as a test and thus was not included in the score plot calculations. Note: all samples were run in triplicate. Numbered crosses represent the direction of the m/z loadings in this score plot.

years was due to the fatty acid, sterol, and pentose sugars along with the suite of lignin-like pyrolysis products present in the 2003 HMW-DOM samples compared to the enriched presence of the aromatic proteins along with the benzene and phenol signature (Fig. 20) for the 2002 HMW-DOM samples.

3.4. DISCUSSION

3.4.1. Environmental Parameters

In 2003, the temperature of the surface water during the brown tide period (> 10,000 cells ml^{-1}) at both sampling sites was 3 to 5 °C lower than during the brown tide in 2002. The temperature range at both sites was within the ranges in which brown tides have been reported by other studies (Cosper et al. 1989; Glibert et al. 2001; Gobler et al. 2002).A greater difference was apparent between the salinity of the surface water in 2002 and 2003. The salinity at both sampling sites was on average 7 to 10 units lower in 2003, at the lower end of ranges reported in other studies of brown tide blooms (Cosper et al. 1989; Glibert et al. 2001; Gobler et al. 2002). Precipitation during the sampling period in2002 was below average while in 2003 the precipitation was above average (http://cdo.ncdc.noaa.gov/ancsum/ACS; http://nwis.waterdata.usgs.gov/md/nwis/discharge), thus the lower salinity in 2003 is believed to be caused by freshwater input due to rain.

The lower salinity in could be the cause for the slower bloom development at Public Landing and the lower brown tide cell density maximum at both sampling sites in 2003 as was shown earlier using culture growth studies by Cosper et al. (1989).

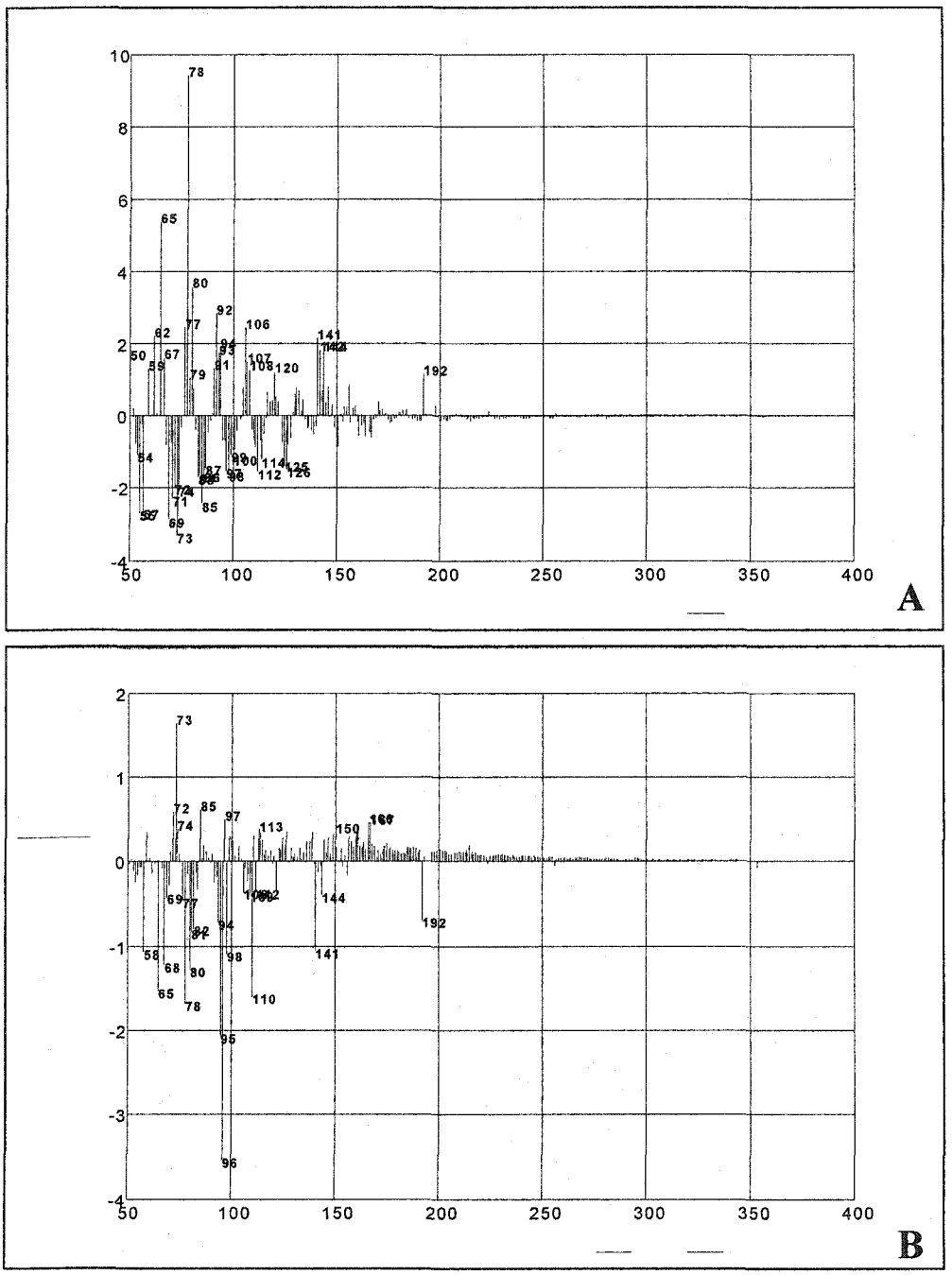

Fig. 21. Difference spectrum reconstructed from discriminant function 1 (A) and discriminant function 2 (B) loadings. The discriminant analysis of HMW-DOM samples pre-brown tide bloom, maximum brown tide bloom, and post-brown tide bloom from Public Landing, MD and Greenbackville, VA in 2002 and 2003 is the same as shown in Fig. 19.

3.4.2. Bloom Events

In contrast to 2002, when only Public Landing experienced a brown tide bloom, both Public Landing and Greenbackville experienced a brown tide bloom in June and July 2003. However, in 2003 the brown tide blooms at both sites differed in intensity and duration. Public Landing experienced an earlier increase in *A. anophagefferens* cell abundance and reached bloom conditions slightly earlier than Greenbackville. In Public Landing in 2003, between June 4 (max bloom) and June 12, *A. anophagefferens* diminished in abundance while at Greenbackville no interruption in the brown tide bloom was apparent.

In 2003 the spring phytoplankton growth occurred earlier and extended over a longer period than in 2002. These spring phytoplankton growths possibly made conditions more favorable for brown tide bloom initiation by either lowering the inorganic nutrient levels and/or releasing organic nutrients for *A. anophagefferens*. Gobler and Sañudo-Wilhelmy (2003) observed a higher DOC:DON ratio before a brown tide bloom, likely due to the release of C-enriched HMW-DOM by autotrophic phytoplankton, and suggested this material could promote brown tide growth under low inorganic nutrient conditions. Similar conditions could have occurred at Public Landing in 2002 and 2003 and at Greenbackville in 2003. However, there was also a spring phytoplankton bloom at Greenbackville in 2002 and no brown tide bloom followed this event.

The *A. anophagefferens* blooms at Public Landing in 2002 and 2003 appeared to crash more quickly, with cell density < 10, 000 cells ml^{-1} within two weeks after the maximum bloom, than the bloom at Greenbackville in 2003 (Table 3). It is possible that

different processes regulate the decline in brown tide cell abundance at the two sites. At Public Landing, dominance of virus-induced lysis over cell growth could have been the cause of the decline as reported for New Jersey and New York coastal bay brown tides by Gastrich et al. (2004). The slower decline of *A. anophagefferens* at Greenbackville in 2003 could be due to less dominant viral lysis (Gastrich et al. 2004) or caused by more intense grazing of other phytoplankton (Gobler et al. 2002) or due to a lower density of heterotrophic bacteria competing for the same organic nutrients (Gobler et al. 1997). Supporting these hypotheses is the fact that in both years the post-bloom DOC and HMW-DOC concentrations were higher at Public Landing than for corresponding Greenbackville samples (Fig. 17A and 17B). This difference in DOC numbers and the crash in brown tide cell counts were more dramatic in 2002.

3.4.3. Bulk DOM Characteristics

The range of DOC values measured at both sampling sites in 2002 and 2003 corresponded to DOC concentrations measured in Shinnecock Bay, Long Island, NY (154-442 μM) and the Maryland coastal bays (213-888 μM) from June to August in 1999 (Lomas et al. 2001).

The DOC concentration of the surface water at Public Landing was about 90 to 300 μM higher in 2002 than at Greenbackville and at both sampling sites in 2003. Initially this DOC concentration difference was contributed to the development of a brown tide bloom at Public Landing, and the lack of a bloom at Greenbackville in 2002. However, the occurrence of a brown tide bloom at both sites in 2003, when there were lower DOC concentrations, indicated that a simple relationship with DOC values is not a

consistent predictor of brown tide blooms.

At Public Landing, unlike in 2002 when the crash in *A. anophagefferens* resulted in a dramatic increase in DOC in the surface water, only a small increase in DOC was observed in 2003. The increase in DOC at Public Landing in 2002 amounted to the release of 1 pg DOC $cell^{-1}$ l^{-1} (Simjouw et al. in press). A similar DOC release after the brown tide bloom in 2003 would have resulted in an increase of ~ 40 μM DOC at Public Landing, considerably higher than what was actually seen (Table 4). This could indicate a different mode of termination for this brown tide bloom (see above), or a higher turnover rate of this presumably labile DOC by bacteria.

There was a significant positive correlation between DOC concentration and the chl *a* content of the surface water, $r = 0.732$ ($n = 12$, $p = 0.05$), for Public Landing in 2003 similar to the 2002 Public Landing data (Simjouw et al. in press). In contrast to 2002, however, the HMW-DOC concentrations did not correlate with the chl *a* concentrations at the 2003 Public Landing site. The samples from Greenbackville in 2003 showed no correlation between the chl *a* concentration and either DOC or HMW-DOC concentration. A significant correlation existed between the % of HMW-DOC and chl *a* concentration at Greenbackville for samples before and up to the maximum brown tide bloom in 2003 (GrBk14 to GrBk21) with $r = 0.859$ ($n = 6$, $p = 0.05$); a strong component of this correlation is the chl *a* due to brown tide cell abundance ($r = 0.978$, $n = 4$). Therefore it is possible that the chl *a* increase and the brown tide onset and bloom might have changed the DOM pool, by the addition of HMW-DOM, to the system at Greenbackville. However, other input and removal processes must then have become dominant as the bloom diminished.

The %HMW-DOC values were in general higher in 2003 at both sampling sites and did not show an overall increase from spring to fall. The higher %HMW-DOC for the initial DOM at both sampling sites in 2003 could indicate a greater contribution from allochthonous sources of the DOM. The lighter HMW-DOM $\delta^{13}C$ values for the spring and early summer 2003 samples compared to 2002 also suggest a greater contribution from allochthonous sources for the DOM at the beginning of the sampling period in 2003 (Fig. 19).

The $\delta^{13}C$ values measured for both sampling sites in both years fall within the range of marine DOM, between –18 and –28 ‰ (Bauer 2002), but showed much more variability in 2003. Even though the $\delta^{13}C$ values at the beginning of the sampling period for both years fall within the marine $\delta^{13}C$ values, the lighter $\delta^{13}C$ values for 2003, around -23 ‰, compared to about –21‰ for 2002 could indicate increased input of terrestrial DOM. The $\delta^{13}C$ values converged to a common $\delta^{13}C$ value at the maximum brown tide bloom at each sampling site. The overall $\delta^{13}C$ value of the HMW-DOM at Greenbackville decreased, while the HMW-DOM $\delta^{13}C$ value at Public Landing increased. Even though the HMW-DOC concentration (and %HMW-DOC) increased during the brown tide period at Public Landing in 2002, the $\delta^{13}C$ values did not show a similar change. The 2003 increase in $\delta^{13}C$ values for both Greenbackville and Public Landing after the maximum brown tide could indicate removal of the introduced HMW-DOC from the system, perhaps by heterotrophic bacteria. This removal of HMW-DOC by other organisms was suggested earlier in this study to explain the absence of an increase in DOC after the brown tide blooms in 2003 compared to Public Landing in 2002.

Results from the DOC and isotopic measurements indicate that both sampling sites in 2003, and possibly Public Landing in 2002, might have experienced different DOM inputs into the surface water, most likely due to a different combination of runoff and *in situ* production. This is also apparent looking at the UV/Vis absorbance characteristics of the surface water samples.

3.4.4. Chromophoric Dissolved Organic Matter Characteristics

The range of spectral slope values (between 0.015 and 0.021) were within reported values by Blough and Del Vecchio (2002) and Rochelle-Newall and Fisher (2002) for surface water samples from estuaries and coastal bays. The different S values at the start of the sampling period again indicate different initial DOM at these sampling sites. No significant correlation with salinity was found for either sampling site, signifying that the spectral slope during the spring to fall sampling period was not merely a function of variation in freshwater input /runoff. The fact that the S values converged to a similar value during the maximum brown tide density at the different sites suggests that even though the initial DOM pool was different at these sites, the *A. anophagefferens* bloom impacted the absorbance characteristics of the DOM pools.

In contrast to the SUVA increase at Public Land and Greenbackville in June 2002, we did not measure an increase in SUVA value in June 2003. However, the SUVA values after the *A. anophagefferens* blooms at the sampling sites in 2002 and 2003 were relatively similar, possibly indicating that the release of photoreactive material cannot be measured in 2003 due to the already high SUVA values for samples before and during the brown tide bloom. Production of CDOM has been hypothesized to occur as the result

of initial phytoplankton production of DOM followed by bacterial re-working (Rochelle-Newall and Fisher, 2002). Our 2002 SUVA data would be consistent with this. A positive correlation between SUVA and chl *a* ($r = 0.738$; $n = 11$, $p=0.05$) was found only for the 2003 Greenbackville sampling site.

3.4.5. Changes to the Initial DOM in the Surface Water

Based on the discussion so far, the characteristics of the DOM can be explored further with respect to the total DOM pool from samples before and up to the brown tide bloom maximum in 2003. During this period, the %HMW-DOC at Greenbackville showed significant positive correlations (95% probability level) with the SUVA, a negative correlation with the spectral slope values of the bulk DOM pool, and also a negative correlation with the $\delta^{13}C$ values of the HMW-DOM. These correlations could indicate input of terrestrial, more aromatic, DOM with higher molecular weight at Greenbackville in 2003. Similar correlations did not exist for samples from the Public Landing site in 2003.

