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Preservation of Proteinaceous Materials in Marine Environments

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Abstract of the Dissertation

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Proteinaceous materials are major components of marine organic matter, and can account for up to 80% of the organic nitrogen in marine organisms. Although proteinaceous materials are generally considered to be labile, some of them are protected from heterotrophic attack and remineralization. In this study, we discussed the possible mechanisms how proteinaceous materials are preserved and transported in marine environments. First, the accumulation of refractory prokaryotic cell membranes has been suggested as one of the possible sources of the proteinaceous materials observed in the deep ocean. The refractory nature of these membranes presumably results from the highly diverse and complicated membrane structures, e.g., peptidoglycan and phospholipid bilayer. Surface layer protein (S-layer) is a specific membrane glycoprotein that is widely found in both Eubacteria and Archaea. This heavily glycosylated protein covers the outermost cell surface in a regularly ordered planar crystalline structure. With special attention to changes in S-layer protein and peptidoglycan, we studied the degradation of two species of marine cyanobacteria, *Synechococcus* CCMP2370 and CCMP1334, with and without S-layer structures, respectively. We also studied degradation of both these species after they had been treated with buffers that strip the S-layer from the cell surface. The changes in

degradation biomarkers, elemental composition and cell morphology were followed during the incubation experiments, suggesting that S-layer protein functions as the skeleton to support the cell structures and removal of S-layer accelerates the degradation of cyanobacterial cells in marine environments.

Second, we provide evidence using electron microscopy (EM) and energy dispersive X-ray spectroscopy (EDS) that Si is deposited on the extracellular polymeric substances (EPS) produced by cyanobacteria, particularly when they begin to decompose. We also found that Si associated with organic micro-blebs collected from the deep ocean. Both nano-particle imaging analysis and bulk organic geochemical analysis show a surprisingly similar appearance between EPS-associated Si in cyanobacteria and micro-blebs in the open ocean. Accordingly, EPS-associated Si might be a precursor of the Si-enriched organic micro-blebs observed in the ocean. The previously unexplored source of particulate silicate minerals may play an important role in the oceanic silicate cycling as well as organic matter export from the surface waters.

Third, a new fluorescent analog, Lucifer Yellow Anhydride-alanine-valine-phenylalanine-alanine (LYA-AVFA), was developed to measure extracellular peptide hydrolysis. Peptide hydrolysis was compared to the uptake of various organic nitrogen species (urea, glutamic acid and dialanine) along transects of the James River estuary and lower Chesapeake Bay salinity gradient during the summer of 2008. Changes in the abundance and composition of dissolved amino acids were also examined. Results from the James River estuary suggest that peptide hydrolysis and organic nitrogen uptake are not always tightly coupled to each other along the salinity transects as a response to the changing environmental conditions in the studied area. This is because diverse input and removal processes can influence both peptide hydrolysis and uptake, but not necessarily simultaneously. A change of dissolved amino acid abundance and

composition was observed from the fresh end of the estuary to the mouth of Chesapeake Bay, which are most likely resulting from the mixing of multiple sources and their impacts on hydrolysis and uptake, e.g. terrestrial input, sediment resuspension and local phytoplankton growth.

DEDICATION

To my father, Zhenyuan Tang.

TABLE OF CONTENTS

List of Tables

List of Figures

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Chapter One

Introduction and Background

In organic geochemistry, the term proteinaceous material refers to organic matter that can be identified as amino acids after acid hydrolysis; this includes amino acids originating from proteins, peptides, peptidoglycans, and other macromolecules that have incorporated amino acid fragments of these compounds during diagenesis. Here we frequently use 'proteinaceous materials' instead of 'total hydrolysable amino acids' because our progressively improving understanding of this amino acid-based organic matter has shown us that there is more information than just amino acid abundance and composition necessary to elucidate processes involved in the biogeochemical cycling of marine organic matter.

1.1. Proteinaceous materials in the marine environments

1.1.1. Biogeochemical cycling of proteinaceous materials in the water column

Nitrogen plays an important role in marine biogeochemical cycles as an essential nutrient that is incorporated into nucleic acids, amino acids, porphyrins, and amino sugars in organisms. Most dissolved inorganic nitrogen (DIN) available for organisms exists in the form of nitrate, as bioavailability, solubility, and redox conditions limit the distribution of ammonium and nitrite. DIN occurs in high abundance in the deep ocean. In contrast, most surface water is depleted in inorganic nitrogen as a result of phytoplankton uptake in the euphotic zone. Dissolved organic nitrogen (DON) can be higher than DIN in these waters, although their cycling and bioavailability are not as well known as DIN. This is because DON is a heterogeneous mixture of organic nitrogen compounds that varies widely in space and time within aquatic environments

and has diverse sources and decomposition patterns (Berman and Bronk 2003). Molecular level identification of organic nitrogen composition is one of the most challenging studies in present geochemical research.

Only a small portion of marine organic nitrogen pool has been well characterized; urea, free and combined amino acids, nucleic acids, and amino sugars have been reported, among which amino acids make up the major portion of characterized organic nitrogen (Benner 2002). Some organic nitrogen compounds combine to form large molecular weight components whose structures are difficult to determine using current analytical techniques. Amino acids, the building blocks of proteins, are the largest reservoirs of organic nitrogen in most organisms, and account for 38 - 84% of total N in phytoplankton, 38 - 66% of N in zooplankton, and 50 - 56% in bacteria and fungi (Parson et al. 1977; Cowie and Hedges 1992; Lee et al. 2000). Large amount of proteins are located in cell membranes and have diverse functions there. Membrane proteins can work as transporters to carry nutrients and wastes across the cell membrane. Others locate at the surface as extracellular enzymes to activate various reactions. Some proteins function as part of the cell wall to support the cell structure, and are called structural proteins. However, proteins are not the only source of amino acids in cells. Amino acids are also major constituents of peptidoglycan, an important bacterial cell wall component. Many of amino acids identified in peptidoglycan are D-enantiomers of amino acids, and have been used as a bacterial biomarker in marine environments (Lee and Bada 1977; McCarthy 1998; Kaiser and Benner 2009). The downward transport of particulate amino acids out of the euphotic zone is quantitatively related to primary production (Lee and Cronin 1984; Lee et al. 2000). This flux decreases by several orders of magnitude between the sea surface and deep-sea floor as a result of heterotrophic remineralization (Lee and Cronin 1984), although dissolution and disaggregation are probably

also important (Lee et al. 2000). Some amino acids are preserved during transport and are buried in sediment, where they can make up 1 to 50% of total organic carbon, but varying greatly with sediment type (Cowie and Hedges 1992; Wakeham et al. 1997). How proteinaceous materials escape degradation during transport remains unclear. A strong quantitative relationship between organic carbon flux and ballast minerals (opal, carbonate and dust) emphasized the importance of minerals in regulating the delivery of organic matter into the deep ocean (Wakeham et al. 1997; Klaas and Archer 2002), although the nature of this association is not known. One possibility is that ballast biominerals (like siliceous diatom frustules and carbonate-rich coccolithophore tests) may physically protect proteinaceous materials inside from attack by hydrolytic enzymes, and can also accelerate their sinking velocities, which more quickly removes them from the water column.

Although amino acids contribute a large fraction of organic carbon to both living organisms and sinking particles in the ocean, they are less important to total dissolved organic matter (DOM) than carbohydrates; this is thought to be the result of their labile nature compared with other components of DOM. The low concentrations of dissolved amino acids limit our understanding of their cycling in most marine environments (Kaiser and Benner 2009). However, close coupling of amino acid production and consumption that results in their low concentrations (Fuhrman and Bell 1985; Kaiser and Benner 2009), suggests that they, with other compounds in DOM, are an important contributor to the DOM that fuels the microbial carbon pump (Azam et al. 1983; Jiao et al. 2011). Although dissolved free amino acids can easily be taken up by microbes, larger proteinaceous materials cannot. Extracellular hydrolytic enzymes are required to cleave proteinaceous materials into peptide fragments that are small enough to pass through the cell membrane. Other ‘speed bumps’ that slow down enzymatic hydrolysis of

biomacromolecules are described by Arnosti (2004).

1.1.2. Bacterial membrane proteins in marine environments

In plants and most microorganisms, the cytoplasm in cells is isolated from its ambient environment by membranes that are usually enclosed by cell walls. *Membrane proteins* refer to those proteins that are attached to, or associated with cell membranes (Madigan and Martinko 2006). Proteins from both eukaryotic and prokaryotic cell walls have been widely isolated and studied. It is difficult to distinguish between membrane protein and cell wall protein, so the membrane proteins mentioned hereafter refer to proteins isolated from either or both membrane and cell wall. Membrane proteins are up to 30% of the cellular proteins we known and are fundamental components of the membrane structure (Engel and Gaub 2008); they perform diverse functions such as solute and ion transport, and information processing (Cho and Stahelin 2005). Compared with cytoplasm proteins, membrane proteins are more hydrophobic and are closely associated with other membrane and wall components like lipids and peptidoglycans. It is thought that this association may potentially limit access to microbial attack (Laursen et al. 1996; Nagata et al. 1998).

1.1.3. Glycosylation of proteins

One of the characteristic features of prokaryotic membrane proteins is their extensive post-translational modification after transcription (Schäffer et al. 2001a). Glycosylation is an important enzyme-regulated post-translational modification of proteins by carbohydrates, and is considered to fundamentally influence and modulate structures and functions of proteins (Taylor and Drickamer 2002; Hitchen and Dell 2006). As a product of glycosylation, glycoproteins are those proteins covalently associated with carbohydrates. The carbohydrate chains of glycoprotein

are highly diverse. There are two major types of attachment of oligosaccharides to glycoprotein: N-linked and O-linked. In N-linked attachment, a β-N-acetylglucosamine (NAG) is linked to the amide nitrogen of Asn in the sequence Asn-X-Ser or Asn-X-Thr, where X is any amino acid residue. The NAG is linked with two or three mannoses in all N-linked glycans. In O-linked attachment, the carbohydrate is linked to the OH group of either Ser or Thr (Voet and Voet 2005). Glycoproteins are important constituents of cell membranes and widely exist in both phytoplankton and bacteria. For example, large amounts of membrane-associated glycoproteins are accumulated in iron-starved cyanobacteria (Riethman et al. 1988). Glycoproteins have also been identified in coccolithophore cells. Nrp1, a glycoprotein from *Emiliania huxleyi* was found to be a major membrane or cell wall protein in nitrogen-limited cells (Palenik and Koke 1995). Glycoproteins are involved in many cell activities but are poorly understood. For example, the carbohydrate groups of glycoproteins that are located on the external surface of the membrane are thought to be important information receptors (Voet and Voet 2005).

1.1.4. Surface layer structure

A specific membrane protein found in Eubacteria and Archaea is the regularly ordered, planar array of paracrystalline proteinaceous subunits termed surface layer (S-layer) protein (Sleytr and Beveridge 1999). S-layer proteins cover the outmost surface of cells and can account for up to 15% of the total protein in bacteria (Smarda et al. 2002); they are heavily glycosylated with an overall degree of glycosylation of about 1-15% (Schäffer and Messner 2001). This S-layer structure was also covered by a polysaccharide sheath in some marine *Synechococcus* species (McCarren et al. 2005). One of the most characteristic features of S-layers is their ability to self-assemble. If the structure is broken up, the subunits of S-layer proteins can automatically refold and reform their original secondary structure with various symmetries, generally

hexagonal symmetry. Very little research exists on marine S-layers. S-layers have been found in cyanobacteria and other common bacteria adapted to saline environments. Since 1972, S-layers have been found in 23 species of cyanobacteria. A schematic diagram of S-layer below (Fig. 1.1) shows the schematic diagram of the S-layer in the *Synechococcu*s CCMP2370, also known as WH8102 (McCarren et al. 2005). The subunits of S-layers have been analyzed by electrophoresis in *Synechococcus sp*. This S-layer is thought to induce mineralization of fine-grain gypsum and calcite that can increase the density of the cell and change its buoyancy in seawater (Smarda et al. 2002). Other functions of S-layers are poorly known. As the outermost layer of prokaryotic cells, S-layers likely play a role in protecting cells, as scaffolding for enzymes, and in excluding transport of large molecules (S-layer includes the polysaccharide sheath).

1.2. Preservation of proteinaceous materials

1.2.1. Preservation of proteinaceous materials due to their primary structure or non-covalent cross-linking

Preferential preservation of membrane proteins is suggested by the observation that bacterial membrane protein degrades more slowly than soluble protein from the cytoplasm (Nagata et al. 1998). Not until 1995 was the first integral protein reported in seawater, with an apparent molecular weight of 48 kDa; this protein was thought to be a trans-out-membrane channel protein of a Gram-negative bacterium (Tanoue et al. 1995). Since then, both dissolved and particulate proteins have been identified in oceanic and coastal waters (Yamada and Tanoue 2003, and cited works). Dominant detected proteins include the membrane proteins porin P and OmaA-like protein from several species. Based on this work, membrane proteins, rather than cytoplasm proteins, appear to dominate in marine environments. In spite of the potential input from diverse organisms in the ocean (Yamada and Tanoue 2003), less than 30 proteins were

identified in seawater by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a common technique to separate proteins from each other. Clearly, further work is needed to determine the presence and distributions of other proteins in the marine environment.

The exact mechanisms of preferential protein preservation are not known. Selective preservation can occur when certain proteins or groups of proteins are more refractory as a result of their primary structures. For example, using fluorescent analogs of peptides, Pantoja et al. (Pantoja et al. 1997; Pantoja and Lee 1999) demonstrated that peptides of different lengths and compositions hydrolyze at different rates in seawater, which suggests selective cleavage of certain peptide bonds by proteolytic enzymes. Preferential removal of polar, charged amino acids by proteolytic enzymes has been observed during the degradation of intact proteins in coastal seawater (Roth and Harvey 2006). Those studies imply that microbial organisms preferentially attack certain primary structures more than others.

Non-covalent cross-linking is also considered to be an important stabilizing mechanism in long-term preservation of peptide linkages in marine environments. Proteins in the cells are folded non-covalently by hydrogen bonding, and electrostatic and hydrophobic interactions (Voet and Voet 2005). It is not clear whether the native conformation of protein is maintained after cell lysis or if proteins will be refolded in some other way. In addition to non-covalent associations that can occur within a single protein, the association between protein and other macromolecules is also important in preservation. Membrane proteins in the cell are associated with membrane lipids either by hydrophobic bonding or by covalent linking (Voet and Voet 2005). The outsides of the membrane proteins are relatively more hydrophobic and can be tightly associated with the membrane. In a degraded cell, the membrane proteins and other labile compounds will be surrounded by a lipid double layer or by algaenan (Knicker et al. 1996;

Hedges et al. 2000; Nguyen and Harvey 2003). These hydrophobic compounds will prevent the attack of hydrolytic enzymes or other chemical reactants such as hydroxyl radicals (Pogozelski and Tullius 1998). Protection of proteins from enzyme hydrolysis by hydrophobic interaction has been better demonstrated in surface sediment (Borch and Kirchman 1999; Nguyen and Harvey 2003). As mentioned above, slower degradation of membrane proteins than cytoplasm proteins was observed in seawater (Nagata et al. 1998), but this study did not extract membrane proteins from the cell matrix. The matrix may be involved in protecting its associated proteins from degradation. As far as we know, there is no direct observation that protein isolated from the cell membrane is better preserved than protein from cytoplasm.

1.2.2. Preservation of proteinaceous materials by chemical modification

Another possible mechanism to explain selective preservation of proteinaceous materials is chemical modifications of protein that potentially change its availability to microbes. Common covalent modifications include glycosylation, advanced glycation end product (AGE) formation, and oxidative coupling. Glycoproteins have been widely found in S-layer proteins as well as other membrane proteins. Although little is known about the significance of protein glycosylation in the preservation of proteinaceous materials, the distinctive functions of glycoproteins in vivo may give us some useful hints. For instance, proteoglycans undergo non-specific interactions with other carbohydrates or macromolecules in the cell; this may help proteins to associate with aggregates enriched in polysaccharide in the marine environments (Aplin and Hughes 1982; West 1986). Glycans are believed to stabilize protein conformation and protect proteins from proteolysis in the cells, both of which can potentially prevent the degradation of proteins in the environments(Varki 1993). In contrast, some features of glycoproteins may negatively influence protein preservation. For example, bacteria can

recognize specific glycans on the cell surface of a host, which helps them infect the host organisms(West 1986; Varki 1993).

It is not entirely clear what the impact of glycosylation is during protein degradation. The glycosylated modifications usually studied in molecular biological research are mediated by enzymes, and are postal translational modifications, as described above. Another reaction of protein and glycan, the Maillard reaction, refers to non-enzymatic reactions between reducing sugars and amino groups in proteins, or other compounds like lipid and nucleic acids. Maillard reactions proceed by formation of Schiff bases and Amadori rearrangement to produce advanced glycation end products (AGEs) and usually occur outside the cell (Singh et al. 2001). Early studies showed that proteins that were abiotically glucosylated turn over 100 times more slowly in seawater after glycation (Keil and Kirchman 1993). However, Liu et al. (2010) found no significant difference between a tetrapeptide and its AGE parallel.

There are several ways to investigate AGE compounds. In marine surface sediments, Nguyen and Harvey (2001) reported that 14% of the proteins were released after treatment with PTB (N-phenacylthiazolium), a reagent that reverses the glycation process and cleaves glucose-derived protein-protein cross-linking. The impact of glycosylation may vary in the marine environments depending on the size or structure of peptides or proteins. Fu et al.(1998) demonstrated that glycation produces carboxymethyllysine (CML) and oxidative products like 3, 4-dihydroxy-L-phenylalanine (DOPA) and dityrosine. So the presence of glycated products can be traced either by cleaving the glucose-derived cross-linking in the samples, or by detecting representative products like DOPA and dityrosine. Protein oxidation in biological samples can be induced either directly by reactive oxygen species or indirectly by secondary by-products of oxidative stress (Shacter 2000). There are many oxidants that can lead to protein oxidation (Dean

et al. 1997; Shacter 2000). Some oxidation end products are chemically stable, while others are reactive and cause further oxidative reactions. Dityrosine, an oxidative product of tyrosine, has been detected in the gut fluid of marine deposit-feeding invertebrates (Giessing and Mayer 2004). This raises the possibility that protein oxidation products might be released as waste products to natural waters, especially where cells undergo high oxidative stress. In early work (not reported here), however, we were unable to detect dityrosine in seawater.

1.2.3. Preservation of proteinaceous materials by mineral association

Association of organic matter with minerals is common in sediment and soils. In aquatic environments, the importance of this association in water column has been highlighted by the finding that POC flux is quantitatively related to the flux of minerals like opal, $CaCO_3$ and dust, which dominate the mineral input in the vast open ocean(Armstrong et al. 2002; Klaas and Archer 2002). Proteinaceous materials are frequently found in association with biogenic minerals (King 1974; Robbins and Brew 1990; Ingalls et al. 2003). Biogenic minerals like opal and $CaCO_3$ originate from diatom frustules and coccolithophore shells, among other sources. As part of phytoplankton cell structure, opal and $CaCO_3$ are major components of sinking particles in the deep ocean (Honjo et al. 2000). Using transmission X-ray microscopy (STXM) and carbon X-ray absorption near-edge structure (XANES) spectroscopy, Abramson et al. (2009) showed that amino acid-enriched organic matter was found within the frustule of diatoms, and suggested that it would be preserved during transit to the seafloor. But this source does not account for the large amount of proteinaceous material observed in sinking particles. In addition, it probably either adheres in between the inter-granular pores or is trapped inside the mesopores of mineral matrices, thus inaccessible to enzymatic attack, as described for sediments (Mayer 1994; Ransom et al. 1997). Ransom et al. (1997) suggested that organic matter in the form of micro-blebs could

act as a type of glue to hold sediment grains together. The sticky nature of organic matter, particularly when in the form of extracellular polysaccharide polymers, has been postulated as a control on the size of particles during vertical transport (Asper et al. 1992; Ransom et al. 1997). Armstrong et al. (2002) suggested that the degradation of this glue between sinking particles might cause disaggregation and thus an apparent decrease in sinking particle flux.

Macro- and micro- phytoplankton like diatom and coccolithophores have long been known as major primary producers in the open ocean, and are responsible for much of the material exported into the deep ocean, because of their size and mineral skeletons. However, recent research highlights the importance of pico-phytoplankton like cyanobacteria to the export of particulate organic matter. Recent studies in oligotrophic gyres have suggested that pico- and nano-plankton contribute a large portion of the particulate organic carbon export in these regions(Richardson and Jackson 2007; Lomas and Moran 2011). Lomas and Moran (2011) showed that cyanobacteria and other nanoplankton accounted for about one third of the total POC flux. Cyanobacteria-derived organic matter was also found to account for the majority of proteins that were identified in deep ocean suspended particles (Dong et al. 2010). These studies highlight the importance of pico-phytoplankton to particulate export, which was long thought unimportant due to the lack of appropriate observation methods.

1.2.4. Environmentally mediated enzymatic peptide hydrolysis

Degradation of proteinaceous materials in nature largely depends on extracellular enzymatic peptide hydrolysis, because most proteinaceous materials are inaccessible to microbial cells, which have membranes that only allow molecules of less than 600 Da to pass through (Hollibaugh and Azam 1983; Weiss et al. 1991). Therefore, most of proteinaceous materials need

to be hydrolyzed extracellularly before taken up by microbial cells. Most of our understanding of extracellular hydrolysis of proteinaceous material has been acquired through studies using synthetic analogs of amino acids or peptides. Both fluorescently labeled and isotopically-labeled amino acids and small peptides have historically been used to estimate peptide lability (e.g., (Hollibaugh and Azam 1983; Somville and Billen 1983; Hoppe et al. 1993). More recently, fluorescent Lucifer Yellow Anhydride (LYA) peptide derivatives were developed; this method allowed hydrolysis rates of actual peptide structures to be measured, but also allowed identification of hydrolysis products (Pantoja et al. 1997). Using this technique, peptide hydrolysis has been evaluated in estuaries (Mulholland et al. 2003), surface microlayers (Kuznetsova and Lee 2001), coastal seawater (Pantoja et al. 1997; Pantoja and Lee 1999), and an oxygen minimum zone (Pantoja et al. 2009). However, most of these studies used LYA peptides synthesized from alanine only to estimate hydrolysis of DCAA, and this linkage might not be representative of peptide linkages in nature. This could bias the measured rate and degradation pattern of natural DCAA, since hydrolysis rates depend on the size and chemical structure of the peptide (Pantoja and Lee 1999; Liu et al. 2010).

With this problem in mind, Liu et al. (2010) synthesized a new peptide analog, AVFA, which is a peptide fragment found in the common protein, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo), and is thus potentially more representative of natural DCAA. This and previous work cited above has shown that extracellular enzymes show preferences for particular linkages within a peptide. For example endopeptidases that preferentially target linkages within polypeptide chains, may be more important during the early degradation of organic matter, while exopeptidases that act on the terminal ends of polypeptide chains and may be more important at the intermediate and late stage of degradation (Berges and Mulholland

2008). Consistent with this, hydrolysis rates of tetraalanine are two orders of magnitude higher dialanine (Pantoja and Lee 1999).

Estuarine ecosystems are important transit points for the delivery of terrestrial organic matter into marine environments. Sharp chemical and biological gradients are commonly observed where fresh and saline water meet in the estuary. Diverse inputs and their intensive interactions with each other cause complicated nitrogen cycling in the estuarine area. During the river flux in the estuarine area, intensive sediment resuspension, rapid growth of phytoplankton as well as many other biogeochemical processes make the nitrogen cycling even more complicated in this type of ecosystem. Consequently, our understanding of nitrogen cycling in the estuary is very limited even though it has been frequently investigated previously (Mayer and Townsend 1996; Mulholland et al. 2003; Liu et al. 2010; Bronk et al. 2010). A better understanding of transport and dynamics of nitrogen compounds, especially organic nitrogen compounds in the estuarine regions are desired.

1.3. Thesis objective and organization:

In this thesis, preservation of proteinaceous materials was studied with the following perspectives: 1) to further understand the distribution, structure, and degradation of bacterial membrane-associated proteinaceous materials in marine environments, 2) to better understand the importance of mineral association with organic matter, and 3) to better understand extracellular peptide hydrolysis in regulating the degradation of proteinaceous materials in nature.

In Chapter Two, surface layer protein, a unique membrane glycoprotein from the cyanobacteria *Synechococcus* was studied as an example of glycoprotein, with the purpose of

better understanding how glycosylation changes its liability, Degradation of the S-layer protein was compared with membrane protein from another *Synechococcus* species with no S layer.