For the 2002 sampling period, significant correlations were found between the %HMW-DOM and the spectral slope value of the surface water for both sites and between the %HMW-DOM and the SUVA value of the HMW-DOM pool for the Public Landing site. The correlation between the %HMW-DOM and the spectral slope value was negative for both Public Landing and Greenbackville while the correlation between %HMW-DOM and the SUVA value was positive for the Public Landing site. The positive correlation between the SUVA value and the %HMW-DOM for Public Landing, and the timing of their increases, suggested that aromatic photoreactive compounds were

introduced into the system during the 2002 brown tide bloom.

3.4.6. Molecular Level Characteristics of the HMW-DOM

Comparing the molecular-level characteristics of HMW-DOM from 2002 and 2003 using discriminant analysis, a clear separation of the samples from each year (Fig. 20) was found. The pre-brown tide bloom HMW-DOM samples (filled black symbols) were clearly different between the two sampling years. This separation along DF1 suggests higher lignin content in the 2003 HMW-DOM samples as compared to the 2002 sample set, corresponding to a more allochthonous source of the DOM in 2003. The brown tide bloom and post brown tide bloom samples in the 2003 sample set are separate from the pre brown tide bloom samples and have less negative DF1 scores. This points to a decrease in the fraction of the HMW-DOM that is ligninaceous as the bloom develops and declines. This pattern also emerged when the 2003 data was analyzed independently (data not shown). The shift along DF1 to less negative values, comparable to the DF1 value for the *A. anophagefferens* HMW-DOM, suggests that the change in the HMW-DOM for the 2003 samples could be due to the *A. anophagefferens* blooms at these sites. For the 2002 samples a similar shift towards *A. anophagefferens* HMW-DOM was seen by the close location in the score plot of the Public Landing post-brown tide bloom sample (PL11). The more dramatic change in the 2002 Public Landing HMW-DOM after the brown tide bloom compared to the 2003 samples could be due to the longer lifetime of this DOM in the surface water, as could also be seen by the increase in DOC, HMW-DOC and SUVA values. The 2003 samples did not show similar increases, possibly due to a quicker turnover of some of this most likely labile material. The slight separation

along DF2 for the 2003 bloom and post bloom samples appears to be due to an enrichment of lipid compounds, potentially cell membrane products from lysis of the brown tide cells during and after the bloom. Some lignin-like pyrolysis products are also visible for the HMW-DOM from 2002. It is likely that due to the low freshwater input during 2002, a relative dry year, lignin in the DOM pool was more degraded and less prevalent in the HMW-DOM pool compared to the HMW-DOM collected in 2003. Instead of pyrolysis products attributed to syringyl and guaiacyl lignin units, there was an increased signal of benzene and phenol (generic pyrolysis products from aromatic precursors) and m/z values generally attributed to aromatic protein compounds. The 2002 HMW-DOM material therefore looked more autochthonous.

3.4.7. *Aureococcus anophagefferens* and DOM Interactions

If DOM is critical to brown tide initiation as hypothesized, then our results either indicate a preference for some aspect of the DOM pool we cannot yet resolve or a flexibility in the kinds of substrates that can be utilized by *A. anophagefferens*. Supporting the former hypothesis is the fact that a spring phytoplankton bloom occurred prior to all three brown tide bloom events. This spring bloom could either draw down inorganic nutrients or release DOM that can be utilized by *A. anophagefferens*, or both. Such pre-brown tide blooms have also been reported for West Neck Bay, Long Island, NY (Gobler and Sañudo-Wilhelmy 2003). Supporting the latter hypothesis are uptake experiments by Mulholland et al. (in press) showing that *A. anophagefferens* cells from different bloom events use different organic substrates. Therefore, instead of looking for a "smoking gun" that initiates brown tide blooms, perhaps we should be looking for a

physiological factor such as extreme adaptability to different (organic) nutrient substrates relative to other co-occuring phytoplankton species.

This study shows as brown tide develops, the bloom appears to change the characteristics of the DOM pool. Our results indicate that this is due to inputs of aromatic, high-molecular-weight, chromophoric DOM into the surface water at the bloom sites.

SECTION 4

ISOLATION AND CHARACTERIZATION OF ESTUARINE DISSOLVED ORGANIC MATTER: COMPARISON OF C_{18} SOLID PHASE EXTRACTION AND ULTRAFILTRATION TECHNIQUES

4.1. INTRODUCTION

Characterization of dissolved organic matter (DOM) from aquatic environments, such as the deep sea, freshwater, or estuarine surface waters, has always been constrained by the ability to obtain a representative fraction of the DOM pool (Thurman 1985; Hedges 1992; Benner 2002). Direct chemical characterization of compounds such as carbohydrates (Pakulski and Benner 1994; Skoog and Benner 1997) and amino acids (McCarthy et al. 1997) within the total DOM of such samples provides low yields, between 3.7 and 10.5 % of the DOC pool or between 6.8 and 13.7% of the total DON pool (Benner 2002 and references therein). Ultrafiltration (e.g., Benner et al. 1992; McCarthy et al. 1996; Guo and Santschi 1997, Minor et al. 2002) or extraction, generally using XAD or C_{18} resins, (Averett et al. 1994; Liška 2000), are therefore often used to concentrate and desalt bulk DOM samples for further analyses.

In ultrafiltration, a nominal cutoff membrane of 1000 Da is often used and the material obtained is classified as high molecular weight DOM (HMW-DOM) or colloidal DOM. It is not currently known how well the HMW-DOM pool represents the total DOM pool from a sample. Depending upon the environment being sampled, the HMW-DOC fraction can range from 20 to 60 % of the total DOC pool (Benner 2002). How well the HMW-DOM fraction represents the total DOM pool also depends on the compound

classes or functional groups that are analyzed and compared (Benner 2002, and references therein). The low molecular weight DOM (LMW-DOM; <1000 Da), i.e. the filtrate of the ultrafiltration, is generally discarded, but is sometimes collected and specific pools are characterized by chemical analysis (Kepkay et al. 1997; Hernes and Benner 2002).

Another method for isolating organic compounds from aqueous solutions for later analysis is the use of C_{18} solid phase extraction (SPE). C_{18} SPE has been used in studies of specific materials such as trace metals, individual organic compounds, and pesticides as well as investigations into natural organic matter (e.g. Well and Bruland 1998; Louchouarn et al. 2000; Bielicka and Voelkel 2001; Mattice et al. 2002; Castells et al. 2004). Mills and Quinn (1981) and Amador et al. (1990) both used C_{18} Sep-pak cartridges to isolate DOM from seawater and estuarine samples. Mills and Quinn (1981) reported a recovery of 10 to 30% of the organic matter based on DOC measurements. Amador et al (1990) focused on humic material in the samples and reported on the removal of DOM fluorescence, UV/Vis absorbance at 280 nm, and the photoproduction capacity for H_2O_2 after C_{18} extraction. They reported a range of 30 to 64% removal of these signals by C_{18} extraction, depending on the type of measurement. Recently the C_{18} technique has also been used to concentrate and isolate DOM from river water samples for NMR analysis (Kaiser et al. 2003; Kim et al. 2003). In the study by Kim et al. (2003), commercially available C_{18} extraction disks were used instead of the previously popular columns or cartridges. DOM extraction from two river water samples in the study by Kim et al. (2003) resulted in a recovery of over 60% of the initial DOM pool. Using ^1H NMR the investigators concluded that the C_{18} SPE disk did not contaminate the sample and that

extracted samples retained large portions of the functional group distribution of the isolated DOM. These observations by Kim et al. (2003) suggest the C_{18} SPE disk extraction can be used for isolating a major fraction of estuarine/marine DOM for molecular-level analyses.

In this study we compare ultrafiltration and C_{18} disk extraction as DOM isolation methods using estuarine samples. The isolates from both methods were characterized using Fourier transform infrared spectroscopy (FTIR) and direct temperature-resolved mass spectrometry (DT-MS). The results from the DT-MS analysis were compared using discriminant analysis. We also evaluated the use of C_{18} solid phase disks to isolate LMW-DOM in the filtrate from ultrafiltration. In addition to mass balance studies to determine the efficiency of the technique, LMW-DOM isolates from ultrafiltration were also analyzed using FTIR and DT-MS. The DT-MS characteristics of size-fractionated extracts were compared with those of bulk DOM (<0.2 μm) extracts using discriminant analysis.

4.2. MATERIALS AND METHODS

4.2.1. Experiment Setup

For the molecular level studies to compare the C_{18} disk extraction and ultrafiltration techniques, we used samples from the Chesapeake Bay Bridge Tunnel at the mouth of the Chesapeake Bay (CBM) and the Elizabeth River (ER), a tributary to the Chesapeake Bay that passes through Norfolk, VA. For both sites, ultrafiltration and C_{18} SPE was performed on replicate 900 ml < 0.2 μm filtered samples. The filtrate from the ultrafiltration was collected into a 1 l amber glass bottle, and stored at 4 °C for later C_{18}

SPE. All samples (ultrafiltration isolates and C_{18} disk extracts) were freeze dried and analyzed by FTIR and DT-MS. Using this approach we were able to compare the isolated DOM from estuarine/marine samples using both techniques and investigate the use of C_{18} SPE on LMW-DOM samples.

For the mass balance characterization of the C_{18} disks, we used bulk DOM samples from the Chesapeake Bay mouth and the Elizabeth River and LMW-DOM samples collected from earlier ultrafiltration experiments, which were frozen at that time (Table 5). These LMW-DOM samples were from the Great Bridge Locks Park in Chesapeake, VA (GB) and the Chesapeake Bay Bridge Tunnel (Table 5). Great Bridge Locks Park, located at the upstream end of the southern branch of the Elizabeth River, is a brackish site where the salinity depends strongly upon rainfall and ranges from 4 to 17 psu at low tide (Johnston unpubl., Miller 2002). The river at this location flows through a heavily wooded region and is fed by numerous marshes.

4.2.2. Sample Collection

Surface water samples were collected using a stainless steel bucket from a dock on the Elizabeth River (ER), a subestuary in the lower Chesapeake Bay and from the Chesapeake Bay Bridge Tunnel fishing pier at the Chesapeake Bay mouth (CBM). The samples were transferred immediately into acid rinsed 4 l amber bottles. In the lab, ~ 1 l aliquots of each sample were filtered into 1 l amber bottles (acid rinsed and combusted at 450 °C, overnight) with a peristaltic pump using a 0.2 μm surfactant-free cellulose acetate Sartorius in-line filter cartridge to remove suspended particles and bacteria. The pump tubing was rinsed before each sample filtration with deionized (DI) water and then

TABLE 5. Summary of the samples used for the different isolation methods in this study. Each ultrafiltration and C18 extraction was performed in duplicate.

Site sampled	Type	Date	Used for	Storage	Name
Mass balance characterization					
Chesapeake Bay mouth	Bulk DOM	Dec. 1, 2003	C_{18} disk characterization	4 °C	CBM
	LMW-DOM	Sept. 13, 2003	C_{18} disk characterization	-20 °C	
Elizabeth River	Bulk DOM	Nov. 6, 2003	C_{18} disk characterization	-20 °C	ER
Great Bridge Lock Park	LMW-DOM	Sept. 23, 2003	C_{18} disk characterization	-20 °C	GB
Molecular-level characterization					
Chesapeake Bay mouth	Bulk DOM	Dec. 1, 2003	Ultrafiltration	4 °C	CBMr1, CBMr2
	Bulk DOM	Dec. 1, 2003	C_{18} disk extraction	4 °C	CBMse1, CBMse2
	LMW-DOM	Collected on Dec. 1, 2003, processed on Dec. 2. 2003	C_{18} disk extraction	4 °C	CBMfe1, CBMfe2
Elizabeth River	Bulk DOM	Dec. 9, 2003	Ultrafiltration	4 °C	ERr1, ERr2
	Bulk DOM	Dec. 9, 2003	C_{18} disk extraction	4 °C	ERse1, ERse2
	LMW-DOM	Collected on Dec. 9, 2003, processed on Dec. 10, 2003	C_{18} disk extraction	4 °C	ERfe1, ERfe2

an aliquot of the sample; the filter was rinsed with about 50 ml sample prior to sample collection. DOC measurements made on 0.2 μm filtered samples before and after inline filtration have shown that the method results in no detectable addition of DOC to the filtered sample (Simjouw and Minor, unpublished results).

4.2.3. DOC and UV/Vis Measurements

Aliquots (6 ml) of sample were taken for DOC concentration and ultraviolet/visible (UV/Vis) absorbance measurements. Samples for DOC measurements were stored in acid- cleaned and muffled borosilicate clear glass vials with Teflon-lined caps, and 50 μl of 6 N HCl was added (pH < 2) to remove inorganic carbon and minimize bacterial activity. DOC samples were stored frozen until further processing. DOC concentrations were measured by high temperature combustion using a Shimadzu TOC-5000 as described in Burdige and Homstead (1994).

Samples for UV/Vis absorbance were also stored in acid-cleaned and muffled borosilicate clear glass vials with Teflon-lined caps. These samples were kept at 4 °C for less than 8 hrs prior to analysis. The UV/Vis absorbance was measured from 190 to 800 nm using a Varian Cary 3 Bio spectrophotometer with DI water as a blank. Absorbance between 250 to 400 nm was used to characterize and compare both isolation techniques. The absorption coefficient a, at wavelength λ, was calculated using $a_{(\lambda)} = 2.303A_{(\lambda)}/L$ where $A_{(\lambda)}$ is the absorbance at wavelength λ and L is the cell path length in meters.

4.2.4. Ultrafiltration

Stirred cells (Amicon 8400), pressurized with ultrapure nitrogen, were used for

ultrafiltration of the samples. To obtain the HMW-DOM fraction, a 1000 Dalton (Da) regenerated cellulose membrane was used to concentrate 900 ml samples by a factor of 30. DOC concentrations from the whole sample, retentate, and filtrate, were used to monitor the efficiency of the ultrafiltration procedure and to calculate the HMW-DOC concentration of the sample as described in Benner (1991) and Klap (1997). The HMW-DOM fraction was desalted by repeatedly rinsing with a total volume of 1500 ml DI water. During the desalting, LMW-DOM that remained in the concentrated sample was also removed. Blank runs using DI water showed that the desalting procedure did not contribute significantly to the sample DOC and did not impact the mass spectrometry measurements.

4.2.5. C_{18} SPE Disk Characterization

Solid phase extraction was performed on 500 or 900 ml samples using C_{18} extraction disks (3M Empore) and a borosilicate-glass 2 l vacuum-filtration unit with a coarse fritted glass holder to support the C_{18} disk. The maximum vacuum was 15 inches of Hg or -50 kPa.

For molecular-level studies, the C_{18} disk was activated and conditioned according the manufacturer's manual. Briefly, the C_{18} disk was rinsed first with 10 ml of MeOH: DI water (90:10), then twice with 10 ml MeOH, and finally with 10 ml of DI water. For complete mass-balance characterization of the C_{18} disk, the disk was further rinsed with 6 l of DI water. We monitored the DOC concentration in the filtrate after every liter of DI water and concluded that 6 l was sufficient to remove the methanol from the C_{18} disk. The retention capacity of the C_{18} disk will most likely be diminished by this extensive DI

rinse; therefore, the calculated recoveries of C_{18} extract by DOC and UV/Vis absorbance analysis will be a lower end value for these samples. Immediately before extraction, all samples, including blanks, were acidified to a pH of 2 to 2.5 with 6 M hydrochloric acid (ACS grade). To elute each sample from the C_{18} disk, we rinsed the disk three times with 10 ml MeOH:DI (90:10) as described in Kim et al. (2003). Eluates from the C_{18} extraction were collected in acid cleaned and combusted glass bottles and dried under vacuum at 40 °C. Dried samples were re-dissolved in DI water and an aliquot was taken for DOC analysis. The re-dissolved sample was frozen and then freeze-dried using a Heto FD4 freeze-drier to obtain dried sample for mass spectrometry.

To investigate the DOM type and concentration that could be retained by C_{18} solid phase extraction disks and the effects of varying size fraction and sample salinity, we performed C_{18} disk extraction of both bulk DOM (<0.2 μm) and LMW-DOM (< 1000 Da, obtained by collecting the ultrafiltration filtrates) from the sites listed in Table 5.

The recovery of DOM, the actual DOM isolated from the initial sample by C_{18} extraction, eluted from the disk, and available for further analysis, was calculated by dividing the integrated absorbance (from 250 to 400 nm), or DOC concentration (corrected to the initial sample volume), of the C_{18} extracted material by the integrated absorbance (from 250 to 400 nm), or DOC concentration, of the initial sample. To calculate the mass balance we also calculated the fraction of the sample in the C_{18} filtrate based on the UV/Vis absorbance or the DOC concentration, as described for the recovery earlier in this section, and added this to the fraction of the C_{18} extracted material. To determine the efficiency of the sample elution of the C_{18} disk, we calculated the loss in UV/Vis absorbance between the initial sample and the filtrate and compared that with the

UV/Vis absorbance of the C_{18} extracted sample obtained from the disk. In this study about 95 – 100% of the sample was recovered from the C_{18} disk.

4.2.6. FTIR Analysis

Fourier-transformed infrared (FTIR) spectroscopy gives information on the presence or absence of particular functional groups in the DOM isolated by the different methods. The freeze-dried DOM samples from ultrafiltration or C_{18} extraction were analyzed as KBr pellets using a Nicolet 5PC FTIR spectrometer (20 scans from 4000 to 400 cm^{-1}, resolution=8, with Happ-Genzel apodization, and CO_2 and H_2O blank correction). A ratio of 1 mg sample with 100 mg KBr was used to ensure maximum resolution of individual peaks.

4.2.7. DT-MS Analysis

The isolated DOM replicates were each analyzed in duplicate by nominal-resolution direct temperature-resolved mass spectrometry (DT-MS) with low voltage electron-impact ionization (EI^+) to obtain a broad overview of the chemical composition. DT-MS provides information on a wide range of chemical substances in marine samples through monitoring the presence of typical molecular ions and fragmentation patterns (Eglinton et al. 1996). It should be emphasized that nominal-resolution EI^+ DT-MS only provides tentative compound identification and that additional characterization is needed to strengthen such identifications; FTIR performs this function here.

Two benefits of DT-MS are the minimal sample manipulation required and the small amounts of sample (micrograms) needed for characterization. For DT-MS analysis,

tens to hundreds of micrograms of the DOM sample was redissolved in 20 to 50 µl DI water and a 1-3 µl aliquot was dried onto a Pt/Rh (90/10) probe (0.125 mm diameter wire). This probe was then inserted into the ionization chamber of a VG AutospecQ magnetic sector mass spectrometer. Desorption of volatile material and pyrolysis (the thermal dissociation of polymeric material) was promoted by resistively heating the sample probe using 0 to 1.1 Amps over two minutes (Boon 1992; Eglinton et al. 1996; Minor 1998). The resulting volatilized components in the chamber were ionized using 16 eV electron impact (EI^+) ionization. Other instrument settings were as follows: acceleration voltage 6.0 kV, direct inlet, mass range 41 – 795, scan rate 1.16 seconds with a 0.5 second delay, resolution 1000. The scans for each temperature region were summed to obtain composite mass spectra (Boon 1992; Eglinton et al. 1996).

4.2.8. Statistical Analysis

Discriminant analysis (DA) was performed on the mass spectra dataset to ascertain molecular-level differences among ultrafiltered HMW-DOM, <0.2 µm DOM C_{18} extracted samples, and LMW-DOM C_{18}-extracted samples. To do this, the mass spectra of the samples were exported to a multivariate statistics program, ChemoMetricks (FOM-AMOLF Institute, the Netherlands). The ChemoMetricks program used a type of discriminant analysis that consists of a two-stage principle component analysis (see Hoogerbrugge et al. 1983 and Minor and Eglinton 1999 for more information). This discriminant analysis was used to transform the dataset and to determine linear relationships (Discriminant Functions) of the initial variables that can be used to summarize the data set. Ideally, a small number of discriminant functions should explain

most of the variance in the data set. In the discriminant analyses performed here, the program was only told which were replicate preparations of the same sample (e.g. CBMr1 and CBMr2 each analyzed twice), and the statistical approach maximized the differences between the samples while minimizing the differences in replicate samples. The significance of the discriminant function was summarized in the B/W ratio, the ratio of the variance between the samples to the variance within replicates, and in the total percent variance (%Var) of the dataset explained by the discriminant function.

Scores for the two major discriminant functions were used to construct a two-dimensional score plot for the different samples from both sites. The score plot was used to visualize relative similarities or differences among the samples. Using the loadings of a discriminant function for the m/z values multiplied with the respective standard deviations we were able to reconstruct difference spectra. The difference spectra show enrichments or depletions of the m/z values along the discriminant function axis (Minor 1998; Minor and Eglinton 1999). This indicates the mass spectral components of the isolated DOM samples responsible for the separation in the discriminant analysis plot.

4.3. RESULTS

4.3.1. Mass Balance and Blank Measurements of the C_{18} SPE Disk

The percentage of chromophoric DOM (as determined by the sum of the UV/Vis absorbance from 250 to 400 nm) recovered using C_{18} disk SPE clearly differed from the percentage of DOC (determined by DOC analysis) (Table 6) although both measurement techniques yielded total mass balances of around 100%. The LMW-DOM samples in Table 6 had somewhat higher total mass balances (slightly greater than 100%); these are

TABLE 6. Mass balance and recovery of DOM for the C_{18} disk characterization samples based on UV/Vis and DOC measurements. %Recovery = $(X_{C18\ extract} / C_{initial\ sample})*100$ % and %mass balance = $((X_{C18\ extract} / C_{initial\ sample}) + (X_{C18\ filtrate} / C_{initial\ sample}))*100\%$, where X = the integrated $Abs_{(250\text{-}400nm)}$ or DOC concentration corrected to initial sample volume and C = the integrated $Abs_{(250\text{-}400nm)}$ or DOC concentration of the initial sample.

Sample	UV/Vis (%)		DOC (%)	
	Mass balance	Recovery	Mass balance	Recovery
CBM bulk DOM	99.7	49.3	99.6	36.4
ER bulk DOM	95.7	63.4	95.0	44.9
CBM LMW-DOM	102.3	56.3	112.3	36.6
GB LMW-DOM	108.1	65.7	93.3	37.5

probably due to measurement uncertainties as the eluates (material removed by and recovered from the C_{18} disk) had low absorbance values (≤ 0.1) and DOC concentrations (< 50 μM).

To determine the blank characteristics of the C_{18} SPE disks we also ran de-ionized (DI) water and artificial seawater (ASW) as samples (also acidified to pH 2-2.5 immediately before extraction). Aliquots of the resulting SPE eluates had DOC concentrations between 10 to 20 μM, similar to the initial concentrations in the unconcentrated natural water samples and considerably lower than the concentrations in the natural-sample eluates. This indicates that in terms of DOC concentrations, the isolation method does not significantly contaminate the resulting sample.

The remainder of the eluate from each blank was freeze-dried and prepared for DT-MS analysis. For the DI water blank, 6 μl of ultrapure water was added to the container of freeze- dried eluate. The water drop was then swirled around the bottom to ensure contact with all surfaces and to dissolve any material from the blank extraction. After extensive swirling, 3 μl was then used for DT-MS analysis as described previously. In case of the ASW blank, some white residue remained in the eluate and appeared on the bottom of the container after the freeze-drying process. Some of this material was used for the DT-MS analysis. Results from both blank runs and an additional instrumentation blank indicate that there is no significant addition of material to the DT-MS signal due to the C_{18} SPE method (Fig. 22). The blank signals were on average 10 to 20 times lower in intensity than a typical DT-MS spectrum of a DOM sample, except for specific m/z signals clearly associated with the blank analysis. These signals were m/z: 99, 103, 112, 119, 129, 130, 139, 141, 194, 195, 196, 279, 354, and 355. The m/z signals 103, 194,

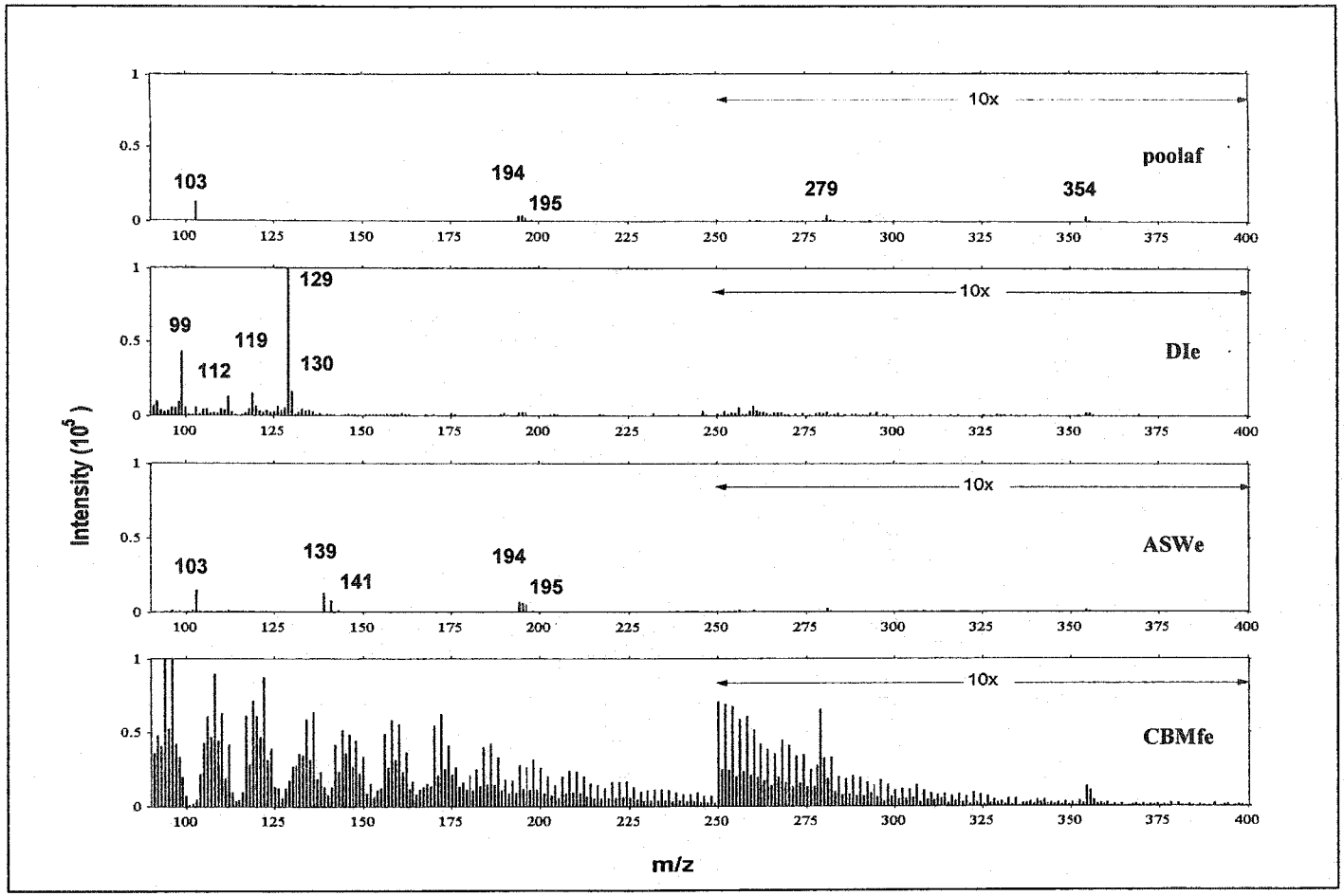

Fig. 22. DT-MS spectra of several blank measurements. Poolaf is an instrument blank where the sample probe is cleaned (flamed) before the measurement. DIe and ASWe are C_{18} extracts from DI water and artificial seawater respectively. The CBMfe spectra is added as a low concentration sample for comparison with the blanks. Note that for ease of comparison the intensity of the m/z region above 250 is multiplied by a factor of 10.