In Chapter Three, mineral association with organic matter was investigated using electron microscopy and chemical analysis, which provided information on chemical composition of marine detrital organic matter at micro- to nano-scales. A new type of authigenic mineral formation was discovered associated with cyanobacteria-derived organic matter. Cyanobacteria-derived organic matter can apparently adsorb Mg and Si from seawater. This process drives the authigenic formation of a Mg-Si enriched amorphous mineral in the seawater. This unique mineral is present throughout the water column and has the potential to facilitate the export of pico-phytoplankton derived organic matter into deeper ocean, as discussed above. This type of organic matter -mineral association may also promote the slower degradation of organic matter, which further facilitates the transfer of cyanobacteria-derived organic matter into the deep ocean.

In Chapter Four, we created a new fluorescent peptide analog, LYA-labeled alanine-valine-phenylalanine-alanine (LYA-AVFA), to evaluate extracellular peptide hydrolysis in the James River estuary and adjacent lower part of the Chesapeake Bay. The mechanism of proteinaceous material degradation was surveyed across an estuarine transect with a focus on how environmental parameters affect the extracellular enzymatic hydrolysis of proteinaceous materials. We also compared hydrolysis of LYA-AVFA with that of LYA tetraalanine (LYA-ALA_4) to examine how differences in peptide structure influence hydrolysis patterns, because extracellular enzymes may show a preference for particular peptide linkages, and these may vary depending on environmental conditions.

References:

Abramson, L., S. Wirick, C. Lee, C. Jacobsen, and J. A. Brandes. 2009. The use of soft X-ray spectromicroscopy to investigate the distribution and composition of organic matter in a diatom frustule and a biomimetic analog. Deep Sea Research Part II 56: 1369-1380, doi:10.1016/j.dsr2.2008.11.031

Aplin, J. D., and R. C. Hughes. 1982. Complex carbohydrates of the extracellular matrix structures, interactions and biological roles. Biochimica et biophysica acta 694: 375-418.

Armstrong, R. A., C. Lee, J. I. Hedges, S. Honjo, and S. G. Wakeham. 2002. A new , mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. Deep Sea Research Part II 49: 219-236.

Arnosti, C. 2004. Speed bumps and barricades in the carbon cycle: substrate structural effects on carbon cycling. Marine Chemistry 92: 263-273, doi:10.1016/j.marchem.2004.06.030

Asper, V. L., W. G. Deuser, G. A. Knauer, and S. E. Lohrenz. 1992. Rapid coupling of sinking particle fluxes between surface and deep ocean waters. Nature 357: 670-672.

Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The Ecological Role of Water-Column Microbes in the Sea. Marine Ecology Progress Series 10: 257-263.

Benner, R. 2002. Chemical composition and reactivity, p. 59-90. In C.A. Carlson and D.A. Hansell [eds.], Biogeochemistry of Marine Dissolved Organic matter. Academic Press.

Berges, J. A., and M. R. Mulholland. 2008. Enzymes and cellular N cycling, p. 1385-1444. In D.G. Capone, D.A. Bronk, and M.R. Mulholland [eds.], Nitrogen in the Marine Environment. Elsevier/Academic.

Berman, T., and D. Bronk. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquatic Microbial Ecology 31: 279-305, doi:10.3354/ame031279

Borch, N. H., and D. L. Kirchman. 1999. Protection of protein from bacterial degradation by submicron particles. Aquatic Microbial Ecology 16: 265-272.

Bronk, D. A., Q. N. Roberts, M. P. Sanderson, E. A. Canuel, P. G. Hatcher, R. Mesfioui, K. C. Filippino, M. R. Mulholland, and N. G. Love. 2010. Effluent organic nitrogen (EON): bioavailability and photochemical and salinity-mediated release. Environmental Science & Technology 44: 5830-5, doi:10.1021/es101115g

Cho, W., and R. V. Stahelin. 2005. Membrane-protein interactions in cell signaling and membrane trafficking. Annual review of Biophysics and Biomolecular Structure 34: 119-51, doi:10.1146/annurev.biophys.33.110502.133337

Cowie, G. L., and J. I. Hedges. 1992. Sources and reactivities of amino acids in a coastal marine environment. Limnology and Oceanography 37: 703-724, doi:10.4319/lo.1992.37.4.0703

Dean, R. T., S. Fu, R. Stocker, and M. J. Davies. 1997. Biochemistry and pathology of radical-mediated protein oxidation. The Biochemical Journal 324: 1-18.

Dong, H.-P., D.-Z. Wang, M. Dai, and H.-S. Hong. 2010. Characterization of particulate organic matter in the water column of the South China Sea using a shotgun proteomic approach. Limnology and Oceanography 55: 1565-1578, doi:10.4319/lo.2010.55.4.1565

Engel, A., and H. E. Gaub. 2008. Structure and mechanics of membrane proteins. Annual Review of Biochemistry 77: 127-48, doi:10.1146/annurev.biochem.77.062706.154450

Fu, S., M.-X. Fu, J. W. Baynes, S. R. Thorpe, and R. T. Dean. 1998. Presence of dopa and amino acid hydroproxides in proteins modified with advanced glycation end products (AGEs): amino acid oxidation products as a possible source of oxidative stress induced by AGE proteins. Biochemical Journal 330: 233-239.

Fuhrman, J. A., and T. M. Bell. 1985. Biological considerations in the measurement of dissolved free amino acids in seawater and implications for chemical and microbiological studies. Marine Ecology Progress Series 25: 13-21.

Giessing, A. M. B., and L. M. Mayer. 2004. Oxidative coupling during gut passage in marine deposit-feeding invertebrates. Limnology and Oceanography 49: 716-726, doi:10.4319/lo.2004.49.3.0716

Hedges, J. I., G. Eglinton, P. G. Hatcher, D. L. Kirchman, C. Arnosti, S. Derenne, R. P. Evershed, and I. Ko. 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Organic Geochemistry 31: 945-958.

Hitchen, P. G., and A. Dell. 2006. Bacterial glycoproteomics. Microbiology 152: 1575-80, doi:10.1099/mic.0.28859-0

Hollibaugh, J. T., and F. Azam. 1983. Microbial degradation of dissolved proteins in seawater. Limnology and Oceanography 28: 1104-1116.

Honjo, S., R. Francois, S. Manganini, J. Dymond, and R. Collier. 2000. Particle fluxes to the interior of the Southern Ocean in the Western Pacific sector along 170W. Deep Sea Research Part I 47: 3521-3548.

Hoppe, H.-G. G., H. Ducklow, and B. Karrasch. 1993. Evidence for dependency of bacterial growth on enzymatic hydrolysis of particulate organic matter in the mesopelagic ocean. Marine Ecology Progress Series 93: 277-283, doi:10.3354/meps093277

Ingalls, A. E., C. Lee, and E. R. M. Druffel. 2003. Preservation of organic matter in mound-forming coral skeletons. Geochimica et Cosmochimica Acta 67: 2827-2841, doi:10.1016/S0016-7037(03)00079-6

Jiao, N., G. J. Herndl, D. a. Hansell, R. Benner, G. Kattner, S. W. Wilhelm, D. L. Kirchman, M. G. Weinbauer, T. Luo, F. Chen, and F. Azam. 2011. The microbial carbon pump and the oceanic recalcitrant dissolved organic matter pool. Nature Reviews Microbiology 9: 555-555, doi:10.1038/nrmicro2386-c5

Kaiser, K., and R. Benner. 2009. Biochemical composition and size distribution of organic matter at the Pacific and Atlantic time-series stations. Marine Chemistry 113: 63-77, doi:10.1016/j.marchem.2008.12.004

Keil, R. G., and D. L. Kirchman. 1993. Dissolved combined amino acids: Chemical form and utilization by marine bacteria. Limnology and Oceanography 38: 1256-1270, doi:10.4319/lo.1993.38.6.1256

King, K. 1974. Preserved amino acids from silicified protein in fossil Radiolaria. Nature 252: 690-692.

Klaas, C., and D. E. Archer. 2002. Association of sinking organic matter with various types of mineral ballast in the deep sea : Implications for the rain ratio. Global Biogeochemical Cycles 16: 1116-1129, doi:10.1029/2001GB001765

Knicker, H., A. W. Scaroni, and P. G. Hatcher. 1996. 13C and 15N NMR spectroscopic investigation on the formation of fossil algal residues. Organic Geochemistry 24: 661-669.

Kuznetsova, M., and C. Lee. 2001. Enhanced extracellular enzymatic peptide hydrolysis in the sea-surface microlayer. Marine Chemistry 73: 319-332, doi:10.1016/S0304-4203(00)00116-X

Laursen, A. K., L. M. Mayer, and D. W. Townsend. 1996. Lability of proteinaceous material in estuarine seston and subcellular fractions of phytoplankton. Marine Ecology Progress Series 136: 227-234.

Lee, C., and J. L. Bada. 1977. Dissolved amino acids in the equatorial Pacific, the Sargasso Sea, and Biscayne Bay. Limnology and Oceanography 22: 502-510.

Lee, C., and C. Cronin. 1984. Particulate amino acids in the sea: Effects of primary productivity and biological decomposition. Journal of Marine Research 42: 1075-1097.

Lee, C., S. G. Wakeham, and J. I. Hedges. 2000. Composition and Flux of particulate amino acids and chloropigments in equatorial Pacific seawater and sediments. Deep-Sea Research I 47: 1535-1568.

Liu, Z., M. E. Kobiela, G. A. McKee, T. Tang, C. Lee, M. R. Mulholland, and P. G. Hatcher. 2010. The effect of chemical structure on the hydrolysis of tetrapeptides along a river-to-ocean transect: AVFA and SWGA. Marine Chemistry 119: 108-120, doi:10.1016/j.marchem.2010.01.005

Lomas, M. W., and S. B. Moran. 2011. Evidence for aggregation and export of cyanobacteria and nano-eukaryotes from the Sargasso Sea euphotic zone. Biogeosciences 8: 203-216, doi:10.5194/bg-8-203-2011

Madigan, M. T., and J. M. Martinko. 2006. Brock Biology of Microorganisms, 11th ed. Pearson Education.

Mayer, L. M. 1994. Relationships between mineral surfaces and organic carbon concentrations in soils and sediments. Chemical Geology 114: 347-363.

Mayer, L., and D. Townsend. 1996. Lability of proteinaceous material in estuarine seston and subcellular fractions of phytoplankton. Marine Ecology Progress Series 136: 227-234.

McCarren, J., J. Heuser, R. Roth, N. Yamada, M. Martone, and B. Brahamsha. 2005. Inactivation of swmA Results in the loss of an outer cell layer in a swimming *synechococcus* strain. Journal of Bacteriology 187: 224-230, doi:10.1128/JB.187.1.224

McCarthy, M. D., J. I. Hedges, and R. Benner. 1998. Major bacterial contribution to marine dissolved organic nitrogen. Science 281: 231-234, doi:10.1126/science.281.5374.231

Mulholland, M. R., C. Lee, and P. M. Glibert. 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Marine Ecology Progress Series 258: 3-17.

Nagata, T., R. Fukudal, I. Koikel, K. Kogurel, and D. L. Kirchman. 1998. Degradation by bacteria of membrane and soluble protein in seawater. Aquatic Microbial Ecology 14: 29-37.

Nguyen, R., and R. H. Harvey. 2003. Preservation via macromolecular associations during Botryococcus braunii decay: proteins in the Pula Kerogen. Organic Geochemistry 34: 1391-1403, doi:10.1016/S0146-6380(03)00154-2

Nguyen, Reno T, and R. H. Harvey. 2001. Preservation of protein in marine systems : Hydrophobic and other noncovalent associations as major stabilizing forces. Geochimica et Cosmochimica Acta 65: 1467-1480.

Palenik, B., and J. a Koke. 1995. Characterization of a nitrogen-regulated protein identified by cell surface biotinylation of a marine phytoplankton. Applied and Environmental Microbiology 61: 3311-5.

Pantoja, S., and C. Lee. 1999. Peptide decomposition by extracellular hydrolysis in coastal seawater and salt marsh sediment. Marine Chemistry 63: 273-291, doi:10.1016/S0304-4203(98)00067-X

Pantoja, S., C. Lee, and J. F. Marecek. 1997. Hydrolysis of peptides in seawater and sediment. Marine Chemistry 57: 25-40.

Pantoja, S., P. Rossel, R. Castro, L. A. Cuevas, G. Daneri, and C. Córdova. 2009. Microbial degradation rates of small peptides and amino acids in the oxygen minimum zone of Chilean coastal waters. Deep Sea Research Part II 56: 1055-1062, doi:10.1016/j.dsr2.2008.09.007

Parson, R. T., M. Takahashi, and B. Hargrave. 1977. Biological Oceanographic Processes, 2nd ed. Pergamon.

Pogozelski, W. K., and T. D. Tullius. 1998. Oxidative strand scission of nucleic acids: routes initiated by hydrogen abstraction from the sugar moiety. Chemical Reviews 98: 1089-1108.

Ransom, B., R. H. Bennett, R. Baerwald, and K. Shea. 1997. TEM study of in situ organic matter on continental margins : occurrence and the “monolayer” hypothesis. Marine Geology 138: 1-9.

Richardson, T. L., and G. a Jackson. 2007. Small phytoplankton and carbon export from the surface ocean. Science 315: 838-40, doi:10.1126/science.1133471

Riethman, H. C., T. P. Mawhinney, and L. a Sherman. 1988. Characterization of phycobilisome glycoproteins in the cyanobacterium Anacystis nidulans R2. Journal of Bacteriology 170: 2433-40.

Robbins, L. L., and K. Brew. 1990. Proteins from the organic matrix of core-top and fossil planktonic foraminifera. Geochimica et Cosmochimica Acta 54: 2285-2292.

Roth, L. C., and H. R. Harvey. 2006. Intact protein modification and degradation in estuarine environments. Marine Chemistry 102: 33-45, doi:10.1016/j.marchem.2005.10.025

Schäffer, C., M. Graninger, and L. Boltzmann-institut. 2001. Review Prokaryotic glycosylation. Archaea 248-261.

Schäffer, C., and P. Messner. 2001. Glycobiology of surface layer proteins. Biochimie 83: 591-599.

Shacter, E. 2000. Quantification and significance of protein oxidation in biological samples. Drug Metabolism Reviews 32: 307-26, doi:10.1081/DMR-100102336

Singh, R., A. Barden, T. Mori, and L. Beilin. 2001. Advanced glycation end-products: a review. Diabetologia 44: 129-46, doi:10.1007/s001250051591

Sleytr, U. B., and T. J. Beveridge. 1999. Bacterial S-layers. Trends in microbiology 7: 253-60.

Smarda, J., D. Smajs, J. Komrska, and V. Krzyzanek. 2002. S-layers on cell walls of cyanobacteria. Micron 33: 257-277.

Somville, M., and G. Billen. 1983. A method for determining exoproteolytic activity in natural waters. Limnology and Oceanography 28: 190-193.

Tanoue, E., S. Nishiyama, M. Kamo, and A. Tsugita. 1995. Bacterial membranes : Possible source of a major dissolved protein in seawater. Geochimica et Cosmochimica Acta 59: 2643-2648.

Taylor, M. T., and K. Drickamer. 2002. Introduction to Glycobiology, Oxford University Press.

Varki, A. 1993. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3: 97-130.

Voet, D., and J. G. Voet. 2005. Biochemistry, 3rd ed. John Wiley and Co.

Wakeham, S. G., C. Lee, J. I. Hedges, P. J. Hernes, and M. L. J. Peterson. 1997. Molecular indicators of diagenetic status in marine organic matter. Geochimica et Cosmochimica Acta 61: 5363-5369, doi:10.1016/S0016-7037(97)00312-8

Weiss, M. S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz. 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science 254: 1627-30.

West, C. M. 1986. Current ideas on the significance of protein glycosylation. Molecular and Cellular Bochemistry 72: 3-20.

Yamada, N., and E. Tanoue. 2003. Detection and partial characterization of dissolved glycoproteins in oceanic waters. Limnology and Oceanography 48: 1037-1048.

Figures:

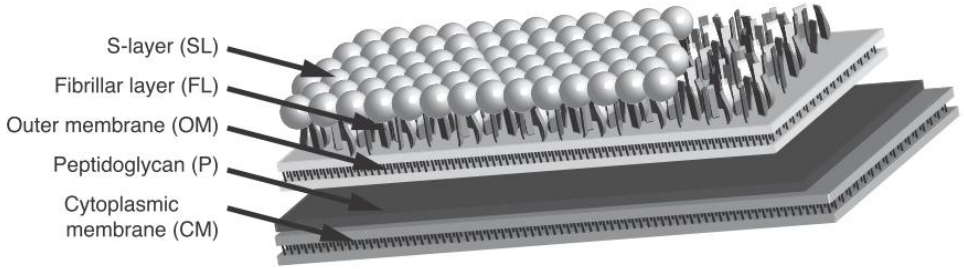

Figure 1.1. Model of Synechococcus sp. Strain WH8102 cell envelope structure (McCarren et al., 2005)

Chapter Two

The role of surface layer proteins in the degradation of a photosynthetic prokaryote, the cyanobacterium *Synechococcus sp.*

Abstract: The accumulation of refractory prokaryotic cell membranes has been suggested as a possible source of dissolved and particulate organic matter in the deep ocean. Surface layer protein (S-layer) is a specific membrane glycoprotein that is widely found in both Eubacteria and Archaea. This heavily glycosylated protein covers the outermost cell surface in a regularly ordered planar crystalline structure. With special attention to the geochemical importance of S-layer in seawater, we studied the degradation of two species of marine cyanobacteria, *Synechococcus sp.* CCMP2370 and CCMP1334, with and without S-layer structure, respectively. Based on evidence from bulk chemical and molecular analysis as well as electron microscopy, we found that glycoproteins like S-Layer were as rapidly degraded as other non-glycosylated proteins. We also studied degradation of both *Synechococcus* species after they had been treated with buffers that can strip the S-layer from the cell surface. Stripped cells of CCMP2370 lost their cell membrane structure and degraded more rapidly and became more enriched in D-amino acids, while stripped CCMP1334 cells maintained their membrane structures and degradation was not enhanced. These results suggest that the rigidity of membrane glycoproteins like S-layer has limited contribution to preferential preservation of these proteinaceous materials during the degradation of the cyanobacterium *Synechococcus*, but S-layer structure functions as a defensive

barrier on the cell membrane like peptidoglycan to protect cyanobacterial cellular materials from degradation.

2.1.Introduction

Proteinaceous materials are major components of marine organic matter, and can account for up to 80% of the organic nitrogen in marine organisms (Lourenc et al. 1998). Although proteinaceous materials are generally considered to be labile, some of them are resistant to heterotrophic attack and remineralization; these resistant compounds may become enriched in seawater and particles during decomposition (Wakeham et al. 1997; Lee et al. 2000).

Recent studies have suggested that bacterial membrane components like membrane proteins and peptidoglycan, are selectively preserved in marine detrital organic matter (Tanoue et al. 1995; McCarthy et al. 1998; Kaiser and Benner 2008). However, the mechanisms how these components are preserved is not very clear in present, especially for membrane proteins. Two potential mechanisms of preservation of bacterial membrane proteins were hypothesized in these studies, chemical modification and non-covalent interaction between membrane components.

One of the characteristic features of prokaryotic membrane proteins is their extensive post-translational modification after transcription (Schäffer et al. 2001b). Glycoproteins are proteins that are covalently bonded to a variety of glycans. Glycoproteins are widely founded in the cell membranes of both prokaryotes and eukaryotes and are the most common post-translational modification of proteins in vivo (Messner 1997; Schäffer et al. 2001b). Glycosylation is thought to stabilize protein conformation and protect proteins from enzymatic attack within the cell, thus this process may also prevent degradation of proteins in natural environment. In lab experiments, glycosylation reduced turnover of proteins in seawater by a

factor of 100 (Keil and Kirchman 1993). However, Liu et al. (2010) found no significant difference between degradation of Serine-tryptophan-glycine-alanine (SWGA) and galactose-SWGA. The apparent contradiction indicates the lack understanding of mechanisms involved in the degradation of glycosylated proteinaceous materials as well as their role in geochemical cycling of marine organic matter.

The surface of membrane proteins are relatively hydrophobic and can be tightly associated with the membrane by non-covalent binding. Membrane proteins will be surrounded by some resistant structures like lipid bilayer and peptidoglycan (Hedges et al. 2000). These structures can capsulate membrane proteins and thus prevent the attack of hydrolytic enzymes or other chemical reactants such as hydroxyl radicals (Pogozelski and Tullius 1998). Protection of proteins from enzyme hydrolysis by hydrophobic interaction has been demonstrated to be an important process to preserve proteins from degradation (Borch and Kirchman 1999; Nguyen and Harvey 2003).

However, growing evidence suggest that bacterial membrane proteins are highly diverse in their functions and locations on the cell membrane. How these proteins behave during the remineralization of bacterial cellular materials is poorly known.

With the full understanding at this point, we chose one membrane protein specifically to study the behavior of individual protein during the degradation of bacterial cells. The model protein here is Surface Layer protein from the *Synechococcus sp.* CCMP2370. S-layer is a specific membrane glycoprotein found in many Eubacteria and Archaea, which covers the outermost cell surface and consists of a regularly ordered, planar array of paracrystalline proteinaceous subunits of 1 or 2 proteins (Sleytr and Beveridge 1999). S-layer proteins can

account for up to 15% of the total proteins in bacteria; they are heavily glycosylated with an overall degree of glycosylation of 1-15% (Schäffer and Messner 2004). S-layers have been found in cyanobacteria and other common bacteria adapted to saline environments (Smarda et al. 2002). Since 1972, S-layers have been found in 23 species of cyanobacteria. As the outermost layer of prokaryotic cells, S-layers play a role in protecting cells, as scaffolding for enzymes, and in excluding transport of large molecules. The unique ultrastructure of the S-layer is located on top of the outer membrane directly beneath the polysaccharide sheath. Thus S-layer structures are among the first structures exposed to the ambient environment when a cell begins to degrade. Very little research exists on marine S-layers; at present, the only marine S-layers have been identified in various species of the cyanobacterium *Synechococcus* (Brahamsha 1996; McCarren et al. 2005)

Two strains of *Synechococcus sp*. CCMP2370 and CCMP1334 were chosen for this study. *Synechococcus* CCMP2370, also known as WH8102, is motile, and favors strong light intensity. Synechococcus CCMP1334, also known as WH7803, is a non-motile species that is adapted to weak light. *Synecococcoccus* sp. CCMP2370 was used as a representative of species with S-Layer structures in their outer membranes, while CCMP1334 is a representative of non-S-layer species (Brahamsha 1996). *Synechococcus* S-layer structures have been relatively well characterized in previous studies (Schultze-Lam et al. 1992; Brahamsha 1996; McCarren et al. 2005; McCarren and Brahamsha 2009). A heavily glycosylated 130 kDa S-layer protein is enriched in *Synechococcus* S-layer structures and present in the gene sequences of this species. Here we report results of degradation experiments using *Synechococcus* CCMP2370 (S layer) and CCMP1334 (no S layer).

2.2. Methods

2.2.1. Culture preparations: Strains of the *Synechococcus* sp. CCMP2370 and CCMP1334 (Bigelow Laboratory) were grown axenically in L1 medium prepared with 0.2 μm filtered coastal seawater (Stony Brook Harbor). The cultures were incubated in 500-mL sterile glass flasks at 20ºC with a 16:8 illumination cycle of ~10mE/m^2/sec. Cells were harvested at the end of the exponential growth stage or early stationary stage and frozen till analysis. Chlorophyll *a* concentrations in collected cultures were estimated from the whole cell spectra from 600nm to 800nm using a Perkin Elmer UV-VIS spectrophotometer (Collier and Grossman 1992).

2.2.2. Isolation of S-layer from CCMP2370: S-layer was isolated from frozen *Synechococcus* cells by optimizing the method of Brahamsha (1996). Briefly, 450ml of cultures of CCMP2370 were harvested by centrifugation at 5250 × g at 4 ºC for 40 min. The resulting pellets were washed with sterile artificial seawater and then resuspended in a stripping buffer (50 mM Tris·HCl / 50 mM Na_2EDTA / 15% sucrose, pH = 7.25). After immersion in the stripping buffer at 4ºC overnight, stripped cells were collected by centrifugation at 6300 × g for 12min, and rinsed twice with sterile artificial seawater. The supernatant with S-layer inside was ultrafiltered using 3 kDa centrifugal ultrafiltration vials to separate stripping buffer from the S-layer components. The retentate (S-Layer) was rinsed twice with sterile artificial seawater. The S-layer and the stripped cells were then used in the following experiments.