195, and 196 are associated with the Pt/Rh probe while m/z 354 and 355 are most likely due to silicone pump oil from the instrument (as indicated by the instrument blank). M/z 279 is a signal from phthalate usually associated with contamination from gloves and air circulation systems. The other signals, m/z 99, 112, 119, 129, 130, 139, and 141, are most likely due to the presence of some inorganic salts in the concentrated blank samples and contamination from compounds, possibly C_{18} material (e.g. m/z 99, 112, 141), that were eluted off the C_{18} disc. The listed m/z values associated with the blank analyses have been removed from the spectrum prior to the multivariate analysis by discriminant analysis, so that the blank signals would not determine the differences or similarities of the samples. From the m/z 139 and 141 in the ASW spectrum we concluded that the material remaining in the freeze-dried eluate was possibly a chlorinated compound resulting from incomplete desalting of the eluate. This conclusion is strengthened by the measured salinity, 1 to 1.5, of the re-dissolved ASW eluate. The salinity of all re-dissolved samples in this study (as determined by refractive index) varied between 0.5 and 1.5, compared to an average salinity of 0.5 for ultrafiltration retentates, indicating that some sea salt remained on the SPE disk during sample addition and was eluted with the sample. Aside from the above-listed m/z values, the low salt residue present in the samples did not appear to interfere with the DT-MS analysis in this study.

4.3.2. Comparison of C_{18} SPE and Ultrafiltration Characteristics

In order to compare SPE and ultrafiltration, replicate aliquots of Elizabeth River and Chesapeake Bay mouth (Table 5, molecular level characterization) water were processed by the two techniques. The UV/Vis absorbance and DOC concentration mass

balance and recovery results are shown in Table 7. As in the work detailed above, for the C_{18} method the mass balance based on UV/Vis absorbance was around 100%. Because we needed to retain enough sample for later DT-MS analysis, we were unable to take an aliquot of the ultrafiltered HMW-DOM for UV/Vis absorbance analysis. Therefore, the UV/Vis absorbance based recovery of HMW-DOM was calculated by the difference in integrated UV/Vis absorbance (250 to 400 nm) of the initial sample and the filtrate divided by the integrated absorbance of the initial sample. The recovery of the HMW-DOM was about 3 to 7 % higher for the bulk Elizabeth River samples than for the Chesapeake Bay mouth samples. The recovery of the bulk-DOM by the C_{18} SPE method was about 3% higher for the Chesapeake Bay mouth samples than the recovery by the ultrafiltration method. For the bulk Elizabeth River samples the recovery by the C_{18} SPE method was 3 to 7% lower than the recovery by the ultrafiltration method. C_{18} SPE of the LMW-DOM sample (the ultrafiltration filtrate) resulted in the recovery of 40 to 50% of LMW-DOM based on UV/Vis absorbance (Table 6). Based on the recoveries calculated using UV/Vis absorbance, combining ultrafiltration isolation of HMW-DOM with C_{18} SPE extraction of the LMW-DOM leads to total recoveries of between 73 and 76% of the Chesapeake Bay mouth sample and between 76 and 78% of the Elizabeth River sample for molecular-level analyses.

The DOC mass balance for the ultrafiltration method was around 100% for both Chesapeake Bay mouth and Elizabeth River samples (Table 7). The HMW-DOM accounted for 49 to 53% of the Chesapeake Bay mouth sample and 48 to 54% of the Elizabeth River sample based on DOC measurements. DOC mass balance calculations were not possible on samples from the C_{18} SPE method because the eluent included the

TABLE 7. Ultrafiltration and C_{18} SPE sample recovery and mass balance results. %Recovery = $(X_{C18\ extract} / C_{initial\ sample})*100$ % and %mass balance = $((X_{C18\ extract} / C_{initial\ sample}) + (X_{C18\ filtrate} / C_{initial\ sample}))*100\%$, where X = the integrated $Abs_{(250\text{-}400nm)}$ or DOC concentration corrected to initial sample volume and C = the integrated $Abs_{(250\text{-}400nm)}$ or DOC concentration of the initial sample. – means not calculated.

Sample	UV/Vis (%)		DOC (%)	
Ultrafiltration				
	Mass balance	**Recovery HMW-DOM**	**Mass balance**	**Recovery HMW-DOM**
CBMr1	-	53.4	102.2	48.8
CBMr2	-	52.9	100.0	52.7
ERr1	-	60.2	104.5	53.8
ERr2	-	56.5	100.0	47.6
C_{18} solid phase extraction				
	Mass balance	**Recovery C_{18} disk extract**	**Mass balance**	**Recovery C_{18} disk extract**
Bulk DOM				
CBMse1	100.0	56.0	-	38.4
CBMse2	97.4	55.6	-	39.1
ERse1	100.5	52.9	-	29.9
ERse2	99.6	52.7	-	24.9
LMW-DOM				
CBMfe1	93.7	50.4	-	37.5
CBMfe2	108.7	41.8	-	31.1
ERfe1	99.2	40.1	-	25.6
ERfe2	97.3	39.8	-	25.5

methanol used to activate the C_{18} disk. The DOC recovered from the bulk DOM samples by the C_{18} SPE method was about 10 to 13% lower for the Chesapeake Bay mouth site, and 22 to 24% lower for the Elizabeth River site, compared to the DOC isolated using the ultrafiltration method. Using the C_{18} SPE method, 31 to 37% of the DOC in the LMW-DOM (ultrafiltration filtrate) could be recovered for the Chesapeake Bay mouth sample. For the Elizabeth River sample the DOC recovery from the LMW-DOM was around 25 to 26%. Combining the DOC recovery of the ultrafiltration method and the C_{18} SPE method, we isolated between 67 and 69% of the total DOC from the Chesapeake Bay mouth sample and between 61 and 66% of the DOC from the Elizabeth River sample.

4.3.3. Bulk Characteristics of the Isolated DOM using FTIR

FTIR spectra of the isolated DOM from both sites are shown in Fig. 23 and 24. Even though the complexity of the DOM makes it difficult to unequivocally resolve functional groups using FTIR, we can obtain an overview of changes in the isolated DOM using the differences in the FTIR spectra for each isolation technique. We performed FTIR analysis on duplicate samples to investigate the reproducibility of the analysis. Because the peak area at the four major peaks in the FTIR spectra as a percent of the total area was similar between duplicates (Fig. 25 and 26), we concluded that the variation in the FTIR spectra between the DOM obtained by the different isolation techniques were not due to sample heterogeneity. For both the Chesapeake Bay mouth and Elizabeth River sampling sites, three major differences are apparent in the spectra for the bulk DOM and LMW-DOM C_{18} extracts (Fig. 23 and 24) as compared to the HMW-DOM spectra. They are: an increase and sharpening of the peak around 3400 cm^{-1},

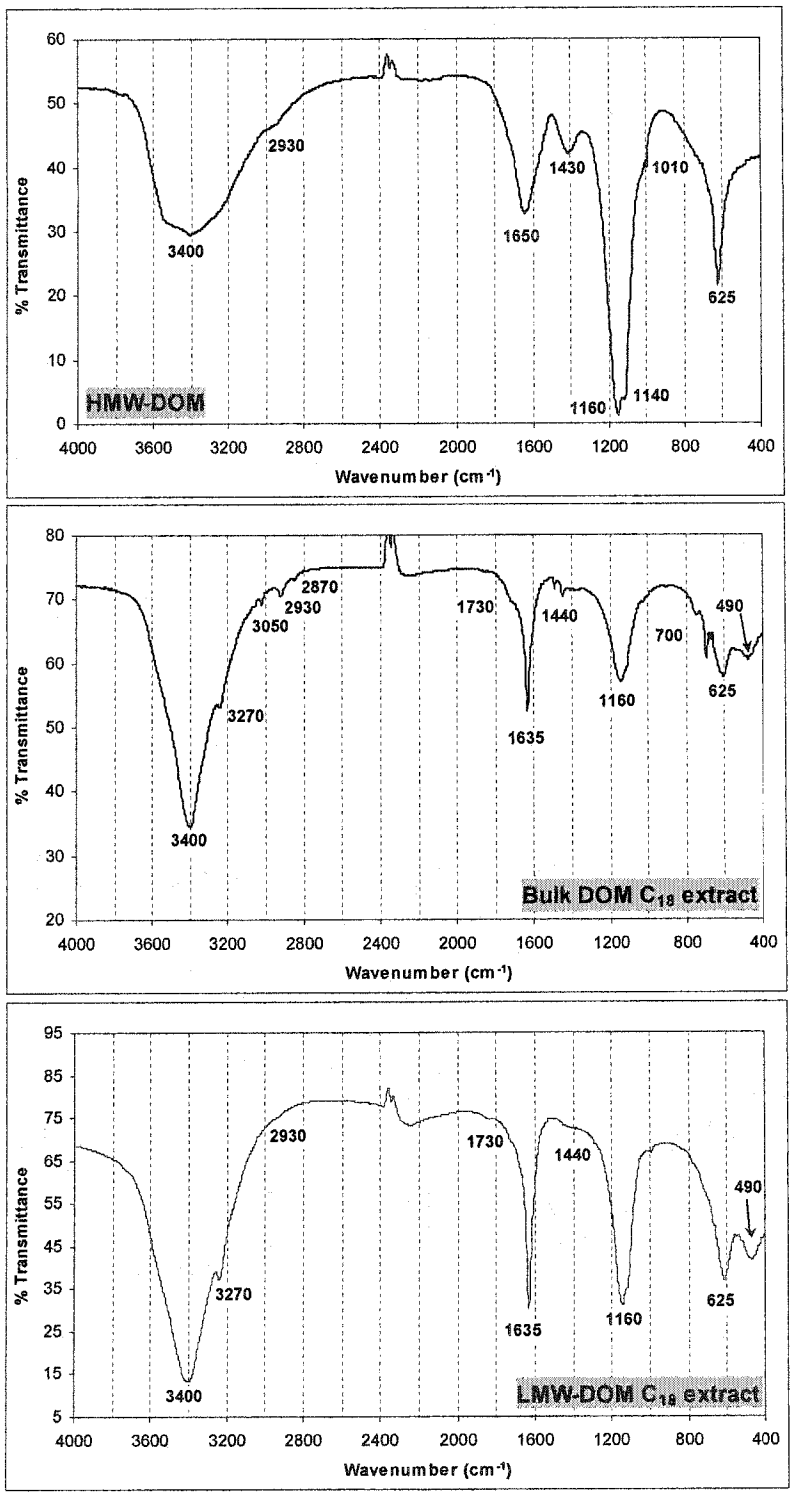

Fig. 23. FTIR spectra of HMW-DOM isolated by ultrafiltration and bulk DOM and LMW-DOM C_{18} disk extracts from the Chesapeake Bay mouth.

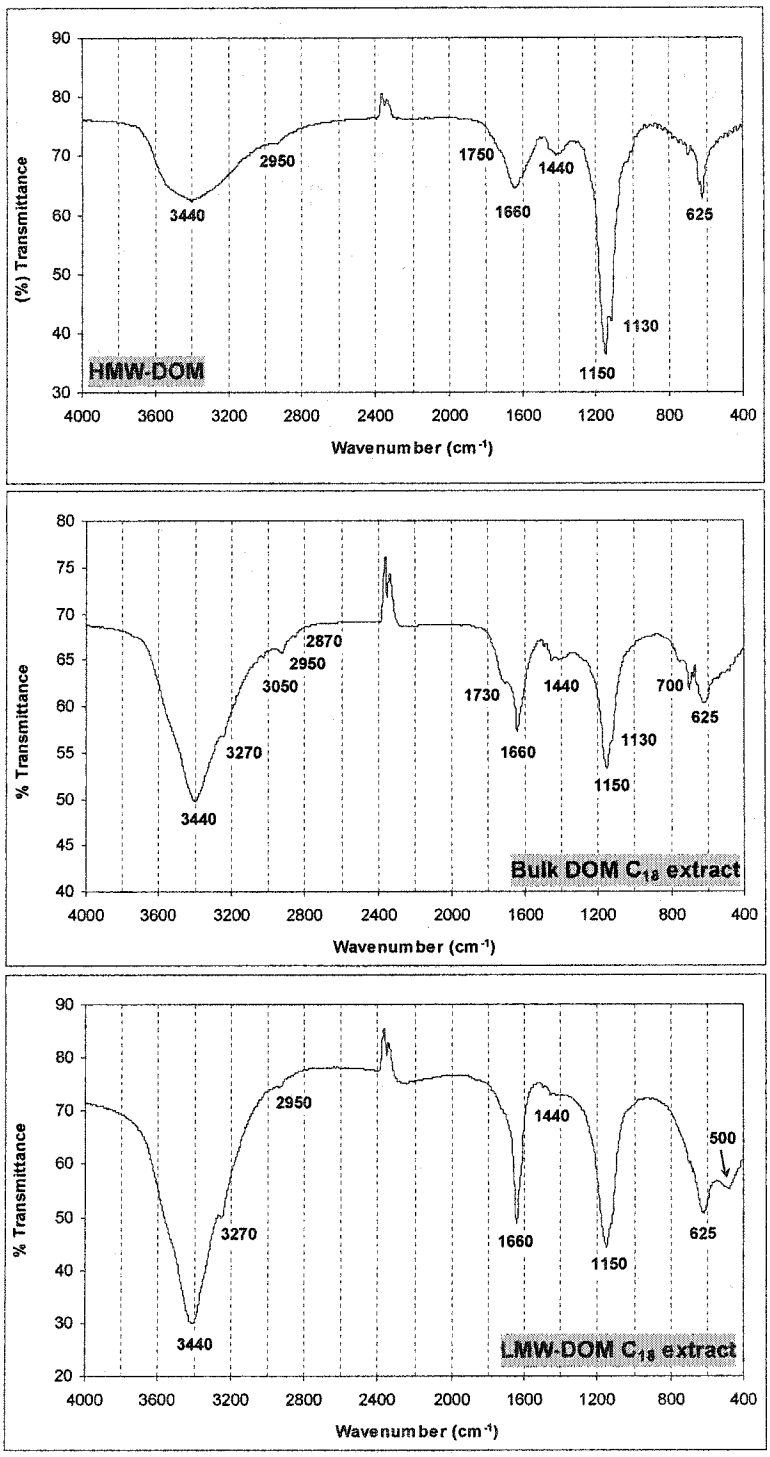

Fig. 24. FTIR spectra of HMW-DOM isolated by ultrafiltration and bulk DOM and LMW-DOM C_{18} disk extracts from the Elizabeth River.