2.2.3. Degradation experiments of stripped and intact S*ynechococcus*: Intact CCMP2370 and 1334 were prepared by harvesting 450 ml of *Synechococcus* culture by centrifugation as above. Although absent in S-layer, stripped CCMP1334 cells from 450 ml culture were also prepared as described above for S-layer isolation. Pellets from all 4 cell treatments were washed twice with sterile artificial seawater, and each type of sample was

resuspended into 200 ml of 200-mesh filtered coastal seawater collected from Stony Brook Harbor at high tide. All cells were incubated in the dark at 22 - 24 ºC for 30 days. A Blank of 200 ml of 200-mesh filtered coastal seawater was incubated in parallel. Subsamples were collected from each incubation after 0, 2, 7, 15 and 30 d. After centrifugation at 5250 × g for 40 min, both the supernatants and pellets were collected, which are referred as surrounding seawater and particles hereafter.

2.2.4. Degradation of commercially available proteins: Ovalbumin (OVA) is a glycoprotein with sugar chains accounting for 3% of its mass, while, bovine serum albumin (BSA) is not glycosylated. Two mgL^{-1} of these commercially available proteins were resuspended in 200 mL of 200-mesh pre-filtered coastal seawater (Stony Brook Harbor) and then incubated in dark. Subsamples were collected during the incubation after 0, 2, 5 and 10 d. Detrital material was rinsed with sterile artificial seawater and collected using 3 kDa centrifugal ultrafiltration.

2.2.5. Gel electrophoresis: Proteins were extracted from the cell pellets and sorted by their size and charge on gel electrophoresis following the methods of (Saijo and Tanoue 2005). Basically, cell pellets collected from each subsampling were resuspended in 62.5 $mmolL^{-1}$ Tris / 2% SDS / 5% 2-mercaptoethanol and boiled for 3 min. After cooling to room temperature, urea was added to a concentration of 8 $molL^{-1}$. The mixtures were stored at 4 ºC overnight, and then mixed with Laemmli sample buffer (Bio-Rad) and boiled for 10 min. After cooling on ice, samples were loaded on 4-20% Ready Gel Tris-HCl gels (Bio-Rad) using a Bio-Rad Mini-PROTEAN system at 20 mA constant AMP. Parallel gels were prepared at the same time; one was stained with Coomassie Brilliant Blue (CBB), and the other one with periodic acid-Schiff stain (PAS). After rinsing with distilled water, the CBB gel was stained for 30 min with CBB

G250 staining buffer at 100gL^{-1} CBB / 10 % acetic acid / 45 % methanol in distilled water (DI), and then rinsed with destaining solution of 10 % acetic acid / 10 % methanol in DI for another 30 min. The PAS gels were stained using Glycoprotein Staining Kits (Pierce) as suggested in the manual protocol. The gel was first rinsed with 50% methanol in DI for 30 min on a shaker, and then rinsed twice with 3% acetic acid in DI for 10 min each. Oxidizing solution (25 mL) was added after the rinse and shaken for 15 min. The gel was then washed three times with 3% acetic acid for 5 min each. Staining solution (25 mL) was then added followed by 25 mL reducing solution and washing thoroughly with 3% acetic acid.

2.2.6. Amino acid analysis: Amino acids were measured in both particles and surrounding seawater from degradation incubations mostly following the method of (Fitznar et al. 1999). Subsamples from each incubation were collected in time series and centrifuged at 5250 × g for 40 min. The pellets were analyzed as particulate amino acids (PAA). The supernatants were collected for analysis of total dissolved amino acids (TDAA) and dissolved free amino acids (DFAA). Both PAA and DTAA were hydrolyzed in 6N HCl (99.999% trace metal basis, Sigma-Aldrich). Ascorbic acid (10μM, final concentration; reagent grade, Sigma-Aldrich) was added to the acidified samples to prevent oxidation. Samples were sparged with dry nitrogen gas for about 15 sec before sealing in glass vials. The prepared samples were hydrolyzed at 110°C on a heating block for 20 hours, and then dried by a stream of N_2. Dried samples were rinsed with drops of DI water and dried again to remove remaining acid. Dried samples were resuspended into DI: methanol of 6: 4 for chromatographic analysis. DFAA samples were directly diluted with DI and methanol to the ratio of 6:4 for chromatographic analysis.

After pretreatment, PAA, TDAA and DFAA were separated on a 5μm Alltima C18 column (length: 250mm; ID: 4.6 mm) with 25 mmol L^{-1} sodium acetate (pH = 7) and methanol :

acetonitrile (13:1, v/v) as mobile phases. Amino acid entantiomers were identified using fluorescent detection (Ex/Em = 330/445 nm) after online chiral derivatization with 3 gL^{-1} *o*-phthaldialdehyde (OPA)(Sigma-Aldrich) and 5 gL^{-1} N-isobutyryl-L-cysteine (>97%, Fluka) in 0.8 $molL^{-1}$ boric acid buffer with a pH of 10.5. For quantification, we used mixed standards prepared by mixing a Pierce H L-amino acid standard with individual D-amino acid as well as beta-alanine (BALA), and gamma-aminobutyric acid (GABA) standards (Sigma-Aldrich). Hydrolysis-derived racemization was calibrated from all the relative abundance of individual D-amino acids (%D = D-amino acids / (D-amino acid + L-amino acid)) using table 1 from Kaiser & Benner (2005). Dissolved combined amino acids (DCAA) was calculated by the difference between TDAA and DFAA.

2.2.7. Electron microscopy: One-mL subsamples were taken from incubations and centrifuged at 4000 x g for 20 min. The pellets were enriched in 0.1 ml of supernatant. One drop of the sample was fixed on 300-mesh, carbon-coated copper grids for 60 s before being air-dried for transmission electron microscopy (TEM) analysis. Prepared grids were viewed on a JEOL JEM-1400 TEM or FEI BioTwinG2 TEM at an accelerating voltage of 120kV. This work was conducted at the Center of Functional Nanomaterials, Brookhaven National Laboratory, Upton, NY.

2.3. Results

2.3.1. Efficiency of S-Layer removal

We found only one major protein, 130 kDa from the stripping buffer using polyacrylamide gel stained with Coomassie Blue (Fig. 2.1d). In previous work, three major proteins 45kDa, 70 kDa and 130 kDa in size were stripped from CCMP2370 (Resch and Gibson

1983; Brahamsha 1996), with 45kDa and 70 kDa having lower concentrations. We did use a stripping buffer pH of 7.5, not 8 as in Brahamsha (1996), but it is unlikely that this accounts for the difference. In our work, the stripped pellets had a protein distribution identical to that of a whole cell SDS-digestion except for the absence of the 130 kDa protein (d1 and d2 in Fig. 2.1d). This suggests that the 130 kDa protein was the only major protein removed from *Synechococcus* CCMP2370 by the stripping procedure. McCarren et al. (2005) demonstrated that the 130kDa protein removed by EDTA stripping buffer is a glycoprotein required for the generation ofsurface layer structure in CCMP2370. Thus we deduce that only surface layer protein was removed by our stripping buffer from CCMP 2370. It is possible that other membrane components, e.g., pigments or polysaccharide sheath, may also be removed with the S-layer proteins; but these molecules were not detected by Coomassie Brilliant Blue after SDS-PAGE.

Extraction of the S-layer structure caused visible changes to the outermost layer of the cell. Under TEM, fresh CCMP 2370 cells have an intact and smooth surface (Fig. 2.1a). In comparison, the stripped CCMP2370 cells still maintain their shape, but the surface of the cell was rough and not as distinct (Fig. 2.1b). The stripping treatment did not cause visible damage on the surface of CCMP1334 as shown in Fig. 2.1c; the surface was still smooth after treatment, indicating that the buffer apparently only targets S layers of cell membranes. Thus, the stripping treatment appears to be an efficient way to remove S-layer proteins from *Synechococcus* cells, while leaving other parts of the cells intact. This allows us to compare degradation of cells with and without S-layer protection.

2.3.2. Changes in CBB-stainable and PAS-stainable materials during degradation of commercial protein and S layer-containing CCMP2370

CBB selectively stains molecules with basic amino acids like ARG, HIS and LYS, while PAS targets molecules with diol functional groups, like sugars. Therefore, proteinaceous materials can be sorted and identified with CB stained gel electrophoresis, while glycoproteins with both peptide and sugar chains are stained by both CBB and PAS. Both OVA (glycoprotein) and BSA (non-glycosylated protein) decomposed rapidly in the 10-day seawater incubation, regardless of the differences in their extent of glycosylation, and both produced significant amounts of lower molecular weight products during the incubations (Fig. 2.2A). This finding indicates that glycoproteins can be as efficiently hydrolyzed as non-glycosylated proteins in coastal seawater.

Change of proteinaceous materials during the seawater degradation of stripped and intact CCMP2370 were traced with both CBB and PAS staining, representing proteins and glycosylated molecules (including glycoprotein), respectively (Fig. 2.2B and Fig. 2.2C). When cultured cells were added to seawater, changes in the protein distribution occurred to the stripped cells, but not the intact cells. Initially, intact CCMP2370 cells (Day 0 at B2 in Fig. 2.2B) in seawater had an identical protein distribution to that in the original culture, including the 130 kDa S-layer protein. In stripped CCMP2370 cells (Day 0 at B1 in Fig. 2.2B) in seawater, however, only two major proteins were identified, implying that a large amount of protein was lost once the cells were resuspended in seawater. During the incubation of both stripped and intact CCMP2370 cells for 30 days, all proteins rapidly decomposed, and no identifiable protein remained after 15 days in incubations.

The 130 kDa surface layer protein was the most abundant PAS stainable glycoprotein at the beginning of the incubation of intact CCMP2370 (C2 in Fig. 2.2C), and was rapidly decomposed along with other glyco-molecules and CBB-stainable proteins over the 30 d of

incubation. Glycoprotein was not observed at any time during stripped CCMP2370 incubations by PAS staining (C1 in Fig. 2.2C). However, a glycosylated molecule of 20 kDa was observed in both stripped and intact samples at the end of incubation. This molecule could not be detected using CBB staining, suggesting that it was not glycoprotein. It may be molecules enriched in glycan but in absence of amino acids.

2.3.3. Change of morphology and elemental compositions during incubation of *Synechococcus sp.*

Synechococcus cell morphology after 2 d of incubation was visualized using transmission electron microscopy (TEM). The cells tended to aggregate together in all four incubations (Fig. 2.3). Cell membranes were still apparent in all cases except cells of CCMP2370 with S-layer stripped off (see arrows in Figure 2.3b-d). However, much of the cellular material lost its integrity and was no longer distinct. In the stripped CCMP2370 cell incubation (Fig. 2.3a), only indistinct cellular organic matter was observed. This difference in appearance suggests that the stripping treatment removed the outer membrane from CCMP2370, but not from the CCMP1334 cells, which had no S layer.

2.3.4. Changes in dissolved and particulate amino acids with incubation time

Fresh cultures of each strain of *Synechococcus* were split in half for use in intact and stripped incubations. The original cell concentrations were estimated from the chlorophyll concentrations in the cultures, assuming a constant Chl/Carbon. Cultures of the two strains were adjusted to have similar chlorophyll concentrations, 1.2 ± 0.1 μg/mL, so that the original cell concentrations from each tank were identical before the stripping treatment, when a large amount of amino acid was lost as a result of S-layer removal and cell lysis. Both dissolved and

particulate amino acids were measured in all incubations. Total particulate amino acid (PAA) concentrations were initially 440 $\mu molL^{-1}$ in seawater with intact CCMP2370, and decreased exponentially over the next 30 d to a concentration of 18 $\mu molL^{-1}$. In contrast, lower PAA concentrations were observed at the beginning of the stripped CCMP2370 incubation, probably due to losses during the stripping treatment and subsequent rinses. However, PAA concentrations in these incubations also decreased rapidly to a concentration of 12 $\mu molL^{-1}$after 30 d. In spite of the rapid loss of PAA concentrations over time, PAA compositions did not vary much over time in either stripped or intact cell incubations of CCMP2370 (Fig. 2.4e,f). No appreciable difference in composition was observed between stripped and intact cells across the incubation. ALA and GLY were the most abundant PAA followed by GLU, GLY and LEU. ALA and GLY progressively increased and GLU decreased during the 30-d incubation.

Initial total dissolved amino acid (TDAA) concentrations ranged from 3.0 (CCMP2370) to 10.5 (CCMP1334) $\mu molL^{-1}$ in seawater of intact cell incubations. In contrast, TDAA concentrations in seawater from stripped CCMP2370 and CCMP1334 incubations were similar but somewhat lower, 1.3 and 1.5 $\mu molL^{-1}$, respectively. In intact but not stripped cell incubations, most of the difference in initial TDAA concentrations was due to free glutamic acid (Fig. 2.4b,d), resulting in DFAA concentrations of 2.3 (CCMP2370) and 6.7 (CCMP1334) $\mu molL^{-1}$. Accordingly, initial combined amino acid (DCAA) concentrations were more similar than dissolved free amino acids (DFAA) in the intact and stripped cell incubations. During the incubation of intact cells, DFAA were rapidly remineralized in the first 2 d, but DCAA increased to 4.0 (CCMP2370) and 7.5 $mmolL^{-1}$ (CCMP1334) after 7 d, before decreasing to 0.93 and 3.18 $mmolL^{-1}$, respectively, after 30 d. The increase may be released from particles, which was

rapidly decomposed in the following incubations. In stripped cell incubations, DFAA remained low, but DCAA concentrations peaked at 15 d, somewhat longer than in intact cell incubations.

TDAA in incubation seawater experienced larger variations in composition over time than did PAA (Fig. 2.4a-d). GLY was usually the most abundant TDAA, and ASP and ALA were also major components. In intact samples of both CCMP2370 and CCMP1334, GLU was initially very high, with relative abundances of 40% and 58%, respectively. As mentioned above, most of them were free glutamic acid, and they lost very quickly during the incubation. Mol% GLY in CCMP2370 incubations progressively increased from less than 20 to more than 50% after 30-d. In the CCMP1334 degradation, mol% GLY increased from less than 10% to more than 20%, although it was decreased after 7 d in intact CCMP1334. The non-protein amino acid, BALA, accumulated during all incubations except that of intact CCMP1334. Another non-protein amino acid, GABA, was below the detection limit for most of the samples during the degradation.

2.3.5. Change in D-amino acid concentrations in particles and surrounding seawater

D-ASP, D-GLU, D-SER and D-ALA concentrations were calculated in both particles and surrounding seawater during the incubations (Fig. 2.5). In particles, %D (=100 * D / (D + L)) of these four amino acids was relatively lower, ranging from 1.3% to 10.1%, than those in dissolved amino acids (Fig. 2.5e and 2.5f). During the incubation of stripped CCMP2370, D% of all the four D-amino acids generally increased with time (Fig. 2.5e). During the incubation of intact CCMP2370, however, this trend was observed only for D-ALA, and to a lesser degree than in stripped cells (Fig. 2.5f). Thus, the proportion of D-amino acids increased with time in stripped cells more than in intact cells. The % D for ALA was greater than other D-amino acids in both

stripped and intact particles after 30 d. D-amino acids were more enriched in TDAA than in particles during the incubations; %D ranged from 0.9 to 44%, which is consistent to those observed in the surface and deep seawater. The %D of each amino acid was generally lower at the beginning of the incubations. Exceptions was observed in TDAA of intact cells, with D-GLU and D-ALA enriched in the intact CCMP2370 and D-SER and D-ALA enriched in the intact CCMP1334 (Fig. 2.5b and 2.5d). Noticeably, these high amounts of D-amino acids can not stay in seawater, but were subject to rapid decomposing in the following two days. This is coincidence with the observation that high amount of free amino acids was observed. Both of them may result from the releasing of labile compounds at the beginning of intact cell incubations. Beside the initial high concentration of D-GLU and D-ALA, both stripped and intact CCMP2370 showed clear increase of %D of TDAA with time for each amino acid, and stripped samples were always more enriched in D-amino acids than their intact counterparts (Fig. 2.5 a &b). Incubations of stripped cells in CCM1334 also showed clear increases in %D of TDAA in the first 15 d (Fig. 2.5c), but followed with a drop at the 30 d. The increasing %D was much less clear in incubations of intact CCMP1334, where only ASP clearly increased in both incubations. D% in CCMP1334 had less difference between stripped and intact subsamples (Fig. 2.5c and 2.5d). The accumulation of D-amino acids during degradation is consistent with the finding that D-amino acids are enriched in dissolved organic matter from the deep seawater, where substantial decomposition of dissolved organic matter is expected (Lee and Bada 1977; McCarthy 1998). However, large variations of %D in TDAA were observed in different species, suggesting that the enrichment of D-amino acids is not identical under all conditions, but rather species specific. Moreover, the higher %D in stripped CCMP2370 may result from the more

degraded cells after removal of S-layer. For CCMP1334 in absence of S-layer, this difference is less.

2.3.6. Statistical analysis

Degradation states were estimated as in (Dauwe et al. 1999) using amino acid composition of particles over the sampling period (Fig. 2.6a). The degradation index (DI) in both stripped and intact CCMP2370 decreased over time, indicating that the incubating cyanobacterial cells indeed degraded over time. During the 30-d incubation, DI of stripped cells decreased faster to a higher degradation state in comparison to intact cells, suggesting that stripped cells degraded faster than intact cells.

Principal component analysis of %D of ASP, GLU, SER and ALA was applied to PAA data from all the subsamples during the incubations of CCMP2370 (Fig. 2.6b). The factor loadings of each subsample from the first principal component were plotted against incubation time (Fig. 2.6b); an increasing loading with time of PAA in stripped CCMP2370 was clearly observed from between 2 d and the end of the incubation. PAA in intact incubations had a similar trend, but to a much smaller extent, suggesting that the stripped cells were subject to more intensive degradation during the 30-d incubation compared with its intact counterpart.

2.4. Discussion:

2.4.1. Enrichment of D-amino acids during the degradation of cyanobacteria

The enrichment of D-amino acids has been frequently reported in bacteria due to their presence in peptidoglycan, teichoic acids, lipopeptides and siderophores (Schleifer and Kandler 1972; Kaiser and Benner 2008 and references therein). Some of these compounds, like

peptidoglycans, add rigidity to the cell membrane, and because of their resistance to decomposition, are accumulated during decomposition of bacterially derived organic matter (Nagata et al. 2003). The accumulation of D-ALA in particulate organic matter with the degradation of CCMP2370 may thus result from the preferential preservation of peptidoglycan during the degradation of cyanobacteria. However, particulate D-ASP, D-GLU and D-SER do not simply increase with time. This may due to the non-simultaneous degradation of peptidoglycan components as suggested by degradation experiments with purified peptidoglycan (Nagata et al. 2003). The %D values of amino acids in the incubation experiments here were generally lower than those reported for suspended particles in natural freshwater and marine environments (Dittmar et al. 2001), suggesting that the incubating *Synechococcus* cells are still fresher than natural particles.

During the degradation experiments, all four D-amino acids measured were more enriched in dissolved organic matter than in particles. Enrichment of D-amino acids in seawater has been reported in previous studies, and is thought to result from the widespread microbial activity present in the marine environment (Lee and Bada 1977; McCarthy 1998). In the experiments here, preferential preservation of cyanobacterial peptidoglycan fragments may have contributed to the transient accumulation of D-amino acids in the surrounding seawater, but these D-amino acids were eventually decomposed by heterotrophic bacteria. It is also possible that growth of heterotrophic bacteria during the incubation was responsible for the increasing proportions of D-ASP and D-SER since these bacteria contain these D-amino acids in their peptidoglycan (Kaiser and Benner 2008). Release of D-amino acids into seawater during the exponential growth of heterotrophic bacteria living on glucose has been observed, and is thought to result from cleavage of peptidoglycan during cell division (Kawasaki and Benner 2006).

At the beginning of the intact cell incubation, a high percent of D-amino acids was observed in the surrounding seawater, while D-amino acids were absent during the incubation of stripped cells. The D-amino acids in DHAA of intact CCMP2370 and CCMP1334 were different in composition, with D-GLU and D-ALA enriched in CCMP2370, and D-SER and D-ALA enriched in CCMP1334. Since stripping buffer is not strong enough to break down peptidoglycan, the high percentage D-amino acid at the beginning of the intact cell incubations may not be from the cleavage of peptidoglycan. In addition to the enrichment of D-amino acids, free glutamic acid concentrations were high at the beginning of intact cell degradation of both CCMP2370 and CCMP1334. Glutamic acid is known to accumulate in the cytoplasm of phytoplankton (Hill and Brest 1988). The initial presence of D-amino acids and glutamic acid suggests that they may be metabolic products of cyanobacterial cells. These compounds were recycled within the first 2 days of degradation in CCMP2370 experiments, but more slowly in CCMP1334, showing that D-amino acids from metabolism are not preferentially preserved. Moreover, metabolically derived D-amino acids appear to be more labile than those from peptidoglycan, which were enriched at the end of the incubation time. The difference in lability of D-amino acids from these two distinctive sources may be the cause of the different distributions of D-amino acids during degradation of the cyanobacteria cells.

Our work strongly suggests that slow degradation of peptidoglycan may not be the only source of accumulating D-amino acids in seawater. Also, as other's showed, D-amino acids can be rapidly removed from the dissolved organic matter pool, and this may vary with their source (Admiraal et al. 1987).

2.4.2. Characteristics of surface layer structures

McCarren et al. (2005) showed that S-layer proteins assemble to form a regularly arranged layer, and together with a polysaccharide sheath, cover the outer cell membrane of *Synechococcus*, effectively isolating the membrane from the ambient seawater. by a layer of polysaccharide sheath.. Therefore, intracellular materials are inaccessible to extracellular enzymes unless the S layer is punctured or removed. In our experiments, the S-layer was removed from *Synechococcus* CCMP2370 cells with stripping buffer. This was apparent from both TEM observation and protein analysis (Fig. 2.1 and Fig 2.2.). The EDTA in the stripping buffer used here is capable of chelating with ions like Mg or Ca in the cell surface. These ions are located at the center of individual subunits of the S-layer and stabilize the S-layer ultrastructure (Engelhardt and Peters 1998). Removal of these ions from the S-layer structures by chelation is a likely cause of the disruption of S-layer structure, since previous studies suggest that subunits of S-layer structure are held together (and held to the underlying cell membrane structure) by weak hydrophobic bonds and ionic interactions (Koval and Murray 1984). However, EDTA apparently lacks the capacity to damage the deeper membrane structure like the lipid bilayer and peptidoglycan, since electron microscopy demonstrated that the stripping buffer efficiently removed the outer membrane of CCMP2370, but had little impact on the membrane of CCMP1334, which has no S layer.

Gel electrophoresis data showed that removal of S-layer proteins from CCMP2370 caused a large portion of proteins to be released from the cells when they were resuspended into coastal seawater. This suggests that removal of the S-layer weakens the cell membrane, and leads to cell lysis as a result of osmotic pressure. The thinner membrane beneath the S layer may not have been strong enough to maintain the cell structure under the osmotic pressure difference between ambient seawater and intracellular cytoplasm. The release of cytoplasmic proteins

suggests that S-layer structure is similar to peptidoglycan in supporting the cell shape and preventing cell lysis. Previous studies indicate that cells with S-layer structures tend to have thinner peptidoglycan (Sleytr et al. 1996), so that cell lysis would be more likely after removal of the S-layer. The role of the S layer in cell degradation clearly needs further study, particularly for those prokaryotes with S layer as their major cell wall components. For example, Archaea have only S-layer in their cell wall, and lack peptidoglycan.

2.4.3. Non-selective degradation of S layer

As described above, the S layer includes one of the most abundant glycoproteins in *Synechococcus* cells. Thus degradation pathways of the S layer help us to understand how glycosylation affects protein degradation. Early work on protein degradation in seawater suggested that abiotic glucosylation of proteins protects them from peptide hydrolysis (Keil and Kirchman 1993). However, this does not appear to be the case for enzymatically produced glycoproteins. In our study, degradation of the commercially available glycoprotein, OVA, was compared to degradation of a non-glycosylated protein, BSA. Both of them degraded rapidly in in coastal seawater. This implies that glycosylation does not necessarily protect individual glycoproteins from enzymatic attack. The degradation behavior of glycoproteins was also studied in the degrading *Synechococcus* cells. We found that the S layer disappeared at the same rate as other proteins, within the first 7 days of incubation. The majority of PAS stainable molecules also disappeared in the first 7 days, suggesting that the majority of the glycoprotein was not preferentially preserved. Liu et al. (2010) also found no difference in resistance to degradation between synthetic glycosylated and non-glycosylated tetrapeptides in a coastal environment. These studies indicate that modification of protein by glycosylation does not always slow protein degradation, whether the glycoprotein is free or part of cellular materials.