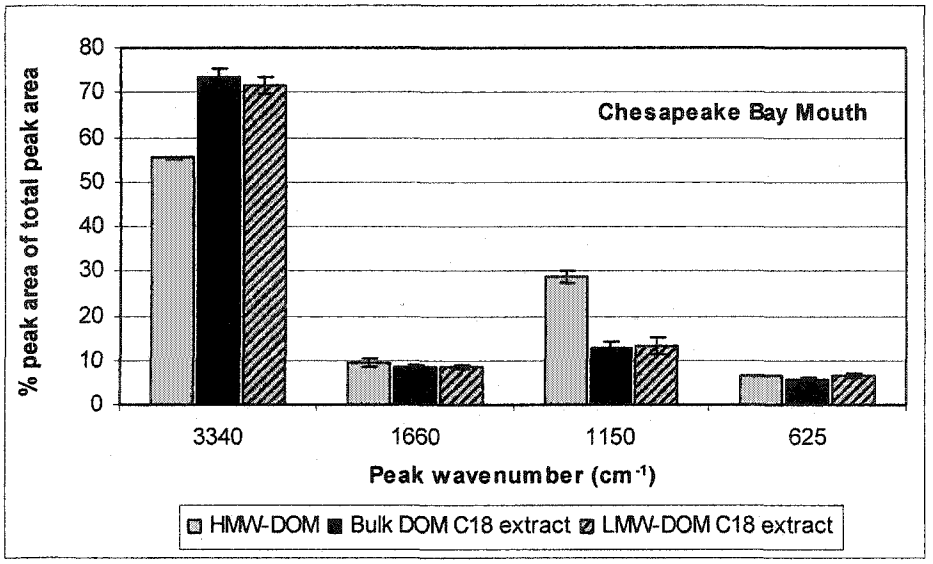

Fig. 25. Bar graph representing the percent of the measured peak area of the four major peaks in the FTIR spectra with respect to the summed peak area of these four peaks for the samples from the Chesapeake Bay mouth.

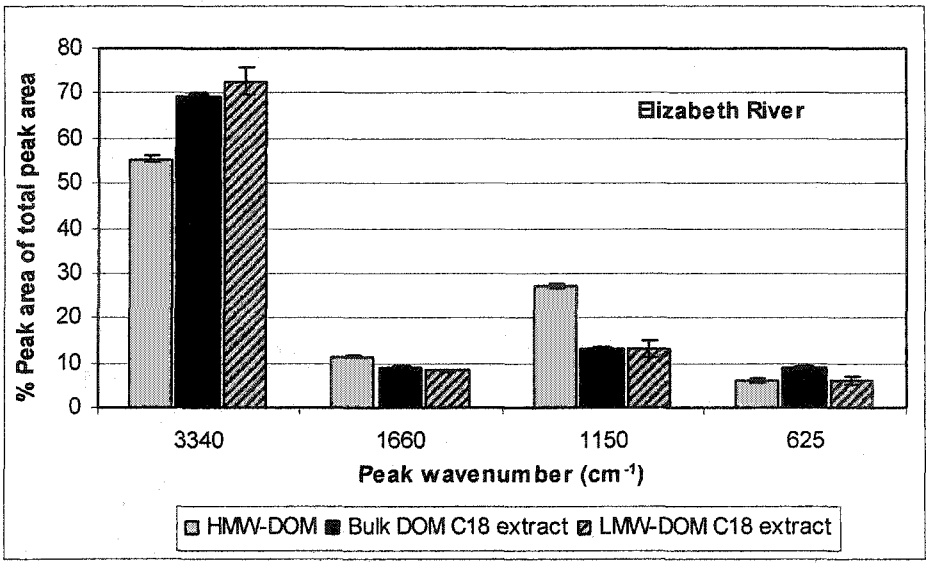

Fig. 26. Bar graph representing the percent of the measured peak area of the four major peaks in the FTIR spectra with respect to the summed peak area of these four peaks for the samples from the Elizabeth River.

an increase, sharpening, and shift in the peak around 1650 cm^{-1}, and a decrease in the peaks at 1160 and 1140 cm^{-1} for the C_{18} isolated DOM compared to the HMW-DOM isolated using ultrafiltration.

The functional groups responsible for such changes can be identified by comparison with tables of characteristic absorption patterns (e.g., Silverstein et al. 1991; Solomon and Fryle 2003). In doing this, the absorption band around 3450 cm^{-1} could be attributed to O-H stretching from the presence of alcohols and/or phenols O-H stretching and N-H stretching from amines and/or amides. The broad shape of this band probably results from the presence of several different alcohols, phenols, amines, and amides as well as various hydrogen bonding interactions within the DOM. This band becomes sharper and stronger in the C_{18} extracts, perhaps indicating preferential concentration of selected compounds within the alcohols, phenols, amines, and amides or a decrease in H-bonding interactions. The appearance of a shoulder at 3270 cm^{-1} in the C_{18} samples might be due to the presence of the amide N-H functional group as primary amides exhibit two N-H stretching bands near 3350 and 3180 cm^{-1} in solid FTIR (Silverstein et al. 1991). The HMW-DOM, bulk DOM C_{18} extract, and LMW-DOM C_{18} extract from both samples show a weak absorption band at 2930, perhaps from alkane C-H stretching. In the bulk DOM C_{18} extracts this band is stronger and additional C-H stretch bands appear at 3050 and 2870. The absorption band around 1660 to 1635 cm^{-1}, which indicates C=C or C=O bonds, is also present in the HMW-DOM samples and the C_{18} extracts, but is stronger and sharper in the C_{18} extracts. The change in this band could be due to amides, which would be consistent with the sharpening and strengthening of the absorption band at 3400 seen in both bulk DOM and LMW-DOM C_{18} extracts, or from an increase in alkene

character, which would be consistent with the presence of a C-H stretch at 3050 cm^{-1} in the bulk DOM C_{18} extracts. The weak absorption band around 1440 cm^{-1} could be associated with bending of O-H in an alcohol or phenol functional group and possibly skeletal aromatic absorption. Aromatic C-H stretching could also explain the weak absorption around 3050 cm^{-1} and the absorption around 700 cm^{-1} in the bulk DOM C_{18} extracts. Stretching of C-O in an alcohol or phenol functional group could cause the absorption band around 1150 cm^{-1}, which is stronger in the HMW-DOM samples than in the bulk DOM and LMW-DOM C_{18} extracts.

The FTIR spectra of the LMW-DOM C_{18} extracts (CBMfe and ERfe) are similar to those of the bulk DOM C_{18} extracts. The bulk DOM C_{18} extracts show more alkene/alkane character in the C-H stretch region (2870-3050 cm^{-1}) and indications of a higher aromatic content (absorptions at 3050 and 700 cm^{-1}).

4.3.4. Molecular-Level Characteristics of Isolated DOM

Differences between DT-MS spectra of samples from ultrafiltration and C_{18} disk SPE were investigated as shown in Fig. 27 and 28 for the Chesapeake Bay mouth samples and Elizabeth River samples respectively. In both samples, the HMW-DOM was characterized by the dominance of m/z values 96, 110, 125, 151, and 160. Both the bulk DOM and LMW-DOM C_{18} extracts contained a different suite of major m/z values: 94, 108, 122, 150, and a suite of what appear to be alkenes/alkanes in the range above m/z 150. Because DT-MS is only semi-quantitative (Minor et al. 2000) and because we did not load precise amounts of DOC on the sample wire for each analysis, we were not able to directly compare the intensity of the signal for each m/z value between the samples.

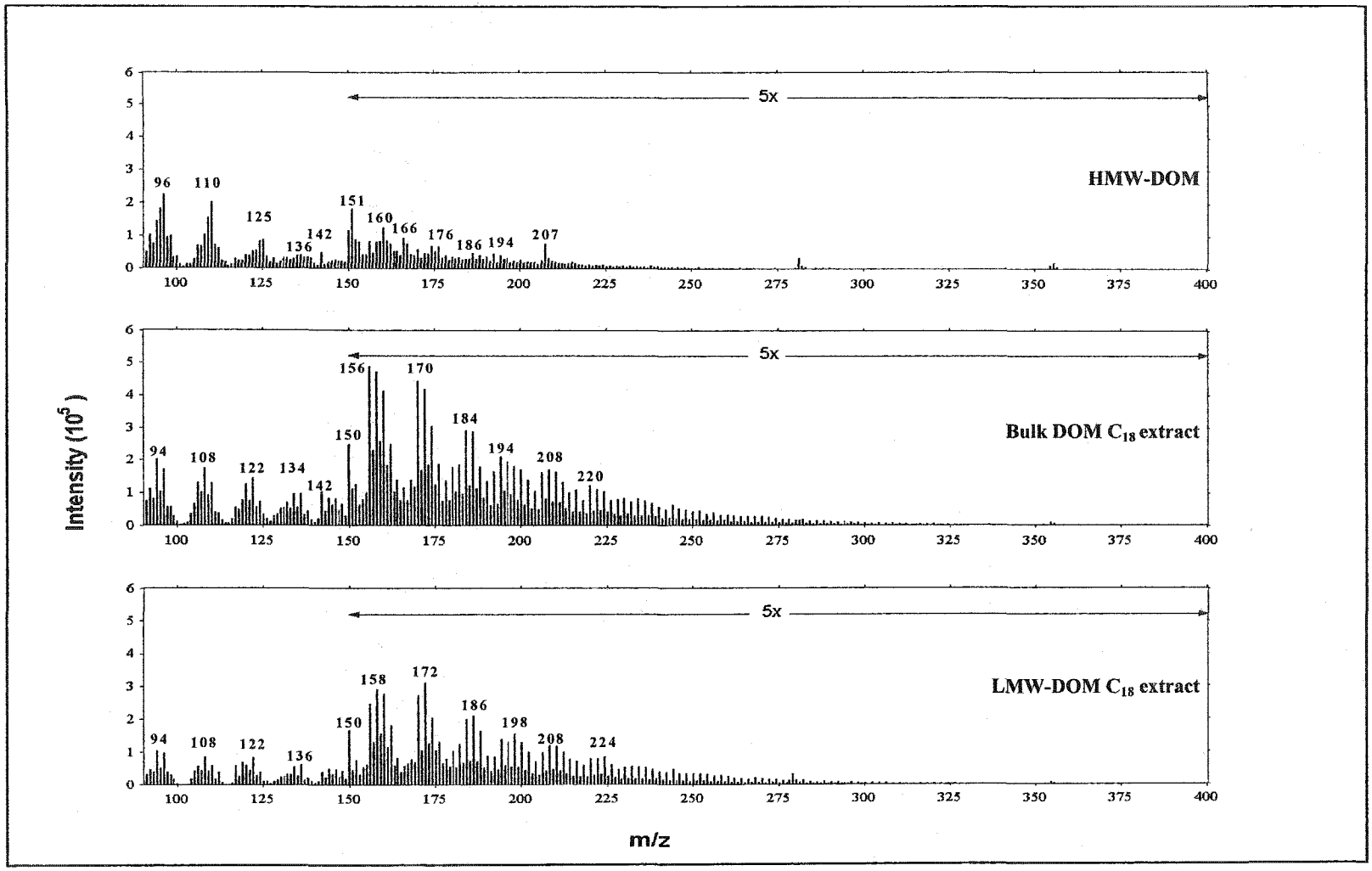

Fig. 27. DT-MS spectra of DOM samples isolated by ultrafiltration and C_{18} SPE from the Chesapeake Bay mouth samples. The signal in the range from m/z 150 to 400 is multiplied by 5 to facilitate identification of the DT-MS signals.

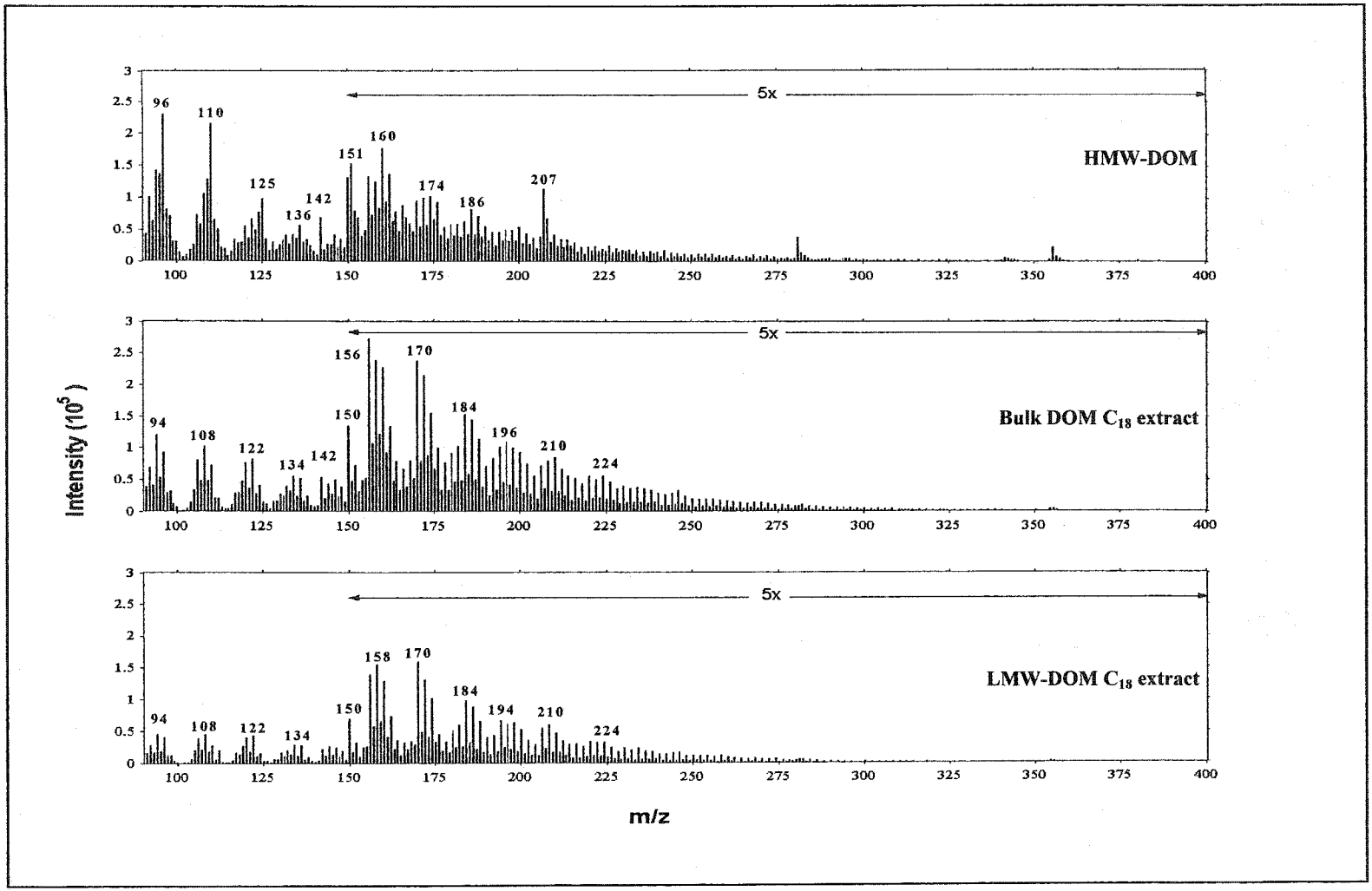

Fig.28. DT-MS spectra of DOM samples isolated by ultrafiltration and C_{18} SPE from the Elizabeth River water samples. The signal in the range from m/z 150 to 400 is multiplied by 5 to facilitate identification of the DT-MS signals.