The apparent difference between our results and those of Keil and Kirchman (1993) may be because the glycosylated proteins in their work were prepared abiotically by heating BSA with glucose at 50ºC for 96 hours, a non-enzymatic mediated Maillard reaction which forms poorly characterized glycosylated products. However, a large portion of the particulate protein in seawater has been identified as glycosylated (Tsukasaki and Tanoue 2010); either individual glycoproteins of great stability must exist, or there are processes other than preferential preservation involved in the accumulation of these glycopeptides in marine particles. As one example, the greater stickiness of glycoprotein may promote the incorporation of dissolved glycoproteins into extracellular transparent polysaccharide (EPS) particles or sorption onto minerals, these particulate glycoproteins may escape bacterial attack and thus be preserved.

Several previous studies have shown that membrane proteins can be better preserved during decomposition (Nagata et al. 1998; Moore et al. 2012). However, non-selective decomposition of S layer proteins demonstrates that not all membrane proteins are preferentially preserved. This is probably because the S layer is located at the outermost cell surface, and protection by the cell membrane structure beneath is limited. Our growing understanding of membrane proteins indicates that they are highly diverse in their structures and functions. Therefore, a study of a single membrane protein cannot represent the degradation behavior of all.

2.4.4. Preservation of proteinaceous materials by S-layer structure

In our experiments, the S-layer structure was efficiently removed from the cyanobacterial cell surface, leaving other parts of the cell intact. This provides us with a novel approach to evaluating the importance of S-layer structures in resistance of cyanobacterial cells to degradation in marine environments. During our incubations, the degradation index of both

stripped and intact cells increased over time, with that of stripped cells increasing more quickly. Correspondingly, the proportion of D-amino acids in particles also increased in both stripped and intact cell incubation, with stripped cells more becoming enriched in D-amino acids than intact cells. Dissolved D-amino acids were also more enriched during degradation of stripped CCMP2370 cells compared to intact cells (this difference was not seen in stripped and intact CCMP1334). These different lines of evidence support the idea that stripping off surface layer proteins causes more intensive degradation of cellular proteins. However, this difference in degradation state is not caused by the accumulation of surface layer structure in the detrital materials, since our results showed that S-layer protein was as labile as other proteins in the cell. It is rather the cell lysis after S layer removal that causes more rapid degradation. Without the protection of the S-layer structure, the cell lysed immediately and cytoplasm proteins were released into seawater as suggested by gel electrophoresis data. Therefore, the S layer functioned as a protective layer.

The importance of bacterial cell membranes to organic matter degradation has been widely discussed. The prevailing view is that the slower degradation of cell membrane is due to the refractory nature of some of its components, e.g., lipid bilayer, membrane protein and peptidoglycan. Lipid bilayer is characterized by its high hydrophobicity, which repels water and protects associated components from the enzymatic attack. Membrane proteins, either imbedded inside the bilayer or anchored on the bilayer like porin and FTR1, are relatively more hydrophobic on the surface (Nunn et al. 2010). Therefore, these membrane proteins may be less accessible to hydrolytic enzymes. Another possibility is that the bilayer can encapsulate the attached membrane proteins, and thus protect them from enzymatic hydrolysis. Membrane proteins are frequently found in deep seawater (Tanoue et al. 1995), marine particles (Moore et

al. 2012) and degraded diatom (Nunn et al. 2010) and cyanobacterial (Miyoshi and Suzuki 2004) cells, suggesting a preferential preservation of these proteins during degradation and transport of organic matter. Peptidoglycan in prokaryotes is another membrane structure that has been recently discussed. Evidence for the accumulation of peptidoglycan fragments in marine organic matter has been shown in various marine environments (Lee and Bada 1977; McCarthy 1998; Kaiser and Benner 2008), and is postulated to be an indicator of slower decomposition of bacterial peptidoglycan as well as the prevailing bacterial activity in diverse marine environments. Slower degradation of purified peptidoglycan was also observed in seawater incubation experiments, consistent with a refractory nature for peptidoglycan (Nagata et al. 2003).

Here we suggest that another membrane structure, S layer, may be important in regulating the degradation of prokaryotes, and contributing to the transport of cyanobacterial cellular materials into the deep ocean (Dong et al. 2010; Lomas and Moran 2011). A better understanding of the S layer is particularly important for prokaryotes like Archaea, in which S layer is a universal cell wall component instead of peptidoglycan as in bacteria. For this reason, the study of S-layer will lead to a better understanding of the biogeochemical contribution of Archaea, which has long been understudied in geochemical research. Our study of S-layer proteins suggests that, even though the S layer itself is fragile to enzymatic attack, and post translational modification has limited impact in protecting the proteins from degradation, its unique planar ultrastructure and location within the cell membrane evidently slows degradation of cellular organic matter by preventing the access of enzymatic hydrolysis to proteinaceous structural materials inside the cell. Our results highlight the importance of diverse function of proteins in the cell, which can also regulate the degradation of cellular materials. How microbes

develop strategies to break down cell membrane structures may be critical for the remineralization of cellular organic matter. Further research is desired to better understand the degradation of proteinaceous materials of various structures within marine prokaryotes as well as in detrital organic matter.

References:

Admiraal, W., C. Riaux-Gobin, and R. W. P. M. Laane. 1987. Interactions of ammonium , nitrate , and D- and L- amino acids in the nitrogen assimilation of two species of estuarine benthic diatoms. 40: 267-273.

Borch, N. H., and D. L. Kirchman. 1999. Protection of protein from bacterial degradation by submicron particles. Aquatic Microbial Ecology 16: 265-272.

Brahamsha, B. 1996. An abundant cell-surface polypeptide is required for swimming by the nonflagellated marine cyanobacterium *Synechococcus*. Proceedings of the National Academy of Sciences of the United States of America 93: 6504-6509.

Collier, J. L., and a R. Grossman. 1992. Chlorosis induced by nutrient deprivation in *Synechococcus sp*. strain PCC 7942: not all bleaching is the same. Journal of bacteriology 174: 4718-26.

Dauwe, B., J. J. Middelburg, P. M. J. Herman, and C. H. R. Heip. 1999. Linking diagenetic alteration of amino acids and bulk organic matter reactivity. Limnology and Oceanography 44: 1809-1814.

Dittmar, T., H. P. Fitznar, and G. Kattner. 2001. Origin and biogeochemical cycling of organic nitrogen in the eastern Arctic Ocean as evident from D- and L-amino acids. Geochimica et Cosmochimica Acta 65: 4103-4114.

Dong, H.-P., D.-Z. Wang, M. Dai, and H.-S. Hong. 2010. Characterization of particulate organic matter in the water column of the South China Sea using a shotgun proteomic approach. Limnology and Oceanography 55: 1565-1578, doi:10.4319/lo.2010.55.4.1565

Engelhardt, H., and J. Peters. 1998. Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. Journal of Structural Biology 124: 276-302, doi:10.1006/jsbi.1998.4070

Fitznar, H. P., J. M. Lobbes, and G. Kattner. 1999. Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with o-phthaldialdehyde and N-isobutyrylcysteine in seawater and fossil samples (mollusks). Journal of Chromatography A 832: 123-132, doi:10.1016/S0021-9673(98)01000-0

Hedges, J. I., G. Eglinton, P. G. Hatcher, D. L. Kirchman, C. Arnosti, S. Derenne, R. P. Evershed, and I. Ko. 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Organic Geochemistry 31: 945-958.

Hill, C., and C. D. Brest. 1988. Interspecific and intraspecific composition and variation of free amino acids in marine phytoplankton. 44: 303-313.

Kaiser, K., and R. Benner. 2005. Hydrolysis-induced racemization of amino acids. Limnology and Oceanography: Method 3: 318-325.

Kaiser, K., and R. Benner. 2008. Major bacterial contribution to the ocean reservoir of detrital organic carbon and nitrogen. Limnology and Oceanography 53: 99-112.

Kawasaki, N., and R. Benner. 2006. Bacterial release of dissolved organic matter during cell growth and decline : Molecular origin and composition. Limnology and Oceanography 51: 2170-2180.

Keil, R. G., and D. L. Kirchman. 1993. Dissolved combined amino acids: Chemical form and utilization by marine bacteria. Limnology and Oceanography 38: 1256-1270, doi:10.4319/lo.1993.38.6.1256

Koval, S. F., and R. G. Murray. 1984. The isolation of surface array proteins from bacteria. Canadian Journal of Biochemistry and Cell Biology 62: 1181-9.

Lee, C., and J. L. Bada. 1977. Dissolved amino acids in the equatorial Pacific, the Sargasso Sea, and Biscayne Bay. Limnology and Oceanography 22: 502-510.

Lee, C., S. G. Wakeham, and J. I. Hedges. 2000. Composition and Flux of particulate amino acids and chloropigments in equatorial Pacific seawater and sediments. Deep-Sea Research I 47: 1535-1568.

Liu, Z., M. E. Kobiela, G. A. McKee, T. Tang, C. Lee, M. R. Mulholland, and P. G. Hatcher. 2010. The effect of chemical structure on the hydrolysis of tetrapeptides along a river-to-ocean transect: AVFA and SWGA. Marine Chemistry 119: 108-120, doi:10.1016/j.marchem.2010.01.005

Lomas, M. W., and S. B. Moran. 2011. Evidence for aggregation and export of cyanobacteria and nano-eukaryotes from the Sargasso Sea euphotic zone. Biogeosciences 8: 203-216, doi:10.5194/bg-8-203-2011

Lourenc, S. O., E. Barbarino, U. M. L. Marquez, and E. Aidar. 1998. Distribution of interacellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factores. Journal of Phycology 811: 798-811.

McCarren, J., and B. Brahamsha. 2009. Swimming motility mutants of marine *Synechococcus* affected in production and localization of the S-layer protein SwmA. Journal of Bacteriology 191: 1111-4, doi:10.1128/JB.01401-08

McCarren, J., J. Heuser, R. Roth, N. Yamada, M. Martone, and B. Brahamsha. 2005. Inactivation of swmA Results in the loss of an outer cell layer in a swimming *synechococcus* strain. Journal of Bacteriology 187: 224-230, doi:10.1128/JB.187.1.224

McCarthy, M. D., J. I. Hedges, and R. Benner. 1998. Major bacterial contribution to marine dissolved organic nitrogen. Science 281: 231-234, doi:10.1126/science.281.5374.231

Messner, P. 1997. Bacterial glycoproteins. Glycoconjugate Journal 14: 3-11.

Miyoshi, T., and S. Suzuki. 2004. Degradation of outer membrane proteins of *Synechococcus sp* . in vitro and in situ. Journal of Oceanography 60: 825-833.

Moore, E. K., B. L. Nunn, D. R. Goodlett, and H. R. Harvey. 2012. Identifying and tracking proteins through the marine water column: Insights into the inputs and preservation mechanisms of protein in sediments. Geochimica et Cosmochimica Acta 83: 324-359, doi:10.1016/j.gca.2012.01.002

Nagata, T., R. Fukudal, I. Koikel, K. Kogurel, and D. L. Kirchman. 1998. Degradation by bacteria of membrane and soluble protein in seawater. Aquatic Microbial Ecology 14: 29-37.

Nagata, T., B. Meon, and D. L. Kirchman. 2003. Microbial degradation of peptidoglycan in seawater. Limnology and Oceanography 48: 745-754, doi:10.4319/lo.2003.48.2.0745

Nguyen, R., and R. H. Harvey. 2003. Preservation via macromolecular associations during Botryococcus braunii decay: proteins in the Pula Kerogen. Organic Geochemistry 34: 1391-1403, doi:10.1016/S0146-6380(03)00154-2

Nunn, B. L., Y. S. Ting, L. Malmström, Y. S. Tsai, A. Squier, D. R. Goodlett, and H. R. Harvey. 2010. The path to preservation: Using proteomics to decipher the fate of diatom proteins during microbial degradation. Limnology and Oceanography 55: 1790-1804, doi:10.4319/lo.2010.55.4.1790

Pogozelski, W. K., and T. D. Tullius. 1998. Oxidative strand scission of nucleic acids: routes initiated by hydrogen abstraction from the sugar moiety. Chemical Reviews 98: 1089-1108.

Resch, C. M., and J. Gibson. 1983. Isolation of the carotenoid-containing cell wall of three unicellular cyanobacteria. Journal of Bacteriology 155: 345-50.

Saijo, S., and E. Tanoue. 2005. Chemical forms and dynamics of amino acid-containing particulate organic matter in Pacific surface waters. Deep Sea Research Part I 52: 1865-1884, doi:10.1016/j.dsr.2005.05.001

Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews 36: 407-77.

Schultze-Lam, S., G. Harauz, and T. J. Beveridge. 1992. Participation of a cyanobacterial S layer in fine-grain mineral formation. Journal of Bacteriology 174: 7971-81.

Schäffer, C., M. Graninger, and P. Messner. 2001. Prokaryotic glycosylation. Archaea 1: 248-261.

Schäffer, C., and P. Messner. 2004. Surface-layer glycoproteins: an example for the diversity of bacterial glycosylation with promising impacts on nanobiotechnology. Glycobiology 14: 31R-42R, doi:10.1093/glycob/cwh064

Sleytr, U. B., and T. J. Beveridge. 1999. Bacterial S-layers. Trends in microbiology 7: 253-60.

Sleytr, U., P. Messner, D. Pum, and M. Sara. 1996. Crystalline Bacterial Cell Surface Proteins, Academic Press.

Smarda, J., D. Smajs, J. Komrska, and V. Krzyzanek. 2002. S-layers on cell walls of cyanobacteria. Micron 33: 257-277.

Tanoue, E., S. Nishiyama, M. Kamo, and A. Tsugita. 1995. Bacterial membranes : Possible source of a major dissolved protein in seawater. Geochimica et Cosmochimica Acta 59: 2643-2648.

Tsukasaki, A., and E. Tanoue. 2010. Chemical qualification of electrophoretically detectable peptides and sugar chains in oceanic surface particulate organic matter. Marine Chemistry 119: 33-43, doi:10.1016/j.marchem.2009.12.004

Wakeham, S. G., C. Lee, J. I. Hedges, P. J. Hernes, and M. L. J. Peterson. 1997. Molecular indicators of diagenetic status in marine organic matter. Geochimica et Cosmochimica Acta 61: 5363-5369, doi:10.1016/S0016-7037(97)00312-8

Figures:

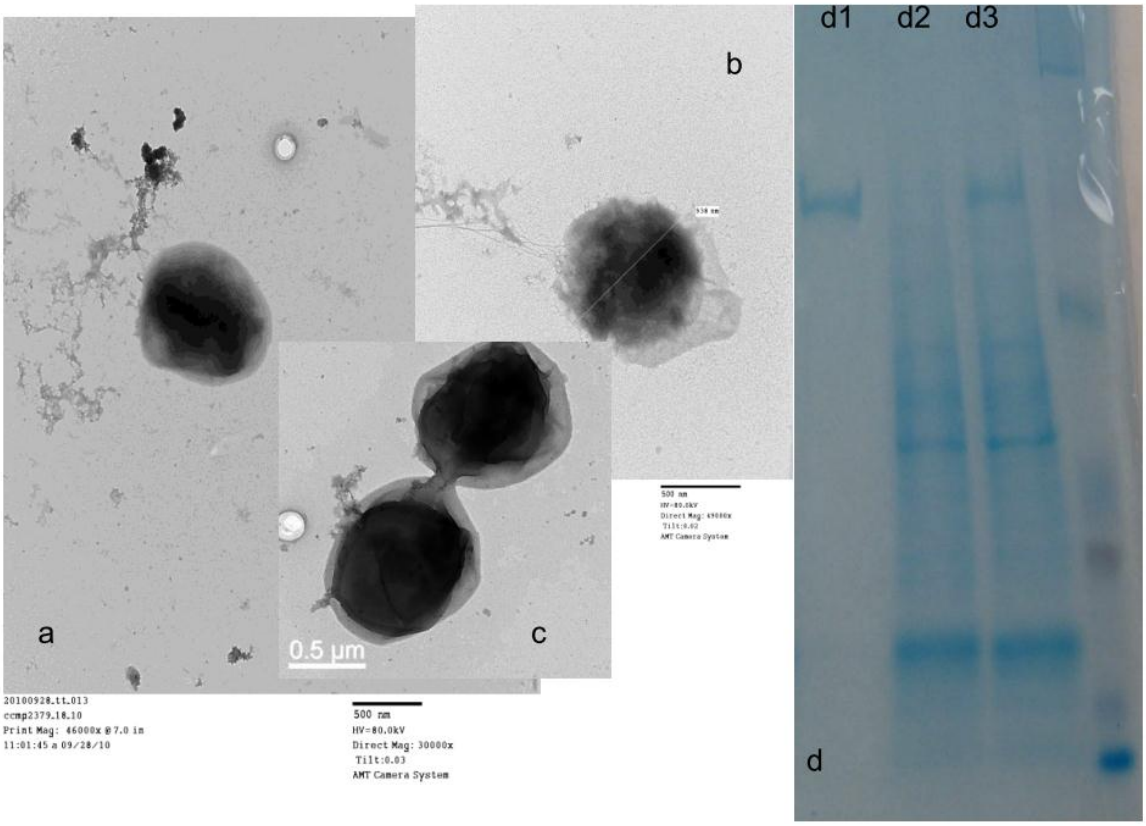

Figure 2.1. Transmission electron micrograph (TEM) of intact cells (a) and stripped cells (b) of CCMP2370 (with S layer); stripped cells of CCMP1334 (without S layer) showing the absence of damage to the surface (c); and CBB-stained SDS-PAGE of the stripping buffer with S layer alone (d1), stripped cells (d2), and intact cells (d3).

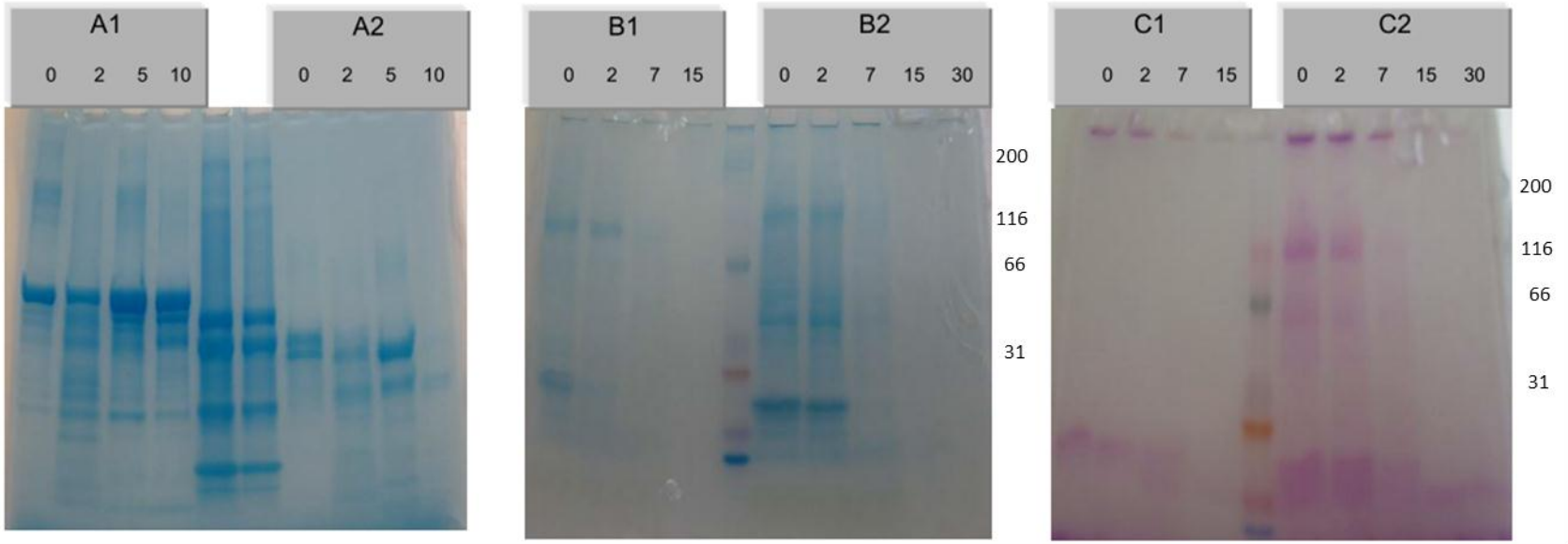

Figure 2.2. SDS polyacrylamide gels of Coomassie blue-stained (A and B) and Periodic acid-Schiff Stained (C) materials. A1 and A2 are incubations of the non-glycosylated protein BSA and the glycoprotein, OVA; B and C are incubations of *Synechococcus* cells. B1 and C1 show stripped cells; B2 and C2 show intact cells.

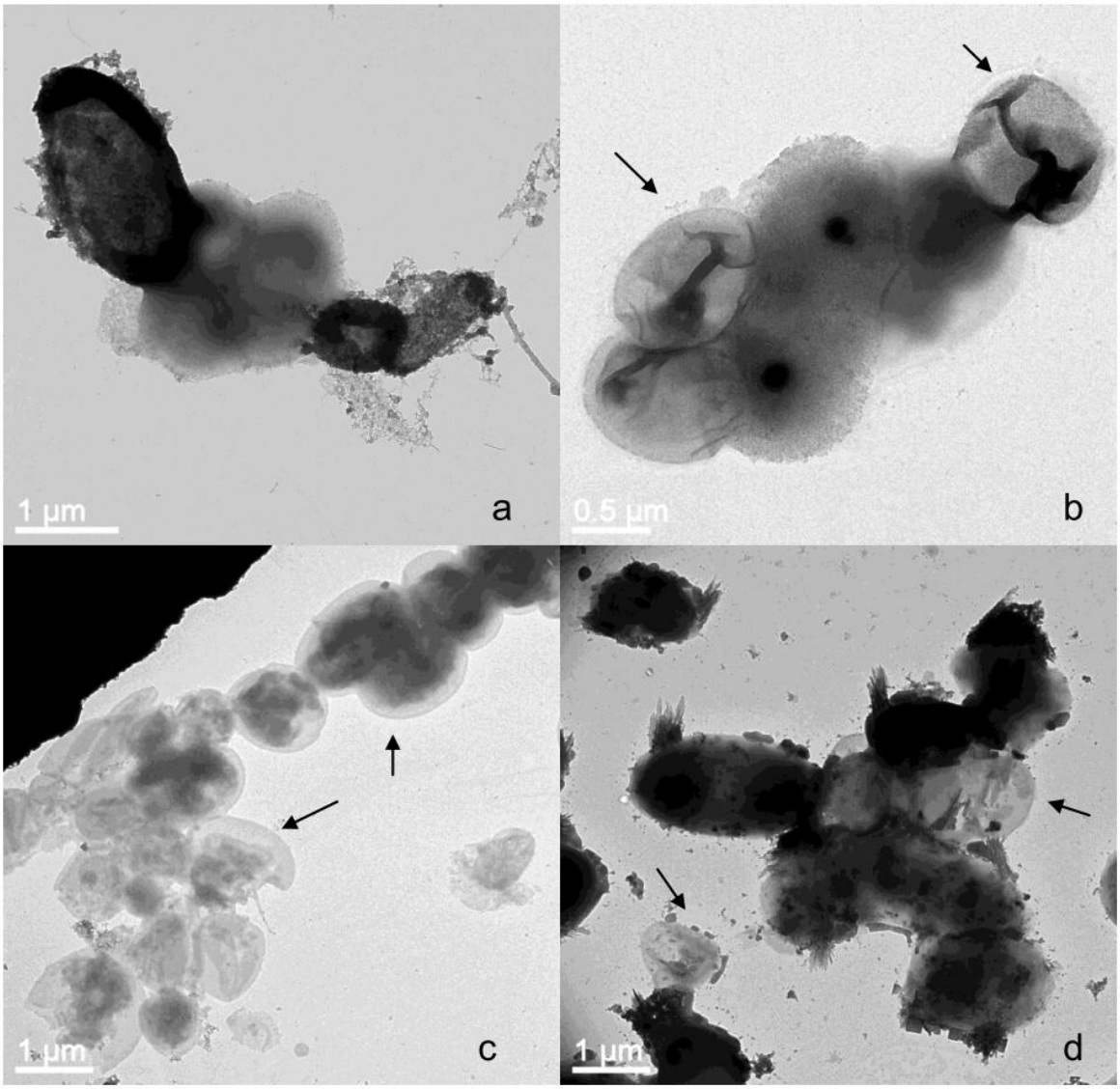

Figure 2.3. TEM of stripped (a) and intact cells (b) of CCMP2370 after 2-d incubation; TEM of stripped (c) and intact cells (d) of CCMP1334 after 2-d of incubation. Arrows show fragments of cell membrane with integrate surface.