However, we can compare the relative intensity of one m/z value to another within one sample as compared to within another sample. In doing so, it appears that the higher m/z values are more dominant in the bulk DOM C_{18} extracts (CBMse and ERse) than in the HMW-DOM samples (CBMr and ERr).

As illustrated in Fig. 27 and 28, the HMW-DOM isolated by ultrafiltration appears different in chemical composition from DOM isolated using the C_{18} disks. Tentative identification of the compounds responsible for these differences was performed by matching sample m/z values with m/z values from analyses of standard compounds (summarized by Klap 1997, and Minor 1998, 2003). The HMW-DOM samples exhibited major peaks at m/z 96, and 110 suggesting the presence of furfurals which are indicative of (degraded) polysaccharides (Boon et al. 1998; Klap 1997; Minor et al. 2001) in the samples. The major peak at m/z 125 can indicate the amino acid alanine (Eglinton et al. 1996) or, in the presence of peaks similar to a chitin standard (m/z 97, 101, 109, 111, 114, 125, and 139; Minor et al. 2003), can be assigned to the presence of aminosugars in the sample. All bulk DOM and LMW-DOM C_{18} extracts (Fig 27 and 28) exhibit major peaks with m/z 94, 108, 122 indicating possible aromatic protein (tyrosine: m/z 94, 108, 186) and phenol ion pyrolysis products (phenol: m/z 94, methylphenol: m/z 108, and ethylphenol: m/z 122) in the samples (Eglinton et al. 1996, Minor et al. 2003). The increased intensity of the combination of m/z 120 and 150 in the DT-MS spectra suggests the presence of vinyl guaiacol and vinyl phenol. These pyrolysis products are the decarboxylated forms of ferulic acid and para-coumaric acid, both thought to play a role in the linkage between lignin and polysaccharides (Jung and Ralph 1990; Klap 1997). They appear to be part of an ion suite that indicates degraded lignin

(m/z 192, 178, 162, 150, 120; Klap 1997) and is present in both bulk and LMW-DOM C_{18} extracts.

The m/z values 210, 194, 167, and 154 in the C_{18} extracted DOM samples also suggest the presence of lignin pyrolysis products, i.e. syringyl units, in the sample (Klap 1997). The DOM isolated by ultrafiltration (HMW-DOM) does not show similar dominant m/z values in the > 150 m/z region.

4.3.5. Discriminant Analysis of DT-MS Results

Discriminant analysis (DA) of the DT-MS spectra of replicate samples from each isolation method showed a clear separation of retentates and extracts along Discriminant Function 1 (DF1; Fig. 29), which explained 46.3 % of the variance between the samples with a B/W of 64.3. The HMW-DOM samples had positive values for the DF1 score, while all the C_{18} SPE extracts had negative DF1 scores. Along with the separation of the HMW-DOM and C_{18} isolated DOM on the DF1 axis, the bulk DOM C_{18} extracts and LMW-DOM C_{18} extracts appeared to cluster in negative DF1, positive DF2 space, and negative DF1, negative DF2 space, respectively (Fig. 29). In addition to separating the whole sample and filtrate extracts, DF2, which accounts for 5.2% of the variance with a B/W of 6.7, also appears to be a function of sample location. For each isolation procedure, the CBM samples plotted more positively along DF2 than corresponding ER samples (Fig. 28). A clear separation by site occurred in the HMW-DOM samples and the bulk DOM C_{18} extracts (CBMr and ERr samples and CBMse and ERse samples), while the LMW-DOM C_{18} extracts (the fe samples) for the two sites overlapped somewhat.

The difference spectrum constructed from the loadings of Discriminant Function

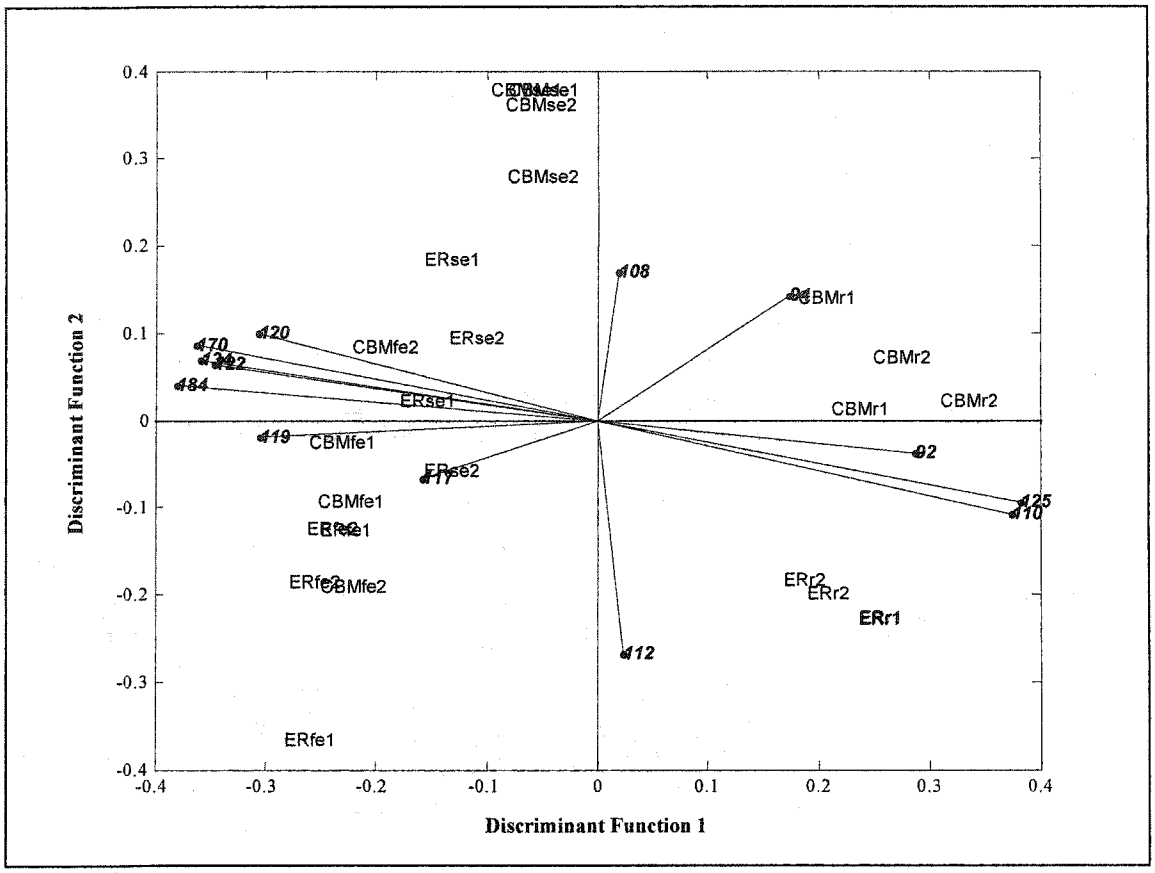

Fig. 29. Discriminant analysis score plot for the DOM samples isolated by ultrafiltration and C_{18} SPE on the < 0.2 μm samples and ultrafiltration filtrates from both the Elizabeth River and Chesapeake Bay mouth sites. Prominent m/z values from the difference spectra are superimposed on the score plot. Sample labels as in Table 5.

1 showed that the HMW-DOM isolated by the ultrafiltration method was more abundant in compounds yielding m/z 92, 96, 110, 125 and 151 (Fig 30). DOM isolated by the C_{18} SPE method was enriched in compounds yielding m/z 119, 122 and > 150. The major peaks that showed up for the positive DF1, m/z 96 and 110, point to the enriched presence of (degraded) polysaccharides in the DOM along with the aminosugar signals m/z 97, 101, 109, 111, 114, 125, 139 and 151. The DOM samples that plotted with negative DF1 appeared to be enriched in lignin-like products, as indicated by the m/z values 120, 150, 156, 170 and 184, and possibly some alkane/alkene compounds as shown by the homologous m/z series above m/z 156 in the difference spectrum.

4.4. DISCUSSION AND CONCLUSIONS

The mass balance for the C_{18} SPE disks was around 100% using both UV/Vis absorbance and DOC measurements (Table 6). Mass balances for both isolation techniques using samples from both sites indicate that no extra material is added based on UV/Vis absorbance and DOC measurements (Table 7). DT-MS analysis of blanks (Fig. 21) also supports the conclusion that no significant amount of material was added during the C_{18} SPE procedure. We observed negligible irreversible absorption onto the C_{18} disk based on UV/Vis absorbance. These results indicate that DOM isolated by C_{18} disk extraction can be characterized by DT-MS as used in this study.

Based on UV-Vis absorbance between 52 and 56 % of the chromophoric material in the bulk DOM samples was isolated using C_{18} disk extraction; for the LMW DOM fraction, between 40 and 50 % of the chromophoric material originally present was isolated by C_{18} extraction. Based on DOC concentration the recoveries dropped to between 25 and 40%

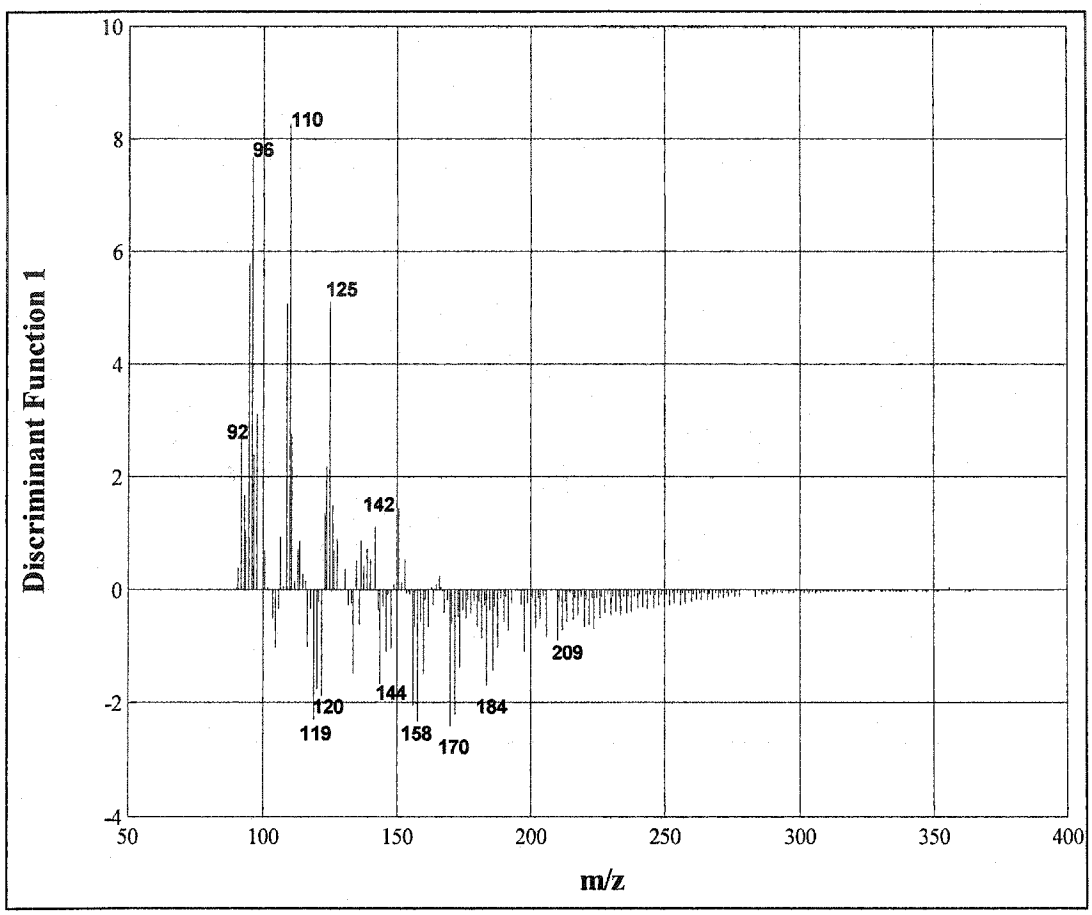

Fig. 30. Difference spectrum based on Discriminant function 1 of the discriminant analysis shown in Fig. 29.

and between 25 and 38% for the bulk DOM and the LMW DOM C_{18} extracts respectively. When ultrafiltration with a 1000 Da membrane was used, the HMW-DOM contained 53 to 60% of the chromophoric material based on UV/Vis absorbance and between 48 and 54% of the organic carbon based on DOC analysis (Table 7). The observed recoveries of DOM using ultrafiltration, based on DOC analysis, from these estuarine/marine sites (Chesapeake Bay mouth and Elizabeth River) are comparable to earlier ultrafiltration experiments using these sites (Simjouw et al. unpublished results) and coastal bay samples from Chincoteague Bay, VA and MD (Simjouw et al. in press). Our recoveries for the C_{18} disk extraction method were lower than reported recoveries for C_{18} disk extraction of river samples (60% based on DOC and 70% based on UV/Vis absorbance, Kim et al. 2003) and closer to the recoveries reported by Amador et al. (1990) for C_{18} Sep-Pak extractions of estuarine and marine DOM (30% of the DOC and 42 % of the absorbance).

For the C_{18} disk extraction method, the recoveries based on UV/Vis absorbance are on average 15 to 20% higher than the yields based on DOC concentrations; when ultrafiltration is used, the UV/Vis absorbance yields are only 5 to 9% higher than yields based on DOC analysis (Table 7). This difference between the recoveries for the different DOM isolation techniques (Table 6 and 7) suggests that more chromophoric material was retained by C_{18} extraction than by ultrafiltration, which is consistent with the observation that DOM isolated using ultrafiltration differs in composition from DOM isolated using C_{18} SPE based on FTIR and DT-MS analyses.