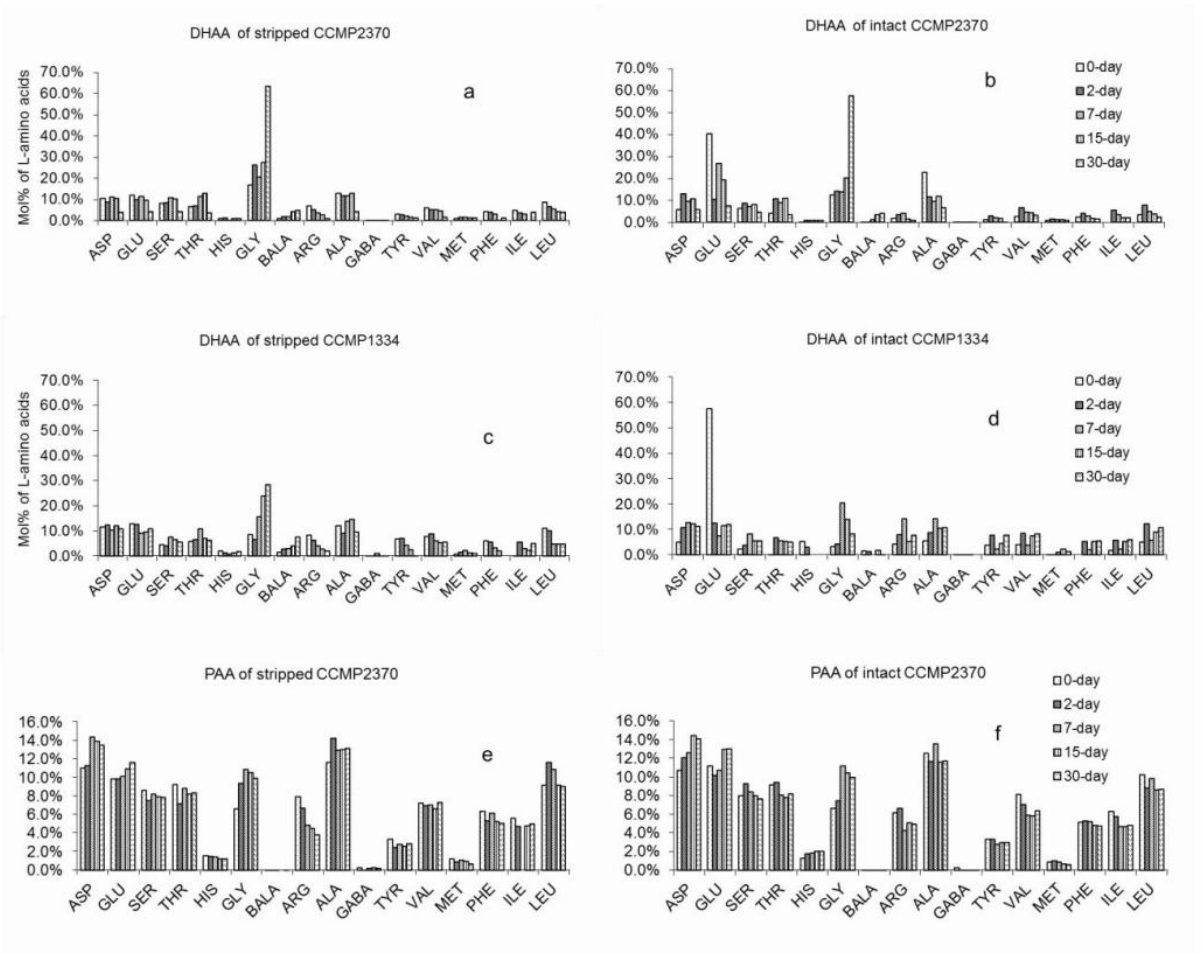

Figure 2.4. Relative abundance of L-amino acids in TDAA in seawater from stripped (a) and intact CCMP2370 (b); relative abundance of L-amino acids in TDAA in seawater from stripped (c) and intact CCMP1334 (d) incubations; in PAA of stripped (e) and intact CCMP2370 (f).

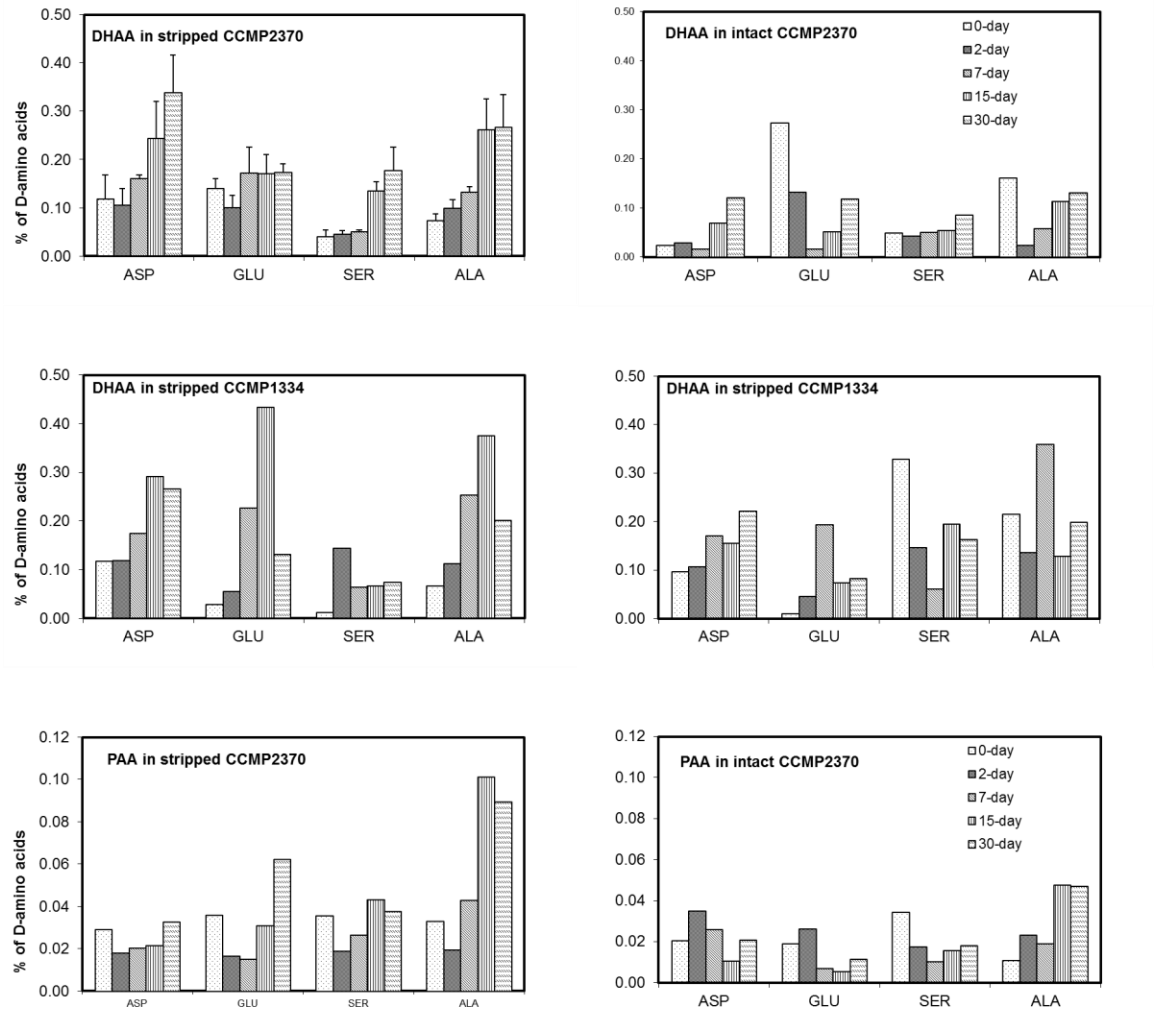

Figure 2.5. Change in the D/L ratios of aspartic acid (ASP), glutamic acid (GLU), serine (SER), and alanine (ALA) during the incubations of stripped and intact cells. Particulate amino acids during the incubations of stripped cells (a) and intact cells (b) of CCMP2370. Dissolved hydrolyzable amino acids during the incubations of stripped cells (c) and intact cells (d) of CCMP2370. Dissolved hydrolyzable amino acids during the incubations of stripped cells (e) and intact cells (f) of CCMP1334.

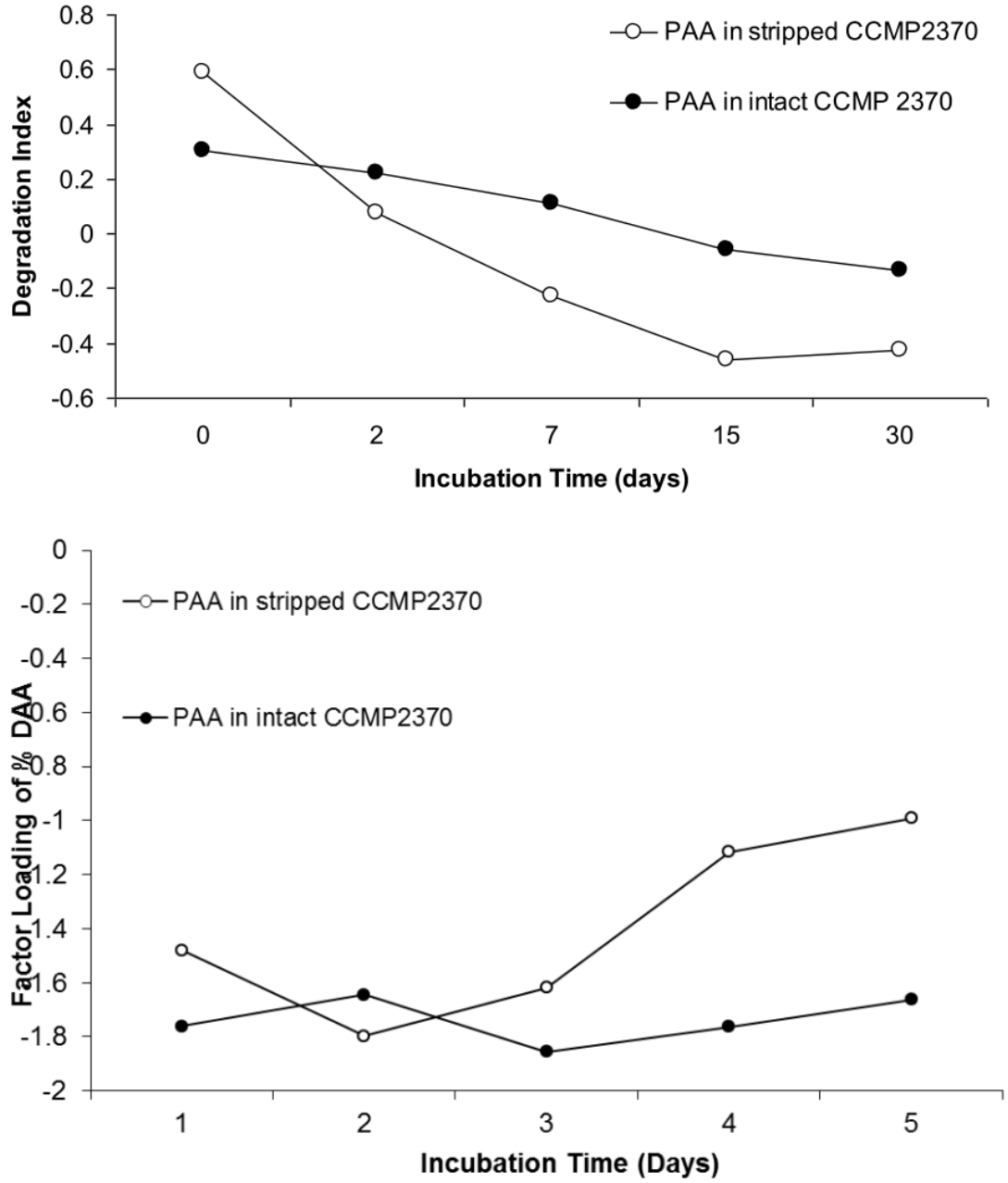

Figure 2.6. Change of Dauwe degradation index of particulate amino acids during the degradation of stripped and intact CCMP 2370 (upper figure); Change of factor loadings of principle component analysis of mol% D-amino acids in particles during the incubation of stripped and intact CCMP2370 (bottom figure).

Chapter Three

Silicate Deposition on Decomposing Cyanobacteria as a Source of Particulate Silicate in the Ocean

Abstract: Marine particles transport organic matter through the oceanic water column to the sediment where the organic matter can be buried. As a net sink of CO_2 over geological time, this pathway is one of the few natural removal mechanisms from the biosphere. Picoplankton like cyanobacteria have not until recently been thought important to particle transport (Ric hardson and Jackson 2007; Lomas and Moran 2010), even though cyanobacteria are one of the major primary producers in the surface waters of the open ocean (Michaels and Silver 1988; Steinberg et al. 2001). Here we provide evidence that silicate deposits on the extracellular polymeric substances (EPS) secreted by cyanobacteria, particularly when they begin to decompose in a natural microbial community. We also found that Si was enriched in micro-blebs that were present in the water column in the Sargasso Sea. The surprising similarity in morphology and composition between Si associated with cyanobacteria-derived EPS and that with open ocean micro-blebs suggests that EPS-associated Si may be a precursor of the Si-enriched organic micro-blebs observed in the ocean. This previously unexplored source of particulate silicate mineral may be as important as diatoms in oceanic silicate cycling and further impact organic matter export in the oceans.

3.1. Introduction:

In marine environments, cyanobacteria are the most abundant picophytoplankton, which are major primary producers that contribute more than 50% of the primary production in

oligotrophic ocean areas (Steinberg et al. 2001). However, picoplankton have been considered to be less important in global particle export than larger micro-level phytoplankton like diatoms and coccolithophores that can produce biogenic minerals as part of their cell. Because of the widely observed and strong quantitative relationship between particulate organic carbon (POC) and ballast minerals (opal, carbonates and dust), biominerals from plankton are thought to contribute greatly to density-driven particle sedimentation (Armstrong et al. 2002, 2009). However, recent studies in oligotrophic areas have suggested that picoplankton are also important in transport to the deep ocean (Richardson and Jackson 2007; Dong et al. 2010; Lomas and Moran 2010). The export mechanism is not known, but transport in fecal pellets has been suggested.

3.2. Methods:

With the purpose of understanding mechanisms of cyanobacterial export in the marine environment, we studied Si deposition on cyanobacteria in culture and in marine particles collected in the field. In the culture studies, we used two species of cyanobacteria, *Synechococcus sp*. CCMP2370 and CCMP1334. Both are open ocean strains originally from the Sargasso Sea where cyanobacteria are a dominant primary producer for most of the year. Cyanobacterial cells grown in culture were added to a concentration of ~10^7 cells/mL in coastal seawater (Stony Brook Harbor, NY) and allowed to decompose in the dark in the presence of natural microbial communities. Zooplankton had been removed from the seawater medium by filtration before addition of the cyanobacteria, so that microbial degradation dominated the incubations. Subsamples were collected over time to track degradation using a variety of biological and chemical analyses.

3.2.1. Culture preparation:

Strains of the cyanobacteria Synechococcus sp. CCMP2370 and CCMP1334 (Bigelow Laboratory) were sterilely grown in L1 medium prepared with 0.2-μm filtered coastal seawater (Stony Brook Harbor, NY). The cultures were incubated at 20ºC with a 16:8 illumination cycle of ~10mE/m^2/sec. Cells were harvested at the end of the exponential growth stage or early stationary stage by centrifugation at 5250xg for 40min. Pellets of cells were rinsed with and resuspended in 0.2 μm filtered coastal seawater.

3.2.2. Incubation of cyanobacterial cells:

Harvested cyanobacterial cells were added to coastal seawater (Stony Brook Harbor) that was filtered through 178-μm mesh to remove zooplankton. The incubations were conducted in sterilized polycarbonate flasks in the dark at 22 - 24ºC. Subsamples were collected after 0, 2, 7, 15 and 30 days. After centrifugation at 5250 x g for 40 min, both the supernatants and pellets were collected as surrounding water and particle samples, respectively. Incubations of coastal seawater without Synechococcus present were also carried out under identical conditions as a blank control. A parallel incubation of autoclaved coastal seawater with Synechococcus CCMP2370 added was used as sterile control. pH was measured in each subsample using a Ross electrode.

3.2.3. Amino acid and nutrient analysis:

Individual amino acids were measured after hydrolysis in both the particles (pellets that contained cell detritus and other particles) and surrounding seawater (supernatant) from the Synechococcus incubation (modified after Fitznar et al. 1999). Samples were hydrolyzed in 6 N HCl (Trace metal basis, Sigma-Aldrich) and 0.11 μmol L^{-1} ascorbic acid (Sigma-Aldrich) for 20 hours. The acid was removed by nitrogen sparging. D- and L- amino acids (DLAA) were chirally derivatized with o-phthaldialdehyde (OPA)(Sigma-Aldrich) and N-isobutyryl-L-cysteine

(IBLC)(Fluka). D and L amino acids were separated by high performance liquid chromatography (HPLC) with fluorescence detection (Ex/Em = 330/445 nm). Only L-amino acids were included in calculations of dissolved and particulate amino acid composition (Fig. 3.3). Pierce H amino acid standard was mixed with individual D-amino acid standards as the mixed standard for both D- and L- amino acids; beta-alanine (BALA) and gamma-aminobutyric acid (GABA) were also added.

Dissolved silicic acid [H_4SiO_4] concentrations were analyzed on 0.2μm filtered water samples using the molybdate blue spectrophotometric method (Mullin and Riley 1965) on a Lachat flow injection colorimeter.

3.2.4. Marine suspended particle collection:

Marine suspended particles were collected from a site northeast of Bermuda (**33.68°N, 57.6°W)** on the R/V Endeavor in October, 2011 (BaRFlux I). About 8L of seawater was collected from various depths using Niskin bottles on a rosette. Seawater was filtered through in-line polycarbonate filter holders with 5 μm and 0.8 μm filters in sequence. The filtrate was further filtered through 0.2 μm PC filters using a Nalgene polysulfone filtration system. Glassware was avoided during the sampling and storage. All filters were frozen until analysis.

3.2.5. Electron microscopy:

One-mL subsamples were taken from incubations and centrifuged at 4000 x g for 20 min. The pellets were washed with distilled water and resuspended in 0.1 ml of distilled water. One drop of the sample was fixed on 300-mesh, carbon-coated copper grids for 60 s before being air-dried for TEM analysis. Prepared grids were viewed on a JEOL JEM-1400 TEM at an accelerating voltage of 120kV. Selected area diffraction mode was applied on interesting parts of the samples.

Thin sections were prepared by centrifuging 45-ml subsamples from cyanobacterial incubations at 5525 x g for 40 min. The pellets were fixed with 3% glutaraldehyde/ 0.05M phosphate buffer (pH=7), and kept at 4 ºC overnight. The samples were then fixed with 2% uranyl acetate and dehydrated with ethyl alcohol. The samples were then stained with lead citrate and uranyl acetate. After being embedded in Epon resin, the samples were cut into ultrathin 80-nm sections with a Reichert-Jung Ultracut E Ultramicrotome. Thin sections were placed on Formvar-coated slot copper grids, and TEM images were taken using an FEI BioTwinG2 Transmission Electron Microscope. The same grid was further analyzed on a Hitachi 4800 scanning electron microscope with an Oxford energy dispersive x-ray spectrometer (EDS) under TEM mode.

Filtered particles from the Sargasso Sea were dried at 55ºC overnight. The filters were then coated with Au, and TEM images were taken using a Hitachi 4800 scanning electron microscope and further analyzed by EDS.

3.3. Results and discussion:

The nanoscale structures of cells were observed using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The spherical or ellipsoidal shapes of *Synechococcus* dominated the initial (t_0) samples of both species (Fig. 3.1A and 3.1B). Other phytoplankton were rarely seen and were considered unimportant in this experimental setting. Also present were extracellular organic-carbon rich fibrillar structures, which are commonly referred to as extracellular polymeric substances (EPS) (Santschi et al. 1998). Similar structures of EPS have been widely observed in cyanobacterial cultures (Strycek et al. 1992). EPS were present in initial cell cultures, but increased greatly in number with increased degradation. After two days, both species of *Synechococcus* had lysed and lost their shapes (Fig. 3.1C and 3.1D). Lysed cells were surrounded by an abundance of EPS, although much of the EPS was not attached to cells.

Most of the EPS was likely to be from cyanobacterial lysis or heterotrophic microbial activity (Pereira et al. 2009), since other organisms were a minor component. TEM pictures of both intact (Fig. 3.1C and 3.1E) and thin-sectioned samples (Fig.3.1E and 3.1F) showed that many of the EPS fibrils were progressively rearranged to form darker, sharp and elongated clusters during the incubation.

In addition, TEM selected area electron diffraction (SAD) patterns showed that polycrystalline structures of several hundred nanometers in length formed after 2 days of degradation (Fig. 3.1C). These structures were commonly observed in both the t_2 and t_{30} incubation samples (Fig. 3.1C and Fig. 3.1E), but were rare in samples with undegraded cyanobacteria (t_0). The polycrystalline structures were morphologically similar to the silicate mineral smectite found in association with organic matter in California continental margin sediments (Bennett et al. 1999). It has been suggested that smectite can form authigenically at low temperatures in association with bacteria (Konhauser and Urrutia 1999). Energy dispersive X-ray spectroscopy (EDS) mounted on SEM demonstrated that these atomically-dense structures contained a high abundance of Si and Mg (Fig. 3.2). We did not detect these elements in fresh cyanobacterial cells.

Chemical analysis provides further support for the idea that Si becomes associated with organic matter during the degradation of cyanobacteria cells. Silicate-associated amino acids in marine particles are commonly enriched in glycine (Ingalls et al. 2003b). An enrichment in particulate glycine has also been observed during the degradation of marine diatoms when their siliceous frustules dissolve (Moriceau et al. 2009). In our *Synechococcus* incubations, glycine was enriched in the dissolved hydrolysable amino acid fraction after 30 d, but not in the particulate amino acid fraction (Fig. 3.3). It is possible that we did not observe the peak in

particulate glycine described by Moriceau et al.(2009), because glycine had already been released to the dissolved phase. Thus, cyanobacterially derived EPS-silicate may be responsible for the high enrichment of dissolved glycine we observed in degraded samples. The finding that glycine can become enriched during the degradation of cyanobacteria suggests that the presence of degraded cyanobacteria may be a potential cause of the enriched glycine often observed in deep ocean particulate matter (Lee et al. 2000).

There are several possible explanations for our observations of EPS-associated Si and Mg. One possibility is that EPS-associated Si and Mg might result from silicate scavenging by brucite [$Mg(OH)_2$], a mineral that forms in seawater when pH increases (Tesson et al. 2008). Although CO_2 is formed when cyanobacteria degrade over time, the pH in our experiments ranged between 7.5 -8.1, which is not favorable for brucite formation (pH >10). No brucite crystals were observed in fresh cyanobacterial cultures either, where the pH was 8.9. A second possible explanation for the presence of EPS-associated Si is that Mg-Si deposits might originate from direct deposition as a result of high silicic acid concentrations due to dissolution of diatoms present in the coastal seawater used in the incubations. However, this is not likely because very few diatoms were present, and silicic acid concentrations in all incubations ranged between 30.3 to 98.5 μM (Fig. 3.5), which is much lower than opal solubility, and is representative of silicic acid levels in most coastal environments as well as deep water of the North Atlantic Ocean (Fig. 3.6).

A third more likely explanation for the presence of EPS-associated Si and Mg is that EPS produced during the degradation of the cyanobacteria provide a template that selectively incorporates Si and Mg. The progressive accumulation of Mg and Si in EPS may be related to the formation of the micro-crystalline particles we observed during degradation of the

Synechococcus. This process might also occur as authigenic smectite formation (Konhauser and Urrutia 1999). One question with this explanation is what the source of the Si and Mg is. Recent studies of both field and culture samples using synchrotron-based x-ray fluorescence microscopy (SXRF) demonstrate that living cyanobacteria cells can accumulate significant amounts of Si (Baines et al. Submitted). As these cells begin to degrade, cell lysis could cause a local elevation of Si concentration near the cells.

Mg-Si deposits were not limited to laboratory samples. To study the existence of EPS-associated silicate in natural particles, samples were collected at a site northeast of Bermuda in October, 2011, as part of the BaRFlux project. This site on the Bermuda rise has very limited terrestrial input. Suspended particles of 0.8 μm to 5 μm in size collected from 2000 m depth were made up of three major types of particles, organic matter rich particles here called "micro-blebs" (Fig. 3.4A) (Ransom et al. 1997), coccolith fragments (Fig. 3.4A), and diatom frustules (Fig. 3.4B). Fecal pellets were only found in particles larger than 5μm (data not shown). The coccolith and diatom frustules had distinguishable matrix structures, while micro-blebs had no well defined or regularly arranged structures, but appeared to be loosely aggregated organic materials. These micro-blebs were also different from diatom frustules and coccoliths in their elemental compositions. EDS analysis suggests that micro-blebs were characterized by elevated Si and Mg, which is consistent with the EPS associated Si observed in degrading cyanobacteria, while diatom frustules were specifically enriched in Si but not Mg, and coccoliths had a high abundance of calcium and Mg but no Si. Besides being enriched in Si and Mg, neither micro-blebs nor the EPS-Si surrounding degrading cyanobacteria has regularly arranged biogenic matrix structures. These chemical and physical similarities as well as the visual similarity lead us to suggest that EPS-Si produced during the degradation of marine cyanobacteria is one of the

possible origins of the micro-blebs observed in marine particles. An unknown amount (probably most) of the Al seen in the spectra of marine particles was from the aluminum sample holder used in SEM. Al was absent in samples where the aluminum holder was not used (Fig. S2.3 in appendix). Therefore, dust particles, which are usually enriched in Al and Si, probably have a limited contribution to these microblebs.

Cyanobacterial silicification has been observed previously in microfossils and in modern extreme environments like hot springs where amorphous silicate is supersaturated and pH is often extreme (Walter et al. 1972; Michalopoulos and Aller 2004). However, the EPS silicate found in this study is different as it deposited in an environment with mild pH and undersaturated opal. This suggests that EPS-associated Si may be deposited almost anywhere in the ocean. Our finding of Mg-Si deposits on degrading cyanobacteria implies a new source of biogenic silicate to the oceans in addition to diatoms, and must therefore be considered in studies of particle flux. This finding provides a reasonable explanation of recent findings that pico- and nano-phytoplankton can be exported into the deep ocean (Richardson and Jackson 2007; Dong et al. 2010; Lomas and Moran 2010). The close association between Si and EPS may also provide protection from remineralization for the EPS and contribute to density-driven sedimentation (Armstrong et al. 2002). This in turn could be a source of smectite in sediments; further investigation is warranted. This study does not exclude the possibility that silicate minerals can also deposit on EPS secreted by other organisms since silicate deposition appears to be a chemical reaction between EPS and silicon.

References:

Armstrong, R. A., C. Lee, J. I. Hedges, S. Honjo, and S. G. Wakeham. 2002. A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. Deep-Sea Research Part II 49: 219-236.

Armstrong, R. A., M. L. Peterson, C. Lee, and S. G. Wakeham. 2009. Settling velocity spectra and the ballast ratio hypothesis. Deep Sea Research Part II 56: 1470-1478, doi:10.1016/j.dsr2.2008.11.032

Baines, S. B., B. S. Twining, M. A. Brzezinski, J. W. Krause, S. Vogt, D. Assael, and H. McDaniel. A surprising role for picocyanobacteria in the marine silicon cycle. PNAS, Submitted.

Bennett, R. H., B. Ransom, M. Kastner, R. J. Baerwald, M. H. Hulbert, W. B. Sawyer, H. Olsen, and M. W. Lambert. 1999. Early diagenesis: impact of organic matter on mass physical properties and processes, California continental margin. Marine Geology 159: 7-34.

Dong, H.-P., D.-Z. Wang, M. Dai, and H.-S. Hong. 2010. Characterization of particulate organic matter in the water column of the South China Sea using a shotgun proteomic approach. Limnology and Oceanography 55: 1565-1578, doi:10.4319/lo.2010.55.4.1565

Fitznar, H. P., J. M. Lobbes, and G. Kattner. 1999. Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with *o*-phthaldialdehyde and N-isobutyrylcysteine in seawater and fossil samples (mollusks). Journal of Chromatography A 832: 123-132, doi:10.1016/S0021-9673(98)01000-0

Ingalls, A. E., C. Lee, S. G. Wakeham, and J. I. Hedges. 2003. The role of biominerals in the sinking flux and preservation of amino acids in the Southern Ocean along 170°W. Deep-Sea Research Part I 50: 713-738.

Konhauser, K. O., and M. M. Urrutia. 1999. Bacterial clay authigenesis: a common biogeochemical process. Chemical Geology 161: 399-413, doi:10.1016/S0009-2541(99)00118-7

Lee, C., S. G. Wakeham, and J. I. Hedges. 2000. Composition and flux of particulate amino acids and chloropigments in equatorial Pacific seawater and sediments. Deep-Sea Research Part I 47: 1535-1568.

Lomas, M. W., and S. B. Moran. 2010. Evidence for aggregation and export of cyanobacteria and nano-eukaryotes from the Sargasso Sea euphotic zone. Biogeosciences 7: 7173-7206, doi:10.5194/bgd-7-7173-2010

Michaels, A. F., and M. W. Silver. 1988. Primary production, sinking fluxes and the microbial food web. Deep Sea Research Part I: Oceanographic Research Papers 35: 473-490.

Michalopoulos, P., and R. C. Aller. 2004. Early diagenesis of biogenic silica in the Amazon delta: alteration, authigenic clay formation, and storage. Geochimica et Cosmochimica Acta 68: 1061-1085, doi:10.1016/j.gca.2003.07.018

Moriceau, B., M. Goutx, C. Guigue, C. Lee, R. Armstrong, M. Duflos, C. Tamburini, B. Charrière, and O. Ragueneau. 2009. Si–C interactions during degradation of the diatom Skeletonema marinoi. Deep Sea Research Part II 56: 1381-1395, doi:10.1016/j.dsr2.2008.11.026

Mullin, J. B., and J. P. Riley. 1965. The spectrophotometric determination of silicate-silicon in natural waters with special reference to seawater. Analytical Chimica Acta 46: 491-501.

Pereira, S., A. Zille, E. Micheletti, P. Moradas-Ferreira, R. De Philippis, and P. Tamagnini. 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS microbiology reviews 33: 917-41, doi:10.1111/j.1574-6976.2009.00183.x

Ransom, B., R. H. Bennett, R. Baerwald, and K. Shea. 1997. TEM study of in situ organic matter on continental margins: occurrence and the "monolayer " hypothesis. Marine Geology 138: 1-9.

Richardson, T. L., and G. A. Jackson. 2007. Small phytoplankton and carbon export from the surface ocean. Science 315: 838-40, doi:10.1126/science.1133471

Santschi, P. H., E. Balnois, K. J. Wilkinson, J. Zhang, J. Buffle, and L. Guo. 1998. Fibrillar polysaccharides in marine macromolecular organic matter as imaged by atomic force microscopy and transmission electron microscopy. Limnology and Oceanography 43: 896-908.

Steinberg, D. K., C. A. Carlson, N. R. Bates, R. J. Johnson, A. F. Michaels, and A. H. Knap. 2001. Overview of the US JGOFS Bermuda Atlantic Time-series Study (BATS): a decade-scale look at ocean biology and biogeochemistry. Deep Sea Research Part II 48: 1405-1447, doi:10.1016/S0967-0645(00)00148-X

Strycek, T., J. Acreman, A. Kerry, G. G. Leppard, M. V. Nermut, D. J. Kushner, and I. J. Acreman. 1992. Extracellular fibril production by freshwater algae and cyanobacteria. Microbial Ecology 23: 53-74.

Tesson, B., C. Gaillard, and V. Martin-Jézéquel. 2008. Brucite formation mediated by the diatom Phaeodactylum tricornutum. Marine Chemistry 109: 60-76, doi:10.1016/j.marchem.2007.12.005

Walter, M. R., J. Bauld, and T. D. Brock. 1972. Siliceous algal and bacterial stromatolites in hot spring and geyser effluents of Yellowstone national park. Science 178: 402-405.

Figures:

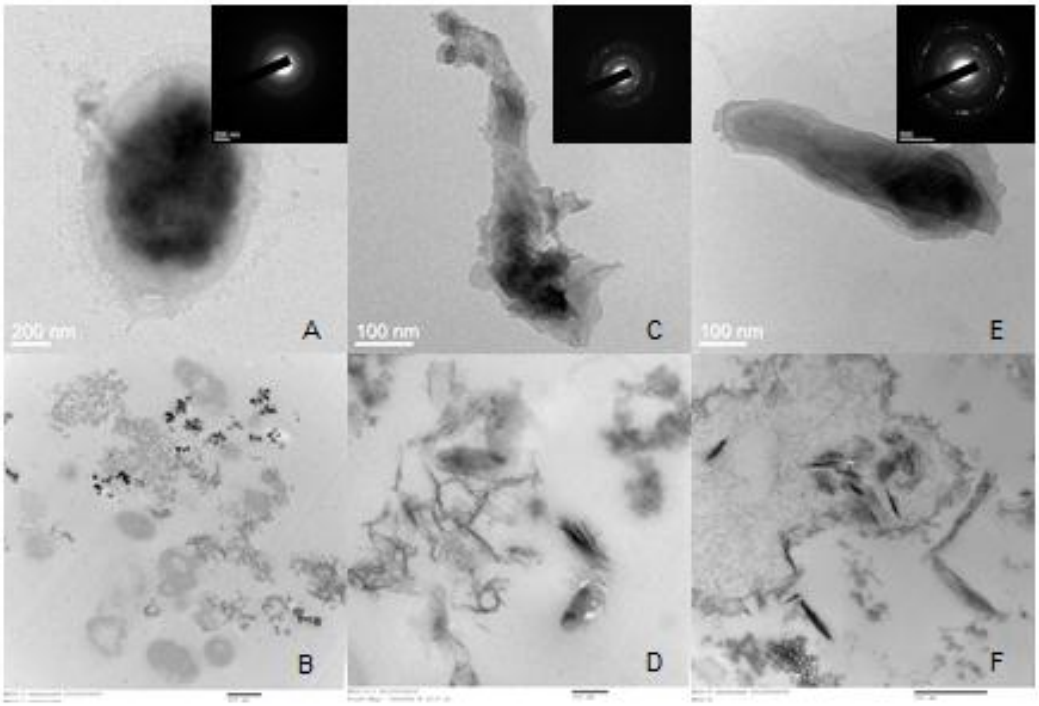

Figure 3.1. TEM images taken during dark incubation of *Synechococcus* cells in coastal seawater with natural microbial communities after 0 day (t_0, A and B), 2 days (t_2, C and D) and 30 days (t_{30}, E and F). Selected area electron diffraction patterns are pictured in A, C, and E, and reflect the crystallographic axis orientation changing across grain boundaries; polycrystalline structures were characterized by rings of luminous points in C and D. B, E and F are images of 80-nm thick thin sections of degrading samples after chemical fixation.

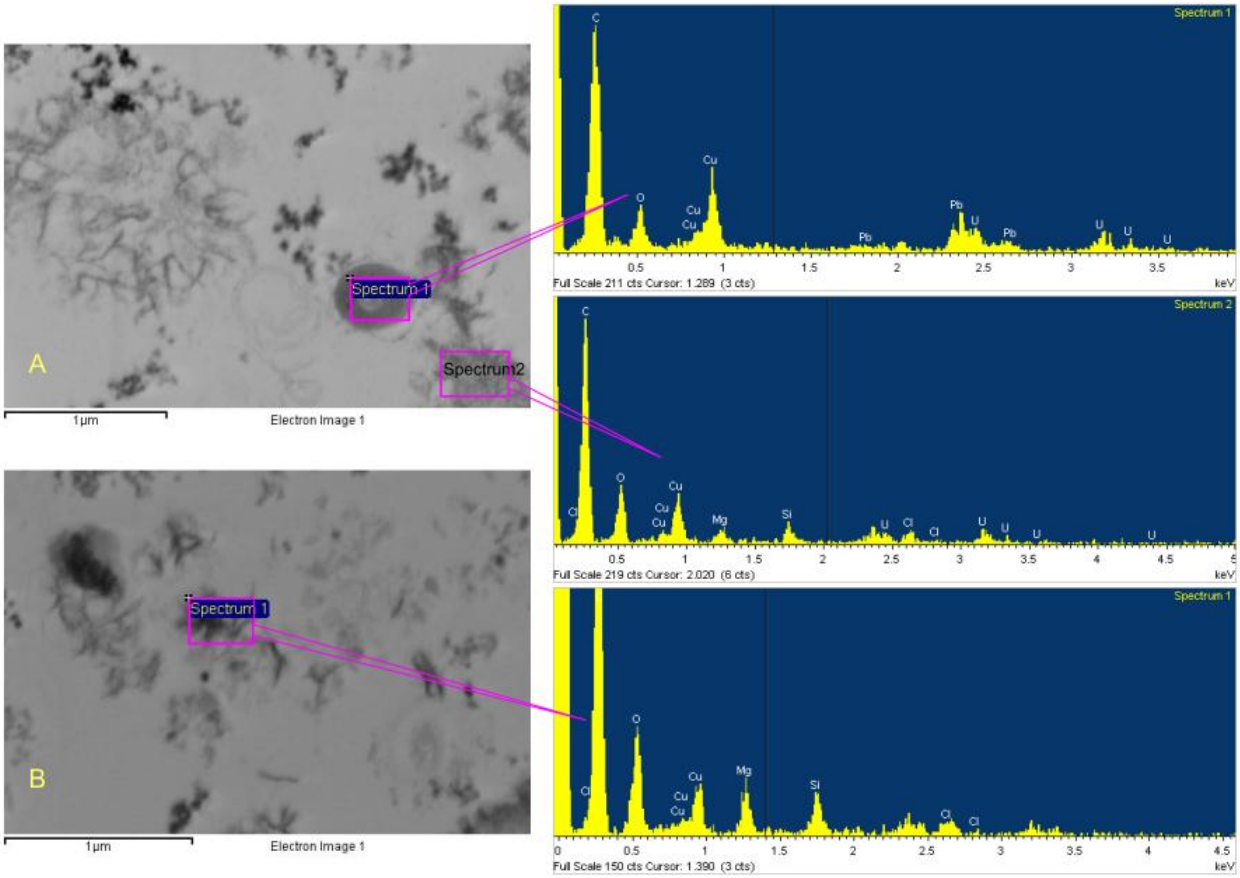

Figure 3.2. STEM images and EDS spectra taken during dark incubation of *Synechococcus* cells after 2 days (A) and 15 days (B). After 2 days, the EDS spectrum of an intact cyanobacterial cell shows the absence of Mg and Si inside the cell. But the EPS adjacent to the cell accumulated both Mg and Si, as did the EPS in the more degraded samples.

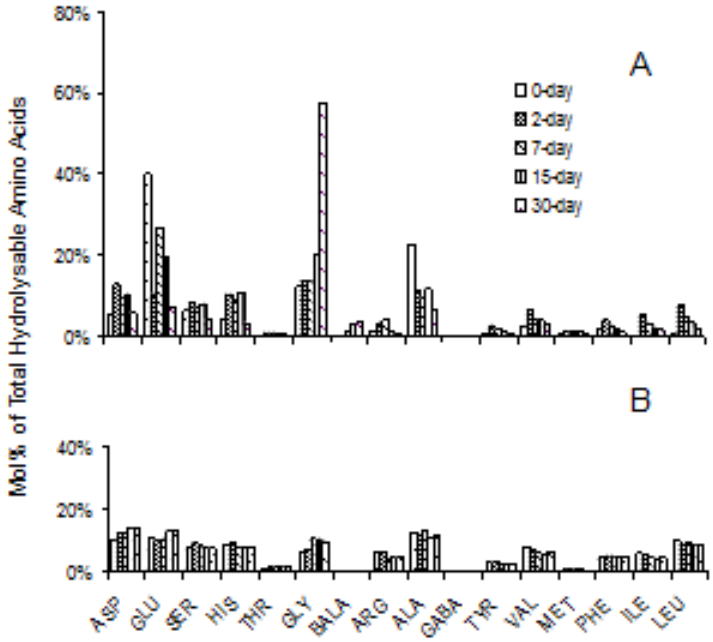

Figure 3.3. Change of relative abundance of dissolved hydrolysable amino acids (A) and particulate hydrolysable amino acids (B) during degradation of *Synechococcus sp.* CCMP2370. An accumulation of glycine was observed at the end of the incubation.

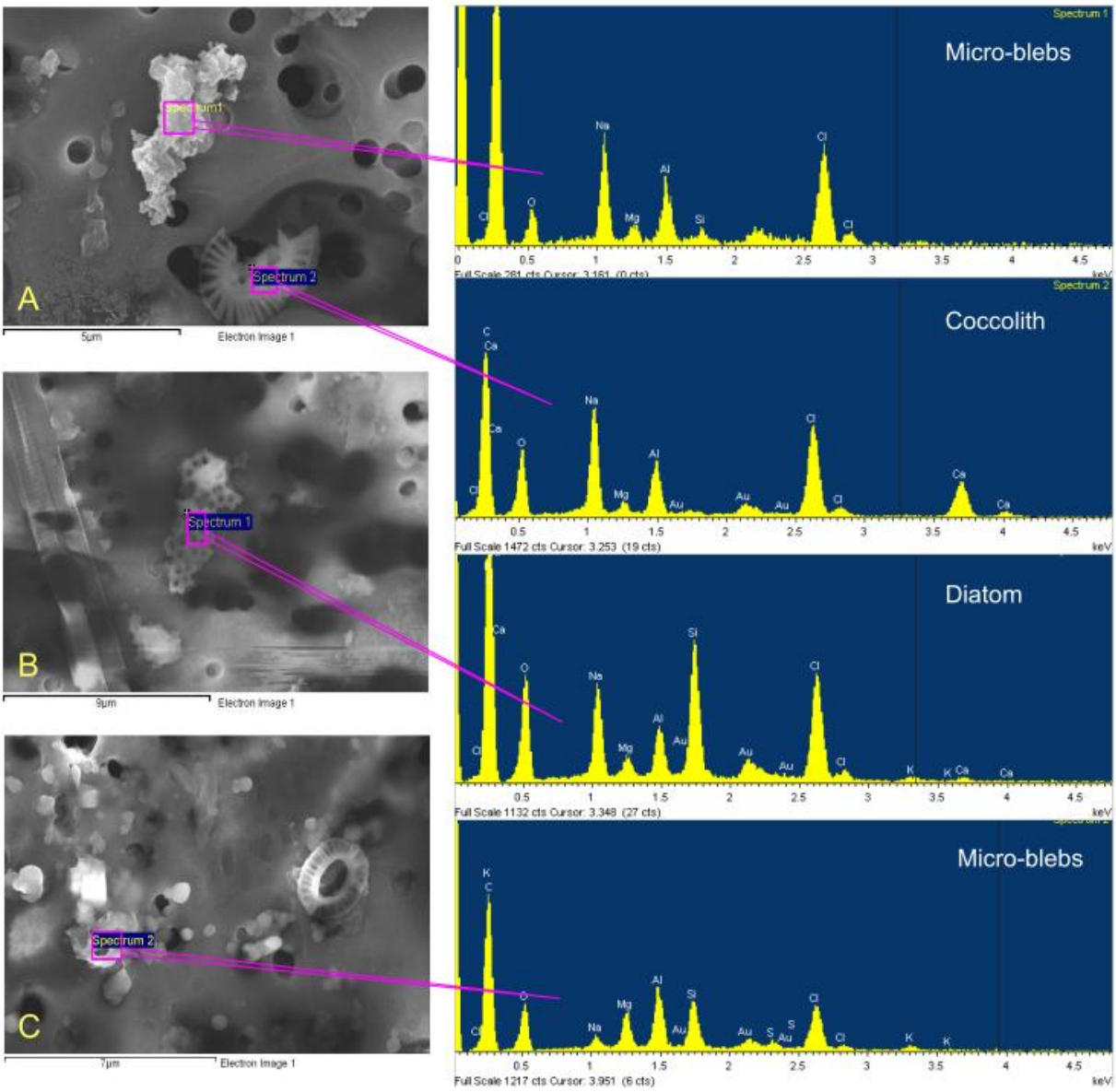

Figure 3.4. SEM images (A, B, and C) and EDS spectra of marine suspended particles collected from 2000 m water depth (A and B) and from 50 m (C) in the Sargasso Sea. A micro-bleb and a coccolith fragment are shown in A, and a fragment of diatom frustule is shown in B. A higher loading of particles is observed in surface water (C). The elemental compositions of micro-blebs were scanned by EDS. Micro-blebs from both surface (G) and 2000 m (D-F) were enriched in both Mg and Si (Enrichment of Al is from aluminum holder).

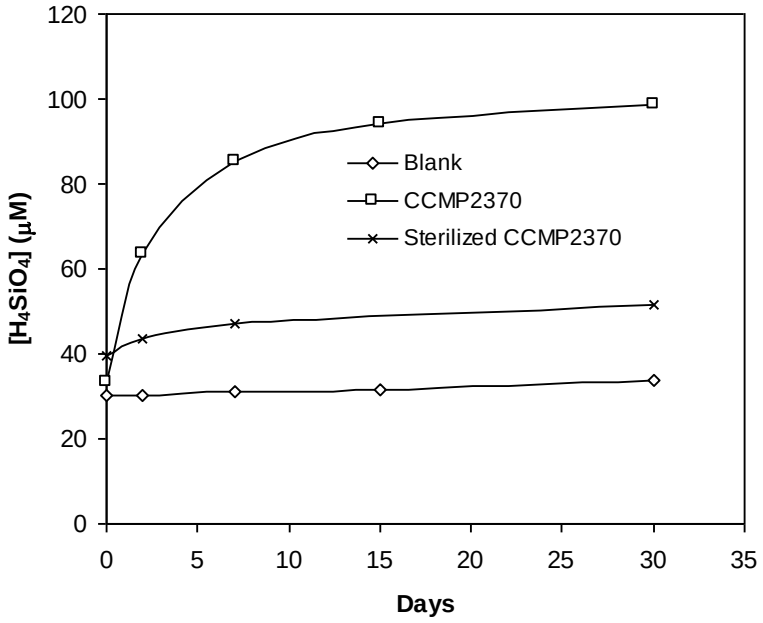

Figure 3.5. Change in $[H_4SiO_4]$ during the incubation of cyanobacteria.

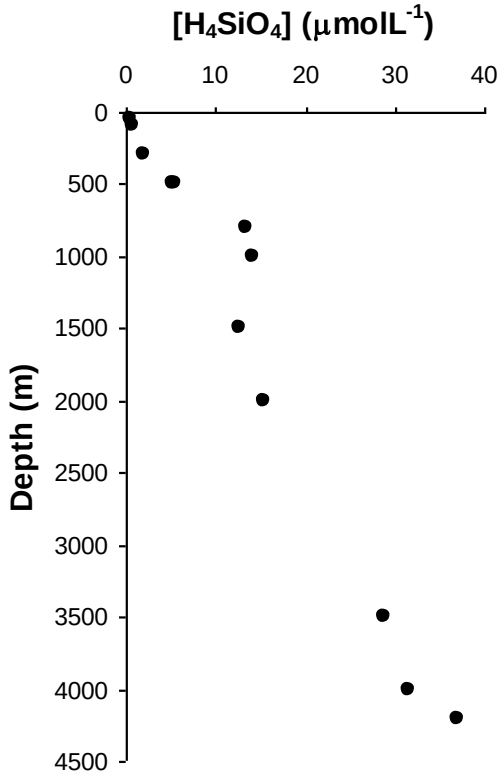

Figure 3.6. Vertical profile of H_4SiO_4 concentrations at the Sargasso Sea station.