FTIR results indicate that DOM isolated by C_{18} SPE contains fewer hydroxyl groups as evident by the sharpening of the absorbance band around 3450 cm^{-1} and the

decrease in the absorbance band around 1150 cm^{-1}. The appearance of the shoulder around 3270 cm^{-1} (which could be attributed to N-H bonds) and the increase of the peak at 1635-1660 cm^{-1} (from C=O or C=C), suggest a relative increase in either amide or unsaturated hydrocarbon functional groups in the C_{18} SPE isolated DOM (Fig. 22 and 23). Increases in absorbance bands at 3050, 2950, and 2870 cm^{-1} and at 700 cm^{-1} imply relative enrichments in aromatic and alkane/alkene structures in the C_{18} extractions of <0.2 μm DOM. The C_{18} SPE method is more likely to preferentially isolate these compounds due to the hydrophobic interactions that govern the extraction of the DOM with the C_{18} disk (Liška, 2000). The FTIR spectra also show that the DOM from LMW-DOM C_{18} extraction (CBMfe and ERfe) was more similar in functional group composition to the bulk DOM C_{18} extracts than to the HMW-DOM isolated by ultrafiltration. Because the LMW-DOM was the filtrate from the ultrafiltration this could indicate that the DOM isolated by C_{18} SPE contained a significant portion of LMW material. This would have to be investigated further by comparison with C_{18} disk extracts of the ultrafiltered HMW-DOM. The FTIR spectra do not indicate significant differences in DOM based upon sampling location.

DT-MS spectra of the different DOM isolates (Fig. 27 and 28) also indicate that molecular-level composition varies with isolation technique. For both the HMW-DOM samples (CBMr and ERr), pyrolysis products from carbohydrate (degraded polysaccharide) compounds and amino sugars dominate the spectrum. The DT-MS spectra for both bulk DOM C_{18} disk extracts (CBMse and ERse) are mostly dominated by aromatic proteins and/or phenolic lignin-like compounds. Again as in the FTIR spectra, the DT-MS spectra for the LMW-DOM C_{18} disk extracts showed more similarity with the

bulk DOM C_{18} extracts than with HMW-DOM isolated by the ultrafiltration method. It is possible that for our sampling sites, CBM and ER, the bulk DOM and LMW-DOM samples contain mostly similar lignin-like compounds, which could account for the comparable FTIR and DT-MS spectra of the C_{18} DOM extracts or that the C_{18} disk SPE preferentially extracts these compounds. Previous studies using C_{18} cartridges on estuarine and marine samples showed that this technique isolates roughly 100 % of the dissolved lignin-derived phenols and that a significant portion of these compounds in the LMW-DOM fraction (as separated using ultrafiltration) can be isolated by C_{18} SPE (Louchouarn et al. 2000).

The differences between the two isolation methods observed in the results from the FTIR analysis (Fig. 23 and 24) and the DT-MS spectra (Fig. 27 and 28) also appear in the results from the discriminant analysis on the DT-MS dataset (Fig. 29). Based on the separation in the DA score plot (Fig. 29) and the difference spectrum from DF1 (Fig. 30), we can again conclude that the C_{18} SPE method preferentially isolates phenolic and lignin-like compounds from the water samples relative to the ultrafiltration DOM isolation method. When we plot the actual m/z values superimposed upon the DA score plot (Fig. 29), we can again see that the separation of the isolation methods is due to the dominance of either polysaccharides (CBMr and ERr samples) or phenolic and lignin-like compounds (all C_{18} SPE DOM samples. It is interesting to note that a separation occurs along DF2 between the two HMW-DOM samples (CBMr and ERr) and similarly between the two bulk DOM C18 disk extract samples (CBMse and ERse). Even though the two isolation techniques yield different types of DOM, molecular level differences between sampling sites can be observed in both of the resulting DOM pools. Therefore,

we propose that both isolation techniques should be used in process studies such as, for example, investigations of DOM bioavailability or the influence of photodegradation on DOM.

It appears that the combination of ultrafiltration and C_{18} SPE methods markedly increases the fraction of DOM that can be isolated from marine estuarine samples. The overall recovery of DOM based on DOC concentration increased from around 50% using ultrafiltration to between 60 and 70 % of the total DOC using both methods. Based on UV/Vis absorbance the recovery increased from between 60 and 70 % to between 70 and 80 % of the total UV/Vis absorbance. Several earlier studies on HMW-DOM characteristics using DT-MS analysis can now be expanded to a portion of the LMW-DOM fraction if sufficient material can be extracted. However, the difference in the isolated material, as shown by FTIR and DT-MS, from the two DOM pools makes direct comparison between HMW and LMW DOM difficult unless C_{18} extraction is also performed on the retentate (HMW-DOM) from ultrafiltration.

SECTION 5

CONCLUSIONS AND FUTURE WORK

5.1. CONCLUSIONS

In this dissertation, the DOM pools in surface water samples from a shallow coastal bay were characterized on a bulk and molecular level over the course of several blooms of the pelagophyte *Aureococcus anophagefferens*. The main goal of this study was to investigate the interactions of the DOM in the surface water and *Aureococcus anophagefferens* during blooms. To this effect, surface water samples were taken at two sites in Chincoteague Bay, Public Landing (Maryland) and Greenbackville (Virginia), over the course of two years prior to, during, and after *Aureococcus anophagefferens* blooms. Environmental parameters of the surface water together with *Aureococcus anophagefferens* densities and chlorophyll *a* concentrations were determined for these surface water samples. Bulk DOM was characterized using DOC analysis and UV/Vis absorbance measurements (spectral slope and specific UV absorbance), while more molecular characteristics of the HMW-DOM pool were determined with mass spectrometry techniques, electron impact (EI) DT-MS and IR-MS. All the results were combined to evaluate the possible influence of the DOM pool on the occurrence of *Aureococcus anophagefferens* blooms and to determine the impact of the bloom events on the DOM pool in the surface water of Chincoteague Bay.

In addition to exploring DOM and *A. anophagefferens* interactions, this dataset allowed further exploration of interactions between allochthonous and autochthonous inputs of DOM within a shallow coastal bay. Sampling in Chincoteague Bay occurred in

2002, an unusually dry year, and 2003, a very wet year, thus providing strong contrasts in DOM sources and fates in the two years.

5.1.1. Interaction of DOM and *Aureococcus anophagefferens* Blooms

Samples collected in 2002, when an *Aureococcus anophagefferens* bloom developed at Public Landing but not at the Greenbackville site, allowed for direct comparison of the characteristics of the DOM between the two sites. The DOC concentration and the C:N ratio at the Public Landing site before the onset of the *Aureococcus anophagefferens* bloom was found to be higher than at Greenbackville along with a lower $\delta^{13}C$ value of the HMW-DOM. Although the Public Landing values could be explained by differences in autochthonous source material, these values are also consistent with a larger terrestrial DOM component at Public Landing.Even though the study revealed differences in the initial DOM pool at both sampling sites, a direct link between the DOM pool and the initiation of the *Aureococcus anophagefferens* blooms at Public Landing in 2002 was not established. The 2002 study did, however, reveal changes in the DOM pool due to the bloom of *Aureococcus anophagefferens*. The brown tide bloom contributed to changes in the DOM pool by a net input of more N enriched aromatic HMW-DOM during the bloom, and more C-enriched HMW-DOM when the *Aureococcus anophagefferens* bloom crashed.

During the 2003 study, rainfall and freshwater input from streams into Chincoteague Bay were well above average, while 2002 was about 50% below average. One of the first clear differences between the two sampling years was the development of a brown tide bloom at both sampling sites in 2003 with DOC concentrations lower than

those when a bloom developed in 2002 (Public Landing only). This indicated that the DOC concentration was not limiting brown tide bloom development at Greenbackville in 2002, but that the quality of the DOM, the molecular level characteristics, might be important. The spring DOM in 2003 had a bigger HMW fraction and was more aromatic, possibly of terrestrial origin, compared to either DOM pool characterized in 2002. Again, no direct link was established between the DOM pool and the brown tide blooms. But if DOM was utilized to out-compete phytoplankton to initiate and sustain bloom densities, *Aureococcus anophagefferens* is either using a portion of the DOM that we cannot currently resolve or must possess adaptability or flexibility regarding utilization of organic compounds.

DOM characterization on a bulk and molecular level indicated that, consonant with results from the 2002 study, the DOM pool changed during the *Aureococcus anophagefferens* bloom in the surface water, even though the DOC and HMW-DOC concentrations did not show a similar increase during and immediately after the bloom. During the brown tide blooms in 2002 and 2003, the bulk and molecular level characteristics of the DOM shifted towards common features which suggest release or transformation of DOM in the surface water by *Aureococcus anophagefferens*. This release or transformation of DOM could be a factor in sustaining brown tide bloom conditions. Further work, as suggested in a later section, could help determine the possible utilization of DOM in the surface water by *Aureococcus anophagefferens*.

5.1.2. DOM in Chincoteague Bay

Even though the focus of this dissertation was to investigate the relationship

between the DOM pool and the occurrence of *A. anophagefferens* blooms in Chincoteague Bay, some results from this study can be used to infer changes in DOM characteristics due to variations in allochthonous and autochthonous inputs. To investigate the importance of freshwater and seawater input sources and internal processes on the various DOM parameters that were measured during the two-year study for both sampling sites, these parameters were plotted against salinity.

By plotting the DOC concentration against salinity for each sampling site (Fig. 31), a significant correlation ($r = 0.798$, $p = 0.05$, $n = 24$) for Public Landing DOC concentration with salinity was found. Surprisingly, the trend at Public Landing is positive, indicating lower DOC concentration when there was a higher freshwater contribution; this result is counterintuitive to the generally higher DOC concentrations measured for freshwater samples (as summarized in Cauwet 2002). This is probably due to high autochthonous inputs of DOC during and just after the brown tide bloom in 2002. The Greenbackville DOC salinity plot, in contrast to the Public Landing plot, shows an overall negative trend, consistent with the usual observation of higher DOC levels in fresher waters. No significant correlations were found for HMW-DOC concentration and the %HMW-DOC with salinity (data not shown).

Because in this study we are looking at time series rather than transects, we cannot use mixing curves to study inputs and losses of DOC. However, we can use UV/Vis absorption characteristics and stable isotope compositions to further constrain potential DOC sources

UV/Vis characteristics of the DOM pool measured in the two-year study (spectral slope and specific UV absorbance) plotted against salinity (Fig. 32) show a significant

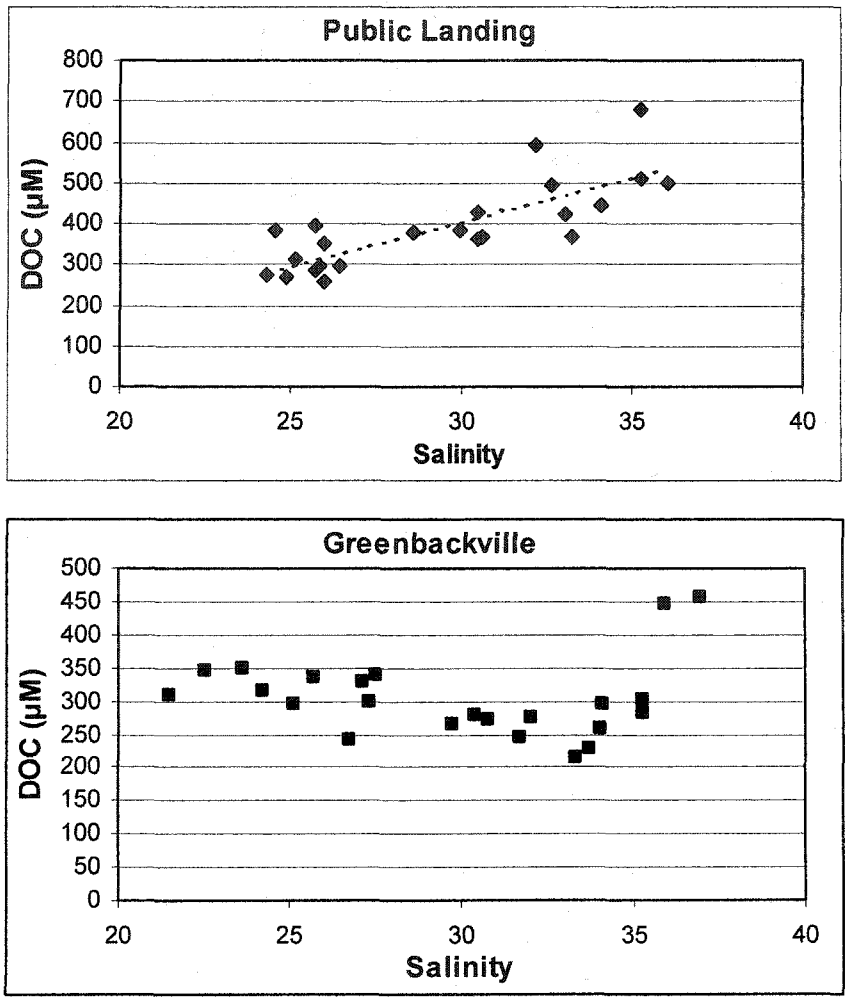

Fig. 31. DOC concentration in µM versus salinity for Public Landing and Greenbackville. The samples were collected during 2002 and 2003. The dotted line indicates the linear trend line.

correlation ($r = 0.587$ and 0.580 respectively, $n = 24$, $p = 0.05$). The spectral slope increases and the SUVA decreases with salinity, strengthening the hypothesis that high molecular weight, aromatic DOM was coming from freshwater inputs as suggested earlier in Section 3. Similar positive trends for the spectral slope were also found along spatial rather than temporal salinity gradients in the Chesapeake Bay (Rochelle-Newall and Fisher 2002); the range of spectral slope values in the Chesapeake Bay were similar to what was observed in Chincoteague Bay. The terrestrial nature of the DOM brought into Chincoteague Bay by freshwater input sources is suggested by the significant correlation ($r = 0.714$, $n = 24$, $p = 0.05$) between the $\delta^{13}C$ values of the HMW-DOM and salinity of the surface water (Fig. 33). Lower salinity surface waters had more isotopically depleted HMW-DOM ($\delta^{13}C \approx -23$ ‰) as compared to those with a higher salinity ($\delta^{13}C \approx -20$ ‰). Most of the $\delta^{13}C$ values measured for both sampling sites in both years fall within the range of temperate marine DOM, between –18 and –23 ‰ (Bauer 2002), with some low salinity HMW-DOM outside this range ($\delta^{13}C \approx -24$ ‰). The lighter $\delta^{13}C$ values of the HMW- DOM with the lower salinity of the surface water indicates an input of terrestrial DOM, $\delta^{13}C$ values between –25 and –30 ‰ (Bauer 2002), by freshwater runoff. While bulk concentrations of DOC and HMW-DOC do not show significant increases with increasing freshwater inputs, the quality of the DOM does appear to differ; showing higher aromaticity, increased molecular weight, and lower $\delta^{13}C$ value.