Chapter Four

Application of a new peptide analog (LYA-AVFA) for characterizing the dynamics of dissolved organic nitrogen in the James River Estuary and lower Chesapeake Bay

Abstract

The cycling of dissolved organic nitrogen was investigated along transects of the James River estuary and lower Chesapeake Bay salinity gradient during the summer of 2008. A new fluorescent analog, Lucifer Yellow Anhydride-alanine-valine-phenylalanine-alanine (LYA-AVFA), was developed to measure extracellular peptide hydrolysis. Peptide hydrolysis from LYA-AVFA and LYA-tetraalanine (LYA-ALA_4), a previously developed peptide analog, was compared to the uptake of various organic nitrogen species (urea, glutamic acid and dialanine) along the transects. Changes in the abundance and composition of dissolved amino acids were also examined. Results from the James River estuary suggest that peptide hydrolysis and organic nitrogen uptake are not always tightly coupled to each other along the salinity transects as a response to the changing environmental conditions in the studied area. This is because diverse input and removal processes can influence both peptide hydrolysis and uptake, but not necessarily simultaneously. For example, hydrolysis potentials of both peptide hydrolysis and uptake of free amino acids were strongly associated with particulate nitrogen, suggesting a strong influence of particle-associated processes on organic nitrogen cycling in the estuary. Hydrolysis

potentials appeared to be more associated with sediment resuspension, while uptake and initial hydrolysis rates were more associated with phytoplankton biomass. As a response to changing hydrolysis and uptake of organic nitrogen, a change of dissolved amino acid abundance and composition was observed from the fresh end of the estuary to the mouth of Chesapeake Bay, which are most likely resulting from the mixing of multiple sources and their impacts on hydrolysis and uptake, e.g. terrestrial input, sediment resuspension and local phytoplankton growth.

Keywords: extracellular peptide hydrolysis, LYA-tetrapeptide, dissolved combined amino acids, dissolved free amino acids, dissolved organic matter, dissolved organic nitrogen, estuary, Chesapeake Bay, James River

4.1. Introduction

Total hydrolyzable amino acids (THAA) are one of the largest identifiable classes of dissolved organic matter (DOM) in various marine environments, and represent 0.8-3% of dissolved organic carbon (DOC) and 4-13% of dissolved organic nitrogen (DON) in the water column (Benner, 2002; Bronk, 2002). Free amino acids (DFAA) only account for a small fraction of THAA (Keil and Kirchman, 1991); the rest are in the form of combined amino acids (DCAA). Compared with bulk DOM, THAA are relatively labile on the time scale of water mass transport in estuarine areas (Coffin, 1989; Kroer et al., 1994). Accumulating evidence suggests that DCAA and DFAA abundances vary greatly among marine environments like coastal regions, and the molecular composition of DCAA and DFAA is also intensively altered (Keil and Kirchman, 1991; Yamashita and Tanoue, 2003; Kuznetsova et al., 2004). The highly dynamic distribution of dissolved amino acids in coastal regions may result from the strong impact of

local removal and input processes during the delivery and mixing of river runoff into coastal regions.

Dissolved amino acids are substantial sources of energy and nutrients for both heterotrophic microorganisms and phytoplankton. The intensive uptake of DFAA by heterotrophic bacteria has been well documented (Williams et al., 1976; Billen and Fontigny, 1987; Fuhrman1987; Berman and Bronk, 2003). Recent studies also suggest direct uptake of dipeptides by phytoplankton (Mulholland and Lee, 2009). However, DCAA, the largest pool of dissolved amino acids, is thought not to be available to most microorganisms because molecules larger than 600 Da cannot pass through cell membranes (Payne, 1980; Weiss et al., 1991). Extracellular hydrolysis of peptides was therefore considered to be the rate-limiting step in the use of THAA by heterotrophic bacteria (Chrost, 1991). The importance of extracellular peptide hydrolysis has been recently examined as a mechanism for making these compounds available to microorganisms, and it is thought that the extent to which enzymatic hydrolysis limits the use of THAA is likely complex and varied in diverse aquatic environments (Arnosti, 2004). In addition, a variety of other biotic and abiotic processes may contribute to transport, input and removal of DFAA and DCAA in coastal regions, e.g., river runoff, phytoplankton growth, sediment resuspension and photodegradation (Hoppe et al., 2002; Guldberg et al., 2002; Rosenstock et al., 2005; Grace and Bianchi, 2010; Arnosti, 2011).

Most of our understanding of peptide hydrolysis has been acquired through studies using synthetic analogs of peptides. Both fluorescently labeled and isotopically labeled amino acids and small peptides have been used to estimate peptide lability (e.g., Hollibaugh and Azam, 1983; Hoppe, 1983; Somville and Billen, 1983). Recently, fluorescently labeled (Lucifer Yellow Anhydride (LYA) peptides were developed, and these not only measured hydrolysis of actual

peptide structures but also allowed identification of hydrolysis products (Pantoja et al., 1997). Using this technique, peptide hydrolysis has been evaluated in estuaries (Mulholland et al., 2003), surface microlayers (Kuznetsova and Lee, 2001), coastal seawater and sediments (Pantoja et al., 1997; Pantoja and Lee, 1999), and an oxygen minimum zone (Pantoja et al., 2009). However, most of these studies used LYA peptides synthesized from alanine only to estimate hydrolysis of DCAA and this linkage might not be representative of the peptide linkages in nature, thereby biasing the degradation pattern of natural DCAA, since hydrolysis rates depend on the size and chemical structure of the peptide (Pantoja and Lee, 1999; Liu et al., 2010). With this problem in mind, we created a new peptide analog, LYA-labeled alanine-valine-phenylalanine-alanine (LYA-AVFA), to evaluate peptide hydrolysis in the James River estuary and adjacent lower part of the Chesapeake Bay. AVFA is a peptide fragment found in the common protein, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo), and is thus potentially more representative of natural DCAA. Here we compare hydrolysis of LYA-AVFA and LYA tetraalanine (LYA-ALA_4) to allow us to examine how differences in peptide structures influence hydrolysis patterns, because extracellular enzymes may show preferences or have specificities for particular peptide linkages and these may vary depending on environmental conditions. In addition, extracellular enzymes may show preferences for particular linkages within a peptide. For example endopeptidases which preferentially target linkages within polypeptide chains may be more important during the early degradation of organic matter versus exopeptidases that target the terminal ends of polypeptide chains (Berges and Mulholland, 2008). Consistent with this, it was previously observed that hydrolysis rates of tetraalanine were two orders of magnitude higher dialanine (Pantoja and Lee, 1999).

To address questions about the dynamics of DCAA and DFAA in estuaries, we compared multiple environmental parameters, e.g. DCAA and DFAA composition, abundance, uptake and hydrolysis, as well as phytoplankton production and biomass, salinity, and nutrient concentrations, during three cruises along the mainstem of the James River estuary to obtain a better view of mechanisms controlling DOM dynamics in estuarine systems.

4.2. Methods

4.2.1. Sample collection and hydrology in James River and lower Chesapeake Bay

The James River is a major tributary to the southern part of Chesapeake Bay, the largest estuarine system in North America, and contributes about 16% of the annual freshwater flow into the Bay (Pritchard, 1952) (Fig. 4.1). The watershed is primarily forested but includes urban and agricultural areas that contribute industrial, municipal, and agricultural inputs. Strong tidal influences extend 150 km upstream in this shallow coastal plain estuary (Wong, 1979).

Two cruises were conducted during July and August along the James River mainstem during 2008 over an area ranging from 1 to 25 in salinity. Samples were collected from the James River along this transect as well as in the southern part of the Chesapeake Bay on July 15 and August 19- 20, 2008, on board the RV *Fay Slover* (Old Dominion University). Sampling stations were named according to their collection time, during July (J) or August (A), and salinity of that station (Fig.4.1). Surface water (2 m) samples were collected from 8-L Niskin bottles mounted on a rosette sampler. Depth profiles of dissolved oxygen concentrations and fluorescence along the transects were collected using a CTD mounted on the rosette sampler.

4.2.2. Dissolved amino acid analysis

Surface samples for particulate and dissolved amino acid analysis were filtered through 0.7μm GF/F filters immediately after collection, and stored at -20ºC until analysis. DFAA concentrations were analyzed by high performance liquid chromatography (HPLC) using fluorescence detection (after Lindroth and Mopper, 1979). Amino acids in thawed samples were derivatized with o-phthaldialdehyde (OPA) and separated on an ODS HYPERSIL C18 column (Supelco, 150mm, 5μm) using a gradient of 10% methanol in 0.04M sodium acetate as solvent A and 10% 0.04M sodium acetate in methanol as solvent B. The gradient was ramped up from 10% to 60% solvent B in the first 22 min, followed by an increase to 85% solvent B in the next 17 min. The gradient was held at 85% solvent B for 2 min, and the solvent B then returned to 10%. The fluorescently labeled amino acids were detected using a Shimadzu RF-10AXL fluorometer with excitation and emission wavelengths of 330nm and 418nm, respectively. Amino Acid Standard H (Pierce) was used as the analytical standard. Non-protein amino acids, β-alanine (BALA) and γ-aminobutyric acid (GABA), from Sigma-Aldrich were added to Pierce standard H before injection.

Total hydrolyzable amino acids (THAA) were determined in water samples after acid hydrolysis using a modification of the methods of Reinthaler et al. (2008). Dissolved samples were mixed with 12N hydrochloric acid (Trace metal basis, Sigma-Aldrich) to a final concentration of 6N. Ascorbic acid (10μM, final concentration, Sigma-Aldrich) was added to the acidified samples to prevent oxidation. Samples were sparged with dry nitrogen gas for about 15 sec before sealing in glass vials with Teflon septa and vortexing. The prepared samples were hydrolyzed at 110°C on a heating block for 20 hours. After hydrolysis, acid was removed by drying under a stream of nitrogen, and redrying after adding a few drops of distilled water. Dried samples were resuspended in distilled water and methanol (v/v 6:4), and analyzed by HPLC as

described above for DFAA. The hydrolysis products of asparagine and glutamine were included in aspartic acid (ASP) and glutamic acid (GLU) measurements. Dissolved combined amino acids (DCAA) were calculated as the difference between THAA and DFAA.

4.2.3. Peptide hydrolysis

Hydrolysis of two fluorescent peptide derivatives was measured: LYA-Ala_4 and LYA-AVFA. Since neither Ala_4 nor AVFA is commercially available, they and their possible hydrolysis products were prepared by Fmoc solid phase synthesis using an automated solid phase peptide synthesizer (Liu et al., 2010). LYA derivatives of these peptides and their possible hydrolysis products were synthesized using the method of Pantoja et al. (1997). Briefly, peptides were condensed with LYA in refluxing aqueous acetate buffer at pH 5 and 105ºC. Raw products were purified by ion exchange column (DOWEX, 50W-X12, 200-400mesh) or HPLC. Peptide hydrolysis was measured by monitoring the loss of the fluorescently labeled peptides (LYA-Ala_4 or LYA-AVFA) and production of hydrolysis products (LYA-Ala, LYA-Ala_2, and LYA-Ala_3 for LYA-Ala_4 or LYA-ala, LYA-ala-val, and LYA-ala-val-phe for LYA-AVFA).

Surface samples for peptide hydrolysis measurements were either not filtered (whole water samples) or syringe-filtered through 0.7 μm pre-combusted GF/F filters (filtered water samples) immediately after collection. Hydrolysis was measured in whole water samples as well as filtered water samples to compare the two particle sizes. Incubation of water samples was begun on board immediately after collection, and samples moved to the lab later in the day; throughout the incubation, samples were held at constant temperature (24 ºC) in 40-ml borosilicate vials that had been combusted and capped with acid-cleaned Teflon caps. To initiate the incubations, LYA-AVFA and LYA-ALA_4 stock solutions were added to both whole water and filtered water samples to a final concentration of 100 nM. Subsamples were collected at

various intervals of time up to 12 hours and 60 hours for LYA-ALA_4 and LYA-AVFA, respectively. A typical LYA-ALA_4 sampling interval was 0, 0.3, 0.6, 1, 2, 4, 6, 8, 10, 12 hours; while a typical LYA-AVFA sampling interval for LYA-AVFA was 0, 2, 6, 10, 12, 16, 20, 24, 28, 30 hour in July or 0, 12, 24, 28, 30, 34, 37, 40, 43, 45, 54 in August. Hydrolysis was stopped by filtering samples though 0.2 μm polycarbonate syringe filters (Millipore) and then freezing them. Frozen samples were transported to Stony Brook for analysis. The loss of LYA-AVFA and LYA-ALA_4 and production of hydrolysis products were measured by HPLC using fluorescence detection with excitation and emission wavelengths of 424 and 550nm, respectively, as described by Pantoja et al. (1997).

Hydrolysis of both LYA-AVFA and LYA-ALA_4 usually follows a two-stage hydrolysis, starting with a slow degradation stage where less than ~10% of the substrate is lost, then followed by more rapid loss and subsequent appearance of hydrolysis products. Therefore, peptide hydrolysis was investigated using two separate approaches. In the first approach, peptide hydrolysis rate constants (k) were calculated after the first 6 -12 h with an assumption of first order degradation. Peptide hydrolysis rates (R) were then calculated as k times the measured DCAA concentrations as in Pantoja and Lee (1994). This first stage is most likely to approach natural conditions.

Using a second approach, we consider the entire length of hydrolysis. It is unlikely that natural conditions prevail for this long (30-50 h), but the degradation does show something about the general potential of the peptides to be hydrolyzed. For calculation of this hydrolysis potential, we do not include the lag time (τ), that is, the length of time of the slow degradation stage before more rapid exponential loss of LYA-analog begins. We define a hydrolysis potential (k_p) as the first-order loss of substrate during the later stage of hydrolysis; we suggest that this is a measure

of the potential for local microbial communities to digest the supplied dissolved organic nitrogen. This potential was measured from the end of the lag time to either when all substrate was gone or to the end of the incubation. Here we report data only for stations where more than 50% of fluorescent substrates were hydrolyzed during the period of sampling. Hence, data for LYA-ALA_4 is reported at only one station (Bloom station in August).

4.2.4. Chlorophyll and particulate carbon and nitrogen measurements

At each station, surface water samples were filtered through a 0.2 μm Supor cartridge filter using a peristaltic pump and collected in sterile Falcon centrifuge tubes. Water was also filtered onto pre-combusted glass fiber (GF/F) filters (nominal pore size of 0.7 μm) for analysis of chlorophyll *a* (Chl-*a*) and total particulate carbon (TPC) and nitrogen (TPN). All filtered samples were immediately frozen until analysis. Chl-*a* samples were analyzed fluorometrically within 5 days of collection (Welschmeyer, 1994). PN and PC samples were dried and pelletized into tin disks prior to analysis using a Europa automated nitrogen and carbon analyzer. This work was done by the Mulholland Lab at Old Dominion University.

4.2.5. Uptake of organic nitrogen and inorganic nutrients

For uptake experiments, water was placed into acid-cleaned PET bottles. Incubations were initiated by adding 0.1 μmol $^{15}N\ L^{-1}$ highly enriched (96-99% ^{15}N and/or ^{13}C) dialanine or glutamic acid stock. While additions were targeted to achieve an atom % enrichment of approximately 10%, actual atom % enrichments varied depending on the concentration of DCAA at stations along cruise transects. All enrichments were > 4%, a level above which reliable uptake rates can be calculated (Mulholland et al., 2009). Uptake rates were measured in triplicate. Except for bicarbonate incubations, which were incubated for 24 hours, uptake experiments were terminated after 30 minutes to 1 hour by gently filtering the entire sample through precombusted

GF/F filters, rinsed with filtered water, and frozen until analysis. Uptake experiments measuring uptake of dialanine were conducted at the same time as the hydrolysis rate experiments and uptake samples were filtered and a peptide hydrolysis sample collected at a common time point for direct comparison. Filters were dried at 40 ºC for two days in a drying oven, then pelletized into tin discs and analyzed using a Europa 20/20 mass spectrometer equipped with an automated N and C analyzer (ANCA) preparation module. Uptake rates were calculated using a mixing model and equations from Montoya (1996) and Orcutt et al. (2001) as explained in detail in Mulholland and Lee (2009). This work was done by the Mulholland lab at Old Dominion University

4.2.6. Statistical analysis

Principal component analysis (PCA), a multivariate regression analysis, reduces a data matrix with a large number of variables to a few variables that explain the majority of the variance in the data. PCA is used frequently in the analysis of complex organic compound datasets (e.g., Yunker et al., 1995; Ingalls et al., 2006; Goutx et al., 2007; Xue et al., 2011). Usually, 60~90% of variance in a dataset can be explained by the first three components using PCA analysis. PCA does not specify the underlying cause of variability, but instead, identifies the factors that mostly related with parameters. PCA was performed here on two types of datasets using MYSTAT (version12, SYSTAT Software, Inc.). The first type includes only the composition of the DFAA and DCAA pools. PCA1 includes relative abundances (mol%) of individual DFAA at all stations, and PCA2 includes relative abundances (mol%) of individual DCAA at all stations. The DFAA dataset includes only ASP, GLU, SER, GLY and ALA. The DCAA data set includes only ASP, GLU, THR, SER, GLY, ALA, BALA, ARG, LEU, ILE, PHE, TYR and HIS. The second type of PCA analysis includes all environmental parameter

measured: DCAA composition, DFAA and DCAA concentrations, peptide hydrolysis rate constants and lag times in both whole and filtered water samples, uptake rates of amino acids and dipeptides, salinity, chlorophyll *a*, oxygen, particulate carbon and nitrogen, nutrient and urea concentrations. PCA graphs plotted here include both the loadings of each variance and the site scores, with values of site scores divided by five for scale.

Pearson correlation analysis was applied to determine the correlation between environmental parameters and the first two PCs from PCA1 and PCA2 (Tables 4.3). PCA analysis was also applied to DCAA compositions in estuarine stations, i.e., excluding J22, A23 and A29 (graph not shown). The first two PCs of this analysis and other environmental parameters measured at the estuarine stations were further analyzed using Pearson correlation analysis.

4.3. Results

4.3.1 Environmental parameters in the James River estuary and lower part Chesapeake Bay

A variety of environmental parameters were monitored during the two summer cruises in 2008 (Table 4.1). In general, Chl-*a* concentrations were higher at the low salinity end-member and decreased with increasing salinity. In the mid-estuary (as defined in Fig. 4.1), however, a local bloom of *Cochlodinium polykrikoides* was observed in August (Mulholland et al., 2009), with Chl-*a* concentrations of up to 31.6 $\mu g\ L^{-1}$. Large chlorophyll maxima have been previously observed at the main turbidity maximum in the James River estuary; however, this phenomenon occurs further upstream in very low salinity waters (Moon and Dunstan, 1990; Bukaveckas et al., 2011). However, local chlorophyll maxima occur in the meso- and polyhaline Chesapeake Bay as a result of algal blooms that are common during summer months (Marshall et al. 2006, Mulholland et al., 2009).

Ammonium was the most abundant form of dissolved inorganic nitrogen (DIN) at most stations during both the July and August sampling periods, with no discernable pattern with salinity. Nitrate was always less than 0.6 μM and decreased slightly as salinity increased. Urea concentrations were always less than 0.5 μM and did not vary substantially over the length of the estuary during the sampling period. Higher phosphate and silicate concentrations were observed in the estuary, with lower concentrations at the mouth of James River and in Chesapeake Bay.

4.3.2. Distribution of free and combined amino acids

DCAA concentrations ranged from 0.46 -1.65 μM during the study period; these values are consistent with previous reports from the Chesapeake Bay drainage area (Mulholland et al., 2003, 0.70~3.87μM in Pocomoke River in May and August 1999 and 2000) as well as other estuaries (Bronk, 2002, and references therein). The lowest DCAA concentrations (0.46μM) were observed in August at the mouth of Chesapeake Bay adjacent to the Atlantic Ocean. These are similar to concentrations observed in open ocean surface seawater (0.2 μM) (Kuznetsova and Lee, 2004; Kaiser and Benner, 2009). There was no clear correlation between DCAA concentration and salinity. Higher DCAA concentrations were observed in the area with higher chlorophyll, likely because DCAA were derived from cell death and decomposition. Comparing the two cruises, DCAA was statistically higher in August (1.12 μM on average) than in July (0.76 μM on average). DFAA concentrations ranged from 0.03 - 0.55 μM, and were in the same range as found in the Pocomoke River (0.07~1.34μM) (Mulholland et al. 2003) and in other estuarine areas (Bronk, 2002, and references therein). Similar to DCAA, DFAA concentrations were also higher in August than in July (Table 4.1). The highest concentrations of DFAA were observed at the lower part of the James River estuary in August. No significant correlations were

observed between DFAA and other environmental parameters, e.g. salinity, chlorophyll *a* or PC/PN. DFAA accounted for 3-31% of THAA in surface waters (Table 4.1). The ratio of DFAA/THAA ranging from 0.03 to 0.31 is slightly higher than observations from the Sargasso sea where DFAA/THAA ratios of 0.04 to 0.09 have been found (Keil and Kirchman, 1999), but similar to those in coastal waters (Kuznetsova and Lee, 2002). For most stations surveyed, DFAA concentrations in both August and July were less than 20% of THAA; two exceptions were from more saline stations in August with a much higher proportion of DFAA, from 24% to 31% of THAA. The DFAA/THAA ratio in August was slightly higher than in July.

DFAA compositions were similar to those reported previously from other estuarine systems (Lee and Bada, 1977; Keil and Kirchman, 1999; Kuznetsova and Lee, 2002), with aspartic acid (ASP), glutamic acid (GLU), glycine (GLY), and alanine (ALA) as the major components (Fig. 4.2). GLY and ALA are the most common components of the DCAA pool in James River estuary and adjacent Bay area with ASP and GLU also important. Hydrophobic amino acids like threonine (THR), tyrosine (TYR), valine (VAL), and leucine (LEU) account for smaller percentages of the DCAA pool. The non-protein amino acids, GABA and BALA were present in all stations surveyed during this study, with BALA clearly present in DCAA. More saline stations showed noticeably different DCAA compositions from those at the lower salinity end-member. The relative abundance of GLU increased along the salinity transect to the open ocean in both July and August. In contrast, decreasing GLY and ALA were observed in July, and less so in August. BALA decreased with increasing salinity in both cruises, while GABA increased with salinity in August (Fig. 4.3). A detailed PCA analysis of DCAA and DFAA composition is presented in section 4.3.4.

4.3.3. Peptide hydrolysis as indicated by loss of LYA-AVFA and LYA-ALA$_4$

As seen in other studies (e.g., Kuznetsova and Lee, 2001; Liu et al., 2010), hydrolysis of LYA-AVFA and LYA-ALA_4 generally followed a two-stage degradation pattern, with slow initial degradation followed by later exponential loss. Hydrolysis rate constants (k) of LYA-AVFA and LYA-ALA_4 were calculated for the slow initial loss over the first 6 to 13 h in both whole water and filtered water samples (Table 4.2). Relatively low rate constants that ranged from undetectable to 0.05 h^{-1} were observed at all stations, except for the bloom station in August, where higher peptide hydrolysis rate constants of 0.15 h^{-1} for LYA-AVFA and 0.03 h^{-1} for LYA-ALA_4 were observed. These rate constants were similar to those using LYA-ALA_4 in the Pokomoke River (0.007 ~0.215 h^{-1}, calculated using data from Mulholland et al., 2003). Rate constants of whole water samples were always larger than filtered counterparts, and rate constants of LYA-AVFA were always larger than those of LYA-ALA_4. There was no clear relationship between the rate constants and salinity, even though the lowest hydrolysis rate constants in whole water were observed at the ocean-most stations (A29). But a strong correlation with chlorophyll *a* was observed by rate constants of both LYA-AVFA ($r = 0.90$, $p<0.05$) and LYA-ALA_4 ($r = 0.75$, $p<0.05$). Hydrolysis rate constants measured here allow us to estimate peptide hydrolysis rates by multiplying rate constants by DCAA concentrations, with the assumption that all the combined amino acids can be hydrolyzed at the same rate as LYA-AVFA or LYA-ALA_4. Peptide hydrolysis rates estimated in this way ranged from undetectable to 0.24 μM h^{-1} for LYA-AVFA, and from undetectable to 0.04 μM h^{-1} for LYA-ALA_4.