To investigate if other phytoplankton along with *A. anophagefferens* could be directly responsible for changes in the DOM pool in Chincoteague Bay, plots of all measured parameters versus chl *a* content of the surface water were constructed. The only

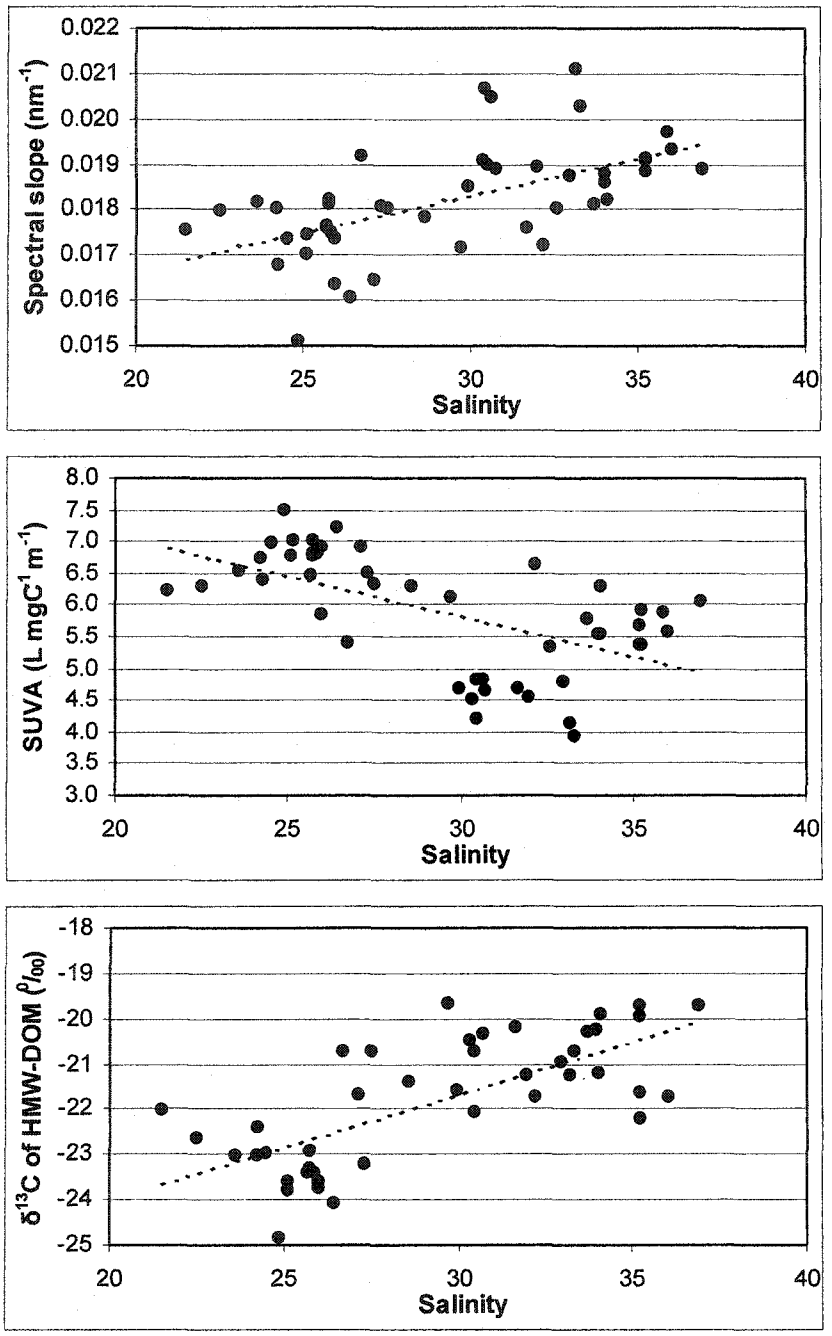

Fig. 32. UV/Vis absorbance characteristics of DOM and $\delta^{13}C$ values of the HMW-DOM versus salinity for Public Landing and Greenbackville. The samples were collected during 2002 and 2003. The dotted line indicates the linear trend line.

significant linear correlation ($r = 0.584$, $n = 24$, $p = 0.05$) was for HMW-DOC concentration and chl *a* content (Fig. 33). For all other DOM characteristics, no significant correlation was found (not shown), indicating that even though HMW-DOC concentration appears to be related to phytoplankton abundance, measured as chl *a*, the change in characteristics of the DOM pool result from more complex processes. The change in DOM characteristics as determined in Sections 2 and 3 were possibly either specific for the type of phytoplankton (*A. anophagefferens*) or due to the presence of bacteria that reworked the available DOM pool. Work by Nelson et al. (1998) and Rochelle-Newall and Fisher (2002a) showed that chromophoric material was produced due to bacterial utilization of the present DOM pool, and was not directly related to phytoplankton growth. Our results are consistent with this.

5.2. UNRESOLVED QUESTIONS AND FUTURE WORK

The research described in this dissertation yields insight into the interaction of *Aureococcus anophagefferens* and the DOM pool. To fully address whether DOM present in surface water initiates and can sustain an *Aureococcus anophagefferens* bloom will require additional research I propose in this section.

5.2.1. Laboratory Culture Studies

In this dissertation the potential interaction between the DOM pool and *Aureococcus anophagefferens* blooms was studied by analyzing DOM present during brown tide growth and bloom. To determine if the DOM pool isolated from surface water samples supports or augments brown tide growth, laboratory enrichment studies using

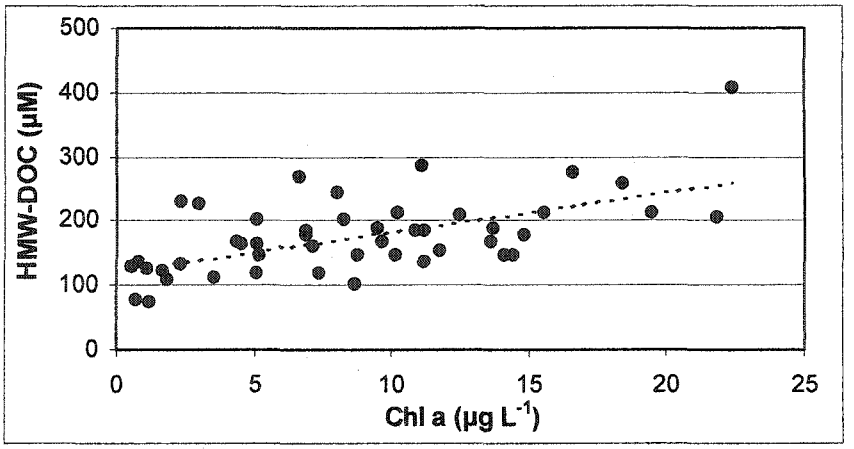

Fig. 33. HMW-DOC concentration in µM versus chl *a* in µg L^{-1} for surface water samples from Public Landing and Greenbackville in 2002 and 2003. The dotted line indicates the linear trend line.

axenic *Aureococcus anophagefferens* cultures should be performed. Berg et al. (2003) conducted similar growth experiments using HMW-DON originating from sediment porewater and found that the *Aureococcus anophagefferens* was capable of using a substantial portion of the HMW-DON. Combining field studies similar to those described in Sections 2 and 3, along with laboratory studies using the characterized field DOM as the only source of C and N in axenic cultures, would be an important continuation of this work.

Using the C_{18} disk extraction technique as described in Section 4 would allow concentration and isolation of approximately 35-45% of the LMW-DOM, so that the culture experiments could involve a substantial portion of the LMW-DOM as well.

Axenic cultures can also be used to investigate the adaptability of *Aureococcus anophagefferens* to grow, or at least to sustain itself, on different types of substrates ranging from urea to HMW compounds. Comparison of the initial DOM pools at the *Aureococcus anophagefferens* bloom sites in 2002 and 2003, as described in Section 3, suggest *Aureococcus anophagefferens* might be capable of using different organic compounds for growth.

During the *Aureococcus anophagefferens* blooms described in Sections 2 and 3, the DOM pool changed toward common features with respect to both UV/Vis absorbance characteristics of bulk DOM and molecular level characteristics of HMW-DOM. This shift in the DOM pool was hypothesized to be due to a release of DOM, and possibly chromophoric DOM (CDOM), by *Aureococcus anophagefferens* into the surface water. Culture studies under axenic conditions could be used to answer the questions if and what kind of DOM, with respect to molecular weight and characteristics, are released by

Aureococcus anophagefferens during bloom conditions and when a bloom crashes. Such studies would also indicate whether bacterial reworking is necessary to make DOM from primary production into CDOM as was suggested by work of Nelson et al. (1998) and Rochelle-Newall and Fisher (2002a).

5.2.2. Additional Areas for Field Studies

The sampling in this study included a drought year and a wet year; precipitation and runoff was lower than average in 2002 and above average in 2003. To elucidate long term patterns with respect to DOM characteristics and the occurrence of brown tide blooms, data from only two years is insufficient. Continuing to sample surface water at both sampling sites in Chincoteague Bay to characterize the DOM pool along with Aureococcus anophagefferens blooms (if they occur) would increase our knowledge of DOM characteristics and thus make more comparisons possible that could reveal patterns between DOM in the surface water and the occurrence of Aureococcus anophagefferens blooms. Using the combination of ultrafiltration and C_{18} techniques for isolating DOM as proposed in Section 4 would increase the total recovery of the isolated DOM for further characterization. When a larger portion (up to ~70%) of the DOM in the surface water can be characterized, a better understanding of DOM cycling with respect to Aureococcus anophagefferens growth and bloom might emerge.

Other unanswered questions that need to be addressed to constrain the study area with respect to occurrences of brown tide blooms involve the residence time of the surface water and with that movement of water, the dynamics of dissolved nutrients and DOM throughout Chincoteague Bay. Many factors are thought to contribute to

Aureococcus anophagefferens blooms. For many of these factors to manifest, such as lowering of the inorganic nutrient concentrations, the surface water needs to remain in place within the Bay system. Even though the residence time of the water in Chincoteague Bay has been estimated to be > 60 hrs (Brumbaugh 1996) and the contrasting bloom conditions at the two sampling sites in Chincoteague Bay in 2002 suggest that the surface waters at each sampling site do not interact prior to and during the brown tide bloom, the actual residence time of the bay water has never been measured nor has the residence time of the surface water at each sampling site. More knowledge about these parameters would increase our understanding about processes occurring at the sampling sites. After the bloom appeared to have crashed at Public Landing in mid-June, 2003 (Section 3), the brown tide cell density increased again to similar levels as were measured at the Greenbackville site. Without knowledge of the surface water advection within Chincoteague Bay, it is impossible to eliminate surface water mixing as a process that diminishes *Aureococcus anophagefferens* cell abundance there.

With respect to our knowledge of sources of DOM, whether allochthonous or autochthonous, more endmembers that can contribute DOM to the sampling sites need to be investigated. Ground water input, pore water diffusion, atmospheric deposition, and coastal runoff (especially from agricultural lands) are potential sources of labile organic matter that can change the local DOM pool along with bacterial reworking of particulate organic matter and bacteria decay products. Therefore the contribution of these input sources needs to be evaluated with respect to DOM concentration and characteristics.

For example, measurements of source-specific biomarkers in the HMW-DOM samples or in the total DOM will be useful to estimate the relative input of these sources

to the DOM pool. Approximation of the relative contribution of terrestrial organic matter to rivers, estuaries and coastal oceans has been done using identification of lignin oxidation products and specific product ratios (Meyers-Schulte and Hedges 1986, Bianchi et al. 1997, Opsahl and Benner 1997, Mitra et al. 2000), and this approach can be useful to determine the impact of freshwater runoff to the DOM pool in Chincoteague Bay. Another example is the analysis of lipids in the HMW-DOM pool to determine the contribution of phytoplankton and bacterial material (Mannino and Harvey 1999, Harvey and Mannino 2001, Zou et al. 2004). The absence or presence of specific poly-unsaturated (like 16:3 and 18:4) fatty acids and mono-unsaturated (like 16:1Δ9 and 18:1Δ9) fatty acids could indicate the contribution of phytoplankton material to the HMW-DOM pool (Mannino and Harvey). Analysis of bacteria specific fatty acids in the ultrafiltered DOM, for instance C10 to C18 normal and branched (hydroxy) fatty acids (Wakeham et al 2003), can be used to determine if bacterial material is an important contributor to the HMW-DOM pool.

The proposed additional research in this section will increase our knowledge and further constrain the sources, sinks, and possible transformation of the dissolved organic matter into the Chincoteague Bay.

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TALKS

Simjouw J. -P., E. C. Minor, and K. Mopper. Isolation and characterization of estuarine dissolved organic matter: a comparison of C18 solid phase extraction and ultrafiltration techniques. Seminar for the Dept of Chemistry and Biochemistry Spring 2004 seminar series.

Simjouw J. -P., E. C. Minor, and M. R. Mulholland. Comparison of dissolved organic matter concentrations and characteristics during *Aureococcus anophagefferens* blooms in 2002 and 2003: A search for possible causes. ASLO Summer meeting in Savannah, GA, USA. Presented by Dr. Minor.

Simjouw J. -P., E. C. Minor, and M. R. Mulholland. Molecular-level characteristics of dissolved organic matter in Chincoteague Bay prior to and during an *aureococcus anophagefferens* bloom, Aquatic Sciences Meeting ASLO 2003, Salt Lake City, UT, USA.

PAPERS

Simjouw J. -P., M. Mulholland, and E. C. Minor. Dissolved organic matter concentration and characteristics during *Aureococcus anophagefferens* blooms in 2002 and 2003: a comparison. Submitted.

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