Lag times at the beginning of LYA-AVFA and LYA-ALA_4 hydrolysis were often longer than the 6-12 h used in the calculation of hydrolysis rate constants above. They were observed in all the filtered samples and most whole water samples except at the Bloom station in August (Table 4.2). In previous studies, hydrolysis of both AVFA and LYA-ALA_4 followed a similar

two-stage pattern with first-order hydrolysis following a short lag time (e.g., Kuznetsova and Lee, 2001; Liu et al., 2010). Lag times in filtered samples (12 to >50 h) were always longer than those in their whole water counterparts (0 – 16 h). Hydrolysis at the August Bloom station was among the shortest lag times measured for both filtered and whole water. There was no clear pattern between lag time and salinity. Compared with LYA-AVFA, LYA-ALA_4 showed a slightly longer lag time in whole water samples at the Bloom station.

Hydrolysis potentials of LYA-AVFA were also measured in both filtered and whole water samples. After the initial slow degradation stage, or lag time, LYA-AVFA decreased exponentially with time and transient hydrolysis products (LYA-AVF and LYA-AV) were usually observed (Fig. 4.4A). LYA-AV was the dominant hydrolysis product in most filtered and whole water samples by the end of incubations. However, occasionally (e.g., filtered A16 and A23 and whole A29 samples), LYA-AVF was the major hydrolysis product. LYA-ala production was not observed, likely due to the short incubation times. Similar patterns were also observed during the degradation of LYA-ALA_4, during which LYA-ala2 appeared to be the dominant production.

Peptide hydrolysis potentials of LYA-AVFA ranged from 0.13~0.41 h^{-1} in whole water samples and from 0.09~0.55h^{-1} in filtered water samples, which were relatively higher than the corresponding hydrolysis rate constants (Table 4.2). The correlation between hydrolysis potentials of whole water samples and salinity was not strong ($r = 0.02$, $p < 0.05$; Table 4.3), although they were generally higher in the more saline Bay stations (A23, A29, and J22) (Table 4.2). Excluding Bay stations (A23, A29 and J22), the highest potential (k_{pw}) appeared at J5 and A22, where highest concentrations of particulate carbon (TPC) and particulate nitrogen (TPN) were observed (Table 4.2). There was a significant correlation between TPN and k_{pw} of LYA-

AVFA hydrolysis in the estuary ($r = 0.70$, $p < 0.05$; Table 4.4). Although the lag phase were longer in filtered than in whole water samples, the hydrolysis potentials were usually similar between whole water samples and their filtered counterparts. Due to the shorter incubation time for LYA-ALA_4 in these experiments (12 h for LYA-ALA_4 vs. 59 h for LYA-AVFA), complete loss of LYA-ALA_4 was observed only in whole water samples from the Bloom station (Fig. 4.4B). Hydrolysis rate potentials of LYA-AVFA (0.19 h^{-1}) and LYA-ALA_4 (0.18h^{-1}) were similar in whole Bloom station water (Table 4.2).

4.3.4 Uptake of organic nitrogen along James River transects

Uptake of organic nitrogen was surveyed in this study using three isotopically labeled compounds, urea, glutamic acid and dialanine, which are small, labile organic nitrogen compounds. Uptake rates of these three compounds have been previously measured in Chesapeake Bay and similar environments (Dzurica et al., 1989; Keil and Kirchman, 1991; Mulholland and Lee, 2009). Our measured uptake rates (Table 4.2) are at the lower end of previous measurement ranges in Chesapeake Bay, 0.002~2.22μM h^{-1} for glutamic acid and 0.02~1.27μMN h^{-1} for dialanine (Mulholland et al., 2002; Mulholland and Lee, 2009). Uptake of urea was generally higher than that of the other two organic nitrogen compounds except at station A16 in August where dialanine and urea uptake were similar. None of the uptake of these three organic nitrogen compounds are correlated with the salinity, But uptake of glutamic acid has a high correlation with TPN and chlorophyll in both estuarine and bay stations ($r = 0.67$ and 0.73, respectively, $p > 0.05$, Table 4.3).

We can compare these measured uptake rates with the peptide hydrolysis rates estimated above. We estimated that uptake of glutamic acid and dialanine was equivalent to 41-550% of peptide hydrolysis rates in whole water samples (Table 4.2). Only 41% of the hydrolyzed peptide

was taken up as amino acids at the Bloom station, and there is no clear trend in this ratio along the salinity transect. Thus, peptide hydrolysis and amino acid uptake are not always tightly coupled at all the stations. It is probable, however, that hydrolysis and uptake are underestimated because the estimation of both rates requires several assumptions. First, peptide hydrolysis rates estimated here are calculated by multiplying first-order hydrolysis rate constants times the DCAA concentrations with the assumption that all dissolved combined amino acids are hydrolyzed at the same rate as LYA-AVFA. This obviously oversimplifies the complex nature of DCAA since differences in hydrolysis rates of various peptides have been well documented (Pantoja et al., 1999). Second, it is assumed that all hydrolysis products are taken up at the same rate as dipeptides. Pantoja et al. (1999) suggested that dipeptides are a major hydrolysis product, since their hydrolysis rate is much lower than that of peptides with more than 4 amino acids. Thus, uptake of dipeptide may be a reasonable indicator for hydrolysis product uptake.

4.3.5. Statistical Analysis

Principal component analyses were performed on three separate datasets. The first two datasets included only the relative abundances of individual amino acids in DFAA (PCA1) or DCAA (PCA2) (Fig. 4.5). PCA of the third dataset investigates the relationship between amino acid composition and concentration and other important environmental parameters (PCA3) (Fig. 4.6). The first two components of these PCA(s) were analyzed with Pearson's correlation analysis to investigate the relationship between DCAA composition and station location along the salinity gradient (Table 4.3).

4.3.5.1. PC analysis of DFAA (PCA1) and DCAA (PCA2) compositions

In PCA1, using the DFAA dataset, the first two components explain 82% of the variance (Fig. 4.5A). PC1 has the highest loadings of GLU and ALA, and lowest of SER. GLY is highest

along the PC2 axis, and ASP is lowest. There is no clear trend with salinity, although there is a tendency for more saline stations to be generally found in the upper half of the graph and lower salinity stations in the bottom half. This places the bloom station with the saline stations, which are generally enriched in GLU, ALA and GLY. Pearson analysis with other environmental parameters indicates that PC1 has the strongest negative correlation with rate constants of LYA-AVFA hydrolysis in whole water samples ($r = -0.74$, $p < 0.05$; Table 4.3), while PC2 has the strongest correlation with uptake rates of free amino acids (as UGLU, $r = 0.74$, $p < 0.05$).

In PCA2, using the DCAA dataset, the first two components of PCA2 explained 45% of the variance (Fig. 4.5B). Amino acid composition changes from fresh to saline water along the axis of PC1 from left to right. Toward the left on the PC1 axis are BALA, ALA and GLY, while GLU and HIS are located to the right. This is consistent with previous work showing terrestrial input of more degraded organic matter from freshwater (GLY, ALA and BALA) and fresher organic matter in salt water (GLU) (Chen et al., 2004). The Pearson correlation coefficient between salinity and PC1 was much higher ($r = 0.85$, $p < 0.05$; Table 4.3) than DFAA, also supporting the importance of salinity as a primary factor influencing DCAA composition. PC1 is also negatively correlated with uptake rates of dialanine, supporting the idea that peptide uptake plays an important role in determining DCAA composition. PC2 is characterized by positive loadings of THR, and negative loadings of LEU and ILE. Pearson analysis shows that PC2 has the highest positive correlation hydrolysis potentials of LYA-AVFA in filtered seawater ($r = 0.60$, $p < 0.05$), and the highest negative correlation with lag time of LYA-AVFA hydrolysis in filtered seawater ($r = -0.47$, $p < 0.05$). This suggests that peptide hydrolysis also influences DCAA compositions to some extent. The Bloom station is in the middle of Fig. 4.5A, implying that the Bloom had little influence on DCAA composition.

4.3.5.2. PC analysis of all the organic nitrogen cycling parameters (PCA3)

To further investigate mechanisms influencing organic nitrogen cycling in the area studied, PCA3 included all parameters measured (Fig. 4.6). PC1 and PC2 explain 41% of the variance. In this plot, freshwater stations with low salinity, high nutrient input and degraded organic matter cluster on the right (b). Open ocean stations with high salinity, low chlorophyll but fresh organic matter cluster on the left (a). The Bloom station with high chlorophyll and high particulate nitrogen (TPN) stands alone (c) at the top of graph, while other stations lie between these three groupings. The hydrolysis potentials for both filtered and whole water are closest to the freshwater cluster. The first principal component is consistent with mixing along the salinity gradient, while the second principal component is characterized by high loading of TPN and Chl-*a*.

4.4. Discussion

4.4.1 Application of LYA-AVFA in evaluating peptide hydrolysis

Fluorescent analogs of peptides and amino acids have long been used in studies of the enzymatic hydrolysis of organic nitrogen compounds (e.g., Hoppe, 1983; Somville and Billen, 1983; Chrost, 1991; Pantoja et al., 1997). An advantage of using fluorescent analogs instead of native compounds is that analytical detection limits of fluorescent analogs are much lower than the native compounds; additions as low as 5 nM, which is close to amino acid concentrations in natural aquatic environments, can be used. Fluorescent analogs also tend to be much cheaper and easier to use than ^{14}C or ^{3}H-labeled compounds. Here we applied a new fluorescent peptide analog, LYA-AVFA to study peptide hydrolysis in estuaries. Rate constants of LYA-AVFA measured here ranged from undetectable to ~ 0.15 h^{-1} in whole water samples and from undetectable to ~ 0.01 h^{-1} in filtered water samples, consistent with results for peptide hydrolysis

obtained by others in this or similar estuarine regions (e.g., Mulholland et al., 2002). These rate constants are calculated over the initial 6-12 h incubation period.

An initial slow hydrolysis of LYA-AVFA and LYA-ALA4 followed by more rapid hydrolysis was observed in our study and many other studies (e.g., Kuznetsova and Lee, 2001; Liu et al., 2010). This initial slow hydrolysis, or lag time, of substrate hydrolysis is often observed before any decrease occurs in either native peptides or fluorescent analogs, but it is not always observed, and the causes of this phenomenon are poorly understood. Several possible explanations have been suggested to explain the lag time. For example, general microbiological theory suggests that bacteria frequently undergo a lag in cell growth when they change environments (Hill and Wright, 1997). That lag could be due to either a change in enzyme concentration or in substrate concentration. Either change could result in a lag in peptide hydrolysis or amino acid uptake. Enzyme concentration can increase due to increased production by bacteria present or after a change in community structure. Lag times might be caused by other factors as well. For example, enzymatic hydrolysis can be inhibited at lower temperatures. However, previous studies have demonstrated that temperature is not directly correlated with lag time in seawater (Kuznetsova and Lee, 2001). In addition Liu et al. (2010) showed that AVFA and SWGA (serine–tryptophan–glycine–alanine) hydrolysis were similar under light and dark conditions, suggesting that light has little influence on peptide hydrolysis.

Our peptide hydrolysis experiments show the influence of both the substrate and enzyme concentrations on lag time. First, we changed the substrate concentrations since we introduce a new substrate into the experimental incubations. Thus, proteolytic enzymes that might be adapted to low concentrations need to be induced (or synthesized) before that substrate can be hydrolyzed. Although we add LYA-AVFA at concentrations of only 10-20% of total DCAA, a

relatively large portion of DCAA may not be available to proteolytic enzymes, either because they are complexed with other compounds, or are intrinsically resistant. The fraction of DCAA that is labile most likely varies in the environments where lag times are different. Second, in our filtration experiment, we changed the enzyme concentrations. Sample filtration appreciably extended measured lag times. Most enzymes are cell associated, so filtration effectively reduces the enzyme concentration. The 0.7μm filters we used do not remove all the organisms capable of synthesizing enzymes, so we see an increase in lag time while the organisms synthesize more enzymes. As further evidence for this idea, Kuznetsova and Lee (2001) observed more rapid LYA-peptide hydrolysis in summer when microbial activity (and presumably enzymatic activity) was higher. The Bloom station in our study is a special case where both enzyme and substrate concentrations are strongly affected by phytoplankton productivity. The shortest lag time was observed at this station in August. Growth of phytoplankton introduces more labile particulate and dissolved organic nitrogen into the water column compared to the relatively refractory organic matter from resuspended sediment and terrestrial inputs. However, the increased phytoplankton biomass, as suggested by the high Chl-*a* concentration at this station, also introduces higher enzyme concentrations (Mulholland and Lee, 2009), which would decrease the lag time. The correlation of hydrolysis rate constant with Chl-*a* suggests that the increase of substrate can enhance the peptide hydrolysis in the estuarine environments. The exponential loss after the slow degradation suggests that extracellular enzymes have the potential to hydrolyze additional substrate more efficiently. This potential is more influenced by the enzyme capability as a response to the ecological setting.

A comparison of our study with that of Liu et al. (2010) allows us to further investigate the effect of enzyme and substrate concentration on peptide hydrolysis. Liu et al. (2010)

measured hydrolysis of AVFA in the Elizabeth River as well as the James River Estuary at the same time as this study, allowing us to compare hydrolysis rates and patterns of the underivatized peptide and its LYA-derivatives. These native peptide experiments required the addition of substrate concentrations two orders of magnitude higher than that used here for the fluorescent peptides. AVFA and LYA-AVFA incubations showed similar two-stage hydrolysis patterns. The lag time of AVFA (24-48 h) was 1-2 days longer than that of LYA-AVFA (0-16 h). First-order hydrolysis potentials were relatively lower for AVFA (0.03-0.08 h^{-1}) than for LYA-AVFA (0.13-0.41h^{-1}). Two explanations might account for these differences in lag times and hydrolysis potentials. The higher substrate concentrations used during the AVFA incubation might result in a delay in hydrolysis while enzymes are synthesized or induced as discussed above. A second explanation could be that the attachment of LYA to the peptide might cause steric hindrance, reducing the access of proteolytic enzymes to the substrate. Previous studies on LYA-ALA_4 suggest that steric hindrance by LYA does not change hydrolysis rates of tetraalanine (Pantoja et al., 1999). Our results also support the idea that steric hindrance is less important since hydrolysis potentials were greater and lag time was shorter in LYA-AVFA incubations. In a comparison between the James and Elizabeth Rivers, AVFA hydrolysis in the highly turbid Elizabeth River had no lag time at all, suggesting that sediment might supply additional enzymes to the water column and reduce the lag time.

Although the LYA-derivatives are structurally more similar to natural peptides than some other fluorescent derivatives, there are clearly limitations to the use of LYA-AVFA hydrolysis as an indicator of proteolytic hydrolysis in aquatic environments, and these should be considered in any interpretation of the data. First, the nature of LYA-AVFA is obviously distinct from the bulk of THAA, which is characterized by heterogeneous and complex chemical structure and

bioavailability (Keil and Kirchman, 1993). Second, the k_p values discussed here are calculated for the exponential decrease that occurs after a lag time of up to 50 hours. A change in the bacterial communities and their resulting enzymatic activities would occur during this period due to changes in ambient THAA substrate, bacterial activity, enzymatic level, etc. As with other fluorescent analogs, the LYA-peptide technique can be used as a measure of the potential capability of THAA hydrolysis in certain environments. However, comparison of LYA-AVFA hydrolysis with organic N uptake suggests that this substrate is a reasonable indicator of peptide hydrolysis in complicated aquatic ecosystems like James River estuary and the adjacent Bay area.

4.4.2 Particle associated hydrolysis and uptake of organic nitrogen in the James River estuary

The influence of particles on organic nitrogen cycling is addressed in our studies in the James River estuary as well as in previous studies in other estuarine areas. In our study, a relatively strong correlation was observed between hydrolysis potentials of whole water LYA-AVFA hydrolysis, k_{pw}, and particulate nitrogen (TPN) and particulate carbon (TPC) concentrations ($r = 0.70$ and $r = 0.63$, $p < 0.05$, respectively; Table 4.4), while k_{pf} had a relatively weaker negative correlation with TPN ($r = -0.47$, $p < 0.05$). In contrast, the k_{pw} of LYA-AVFA is independent of DCAA ($r = 0.13$, $p < 0.05$) and chlorophyll *a* ($r = -0.02$, $p < 0.05$) concentrations. Principal component analysis of all environmental parameters (Fig. 4.6) further demonstrates that TPN explains the second largest variance in the data. Additional evidence for the role of particles is that peptide hydrolysis lag time was much longer after sample filtration, supporting the idea that tetrapeptide hydrolysis is predominantly associated with particles larger than 0.7μm. Furthermore, in addition to increased lag times after filtration, LYA-AVF became the dominant product instead of LYA-AV at some stations (A16 and A23). Thus, the presence of particles may not only reduce enzyme abundance in the water column, but also alter hydrolysis patterns.

Previous studies have also suggested that enzymatic hydrolysis is strongly associated with large particles and aggregates (Pantoja and Lee, 1999; Mulholland and Lee, 2003; Grossart et al., 2007; Mulholland et al., 2009).

There are multiple sources of particles in estuaries, e.g., riverine input, sediment resuspension, and in-situ phytoplankton growth. In spite of a strong correlation with TPN, k_{pw} is poorly correlated with either chlorophyll *a* or primary production (in terms of uptake rate of HCO_3^-, Tables 4.2 and 4.4), suggesting that in-situ phytoplankton growth does not directly influence enzymatic peptide hydrolysis potentials in estuaries. The correlation of k_{pw} with salinity is also low ($r = 0.02$, $p < 0.05$), implying limited influence from terrestrial input. According to previous reports, peptide hydrolysis in sediments is usually much faster than in the water column (Pantoja et al., 1997). If sediment resuspension due to estuarine turbulence releases enzymes from the sediment, peptide hydrolysis would be enhanced in water with more resuspended particles, as we observed.

The uptake of amino acids (as glutamic acid) is also enhanced in stations with high particle (as TPN and TPC) abundance in the James River Estuary (Table 4.4.). Relatively strong correlations were identified between TPC and TPN concentrations and uptake of glutamic acid (UGLU) ($r = 0.74$ and 0.61, $p < 0.05$, respectively, Table 4.4.), similar to the correlation with peptide hydrolysis potentials, k_{pw}. PCA of these parameters also shows the close correlation (Fig. 4.6). In contrast to peptide hydrolysis, however, the correlation between uptake of glutamic acid and chlorophyll concentrations ($r = 0.82$, $p < 0.05$, Table 4.4.) was strong. Meanwhile, Hydrolysis rate constants k_w and k_s were also correlated to Chl-*a* ($r = 0.90$, $p < 0.05$) but independent of TPN ($r = 0.29$, $p < 0.05$). The correlations observed between peptide hydrolysis potential and TPC/TPN, and amino acid uptake and TPC/TPN, suggest that particles influence

both hydrolysis potentials and uptake, most likely because the hydrolytic enzymes are associated with particles. However, initial hydrolysis rates are more influenced by labile organic matter input from local phytoplankton growth, which can also enhance the uptake of amino acids.

Although both peptide hydrolysis and amino acid uptake were strongly associated with particles, the mechanisms of association appear to be different, with k_{pw} more related to sediment resuspension, and peptide hydrolysis rate and GLU uptake rate more related to in-situ biomass. In addition, estimated hydrolysis rates of LYA-AVFA (that assumed all DCAA were available to enzymatic hydrolysis) were sometimes faster than the sum of uptake of glutamic acid and dialanine, suggesting that the supply of free amino acids and dipeptide by peptide hydrolysis is not always be the limiting factor for uptake of these compounds. This is not necessarily contrary to the idea of close coupling of hydrolysis and uptake (Fuhrman, 1987; Hoppe et al., 1993; Kuznetsova et al.2004; Mulholland and Lee, 2009). Diverse input and removal processes can potentially influence both peptide hydrolysis and uptake, but not necessarily simultaneously. For example, higher concentrations of free amino acids were observed at the Bloom station where phytoplankton biomass was high; such a correlation has been noted previously (Coffin, 1989; Sellner and Nealley, 1997). If phytoplankton (or zooplankton) excretion is a significant source of free amino acids, as seems likely, this would be a source of amino acid substrate in addition to peptide hydrolysis.

4.4.3 Changes in DCAA composition and concentrations in the James River estuary and lower Chesapeake Bay

In spite of the different data sets used, both PCA analyses that include DCAA composition (Fig. 4.5B and 4.6) have first principal components that are strongly correlated with distance from the freshest water stations. Two of the major factors that change with distance

along this estuarine gradient are salinity and terrestrial organic matter input. Two end-member mixing of parameters between fresh and saline water results in their distribution being conserved in estuaries (Boyle et al., 1974). Although the relation between composition and distance from the freshest water stations is not strictly linear in our data, the strong correlation ($r = 0.85$, $p < 0.05$, Table 4.3) implies that the mixing of fresh and saline water is the primary factor controlling the variability in DCAA composition. The changing source of the organic matter is likely to be more important to this variability than any difference strictly due to the change in salt content. The river discharges some organic matter from freshwater algae but also considerable material of terrestrial origin into the estuary, while at the ocean end, DCAA originates from primary production by marine algae. Mixing of these sources causes the gradient of DCAA composition in the estuaries.

Evidence for input from degraded material is clear from DCAA compositional data. Elevated mol% ALA and GLY were observed at the freshest water stations. ALA and GLY are frequently enriched in DCAA of coastal bottom water compared with open ocean surface water (Yamashita and Tanoue, 2003). Previous studies have shown elevated mol% ALA and GLY in more degraded samples of marine particles and thus these amino acids (particularly GLY) are considered to be indicators of degradation of POM (Lee et al., 2000; Ingalls et al., 2003) and sediments (Dauwe and Middleburg, 1998). Thus, elevated ALA and GLY in terrestrially derived DCAA might result from the relatively refractory nature of terrestrial organic matter. The refractory nature of terrestrial organic matter has been observed in other estuaries using different geochemical tools (e.g., Repeta et al., 2002). The appearance of the non-protein amino acids GABA and BALA are also usually considered as indicators of amino acid degradation during early diagenesis (Cowie and Hedges, 1994; Ingalls et al., 2003). However, our study in the James

River estuary exhibited contrasting evidence for this, with opposite correlations of GABA and BALA with salinity (Fig. 4.3). This finding agrees with work in the Western Arctic Ocean, where the mol% dissolved BALA decreased with depth, while mol% dissolved GABA increased with depth in the same samples (Davis et al., 2009). These results suggest that using amino acid composition as a degradation index requires caution in evaluating organic matter degradation state in estuarine areas.

Even though DCAA composition was strongly correlated with distance from the Chesapeake Bay (Fig. 4.5B), there was no correlation ($r = -0.12$, $p < 0.05$; Table 4.3) observed between DCAA concentrations and salinity (as an indicator of distance down the estuary). This absence of correlation indicates that the concentration of DCAA is not related to the mixing of fresh and saline end members in the James River estuary. The absence of correlation between concentrations of labile organic components and salinity is frequently observed in estuaries, and is thought to be due to their short retention time in the water column. In-situ primary production may be an important source of labile proteinaceous materials in the estuary; this input has been largely underestimated in the past (Bianchi et al., 2004; Davis et al., 2009). Elevated chlorophyll was observed at the Bloom station in mid-estuary during both July and August (Table 4.1). Pearson correlation analysis demonstrates the relatively strong correlation of DCAA concentration (but not composition) with chlorophyll throughout the estuary ($r = 0.56$, $p < 0.05$; Table 4.3), suggesting that in situ primary production might be a major input of DCAA. As mentioned earlier, the lag time of LYA-AVFA hydrolysis is shorter at the Bloom station where Chl-*a* and DCAA are elevated, consistent with the assumption that shorter lag time is a response to higher ambient combined amino acid concentrations.

References:

Arnosti, C., 2004, Speed bumps and barricades in the carbon cycles: substrate structural effects on carbon cycling. Marine Chemistry, 92: 263-273.

Arnosti, C., 2011, Microbial extracellular enzymes and the marine carbon cycle, Annual Review of Marine Science, 3:401-425

Benner, R., 2002. Chemical composition and reactivity, pp. 59-90. In: C. Carlson and D. Hansell (Ed.), Biogeochemistry of Marine Dissolved Organic Matter. Academic, San Diego.

Berman, T., D.A. Bronk, 2003, Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquatic Microbial Ecology, 31: 279-305.

Berges, J.A. M. Mulholland, 2008, Enzymes and cellular N cycling, pp.1385-1444. In: Capone, D.G., Bronk, D. A., Muhholland, M. R., Carpenter, E. J., (eds.), Nitrogen in the Marine Environment, Elsevier/Academic

Bianchi, T.S., T. Filley, K. Dria, P.G. Hatcher, 2004. Temporal variability in sources of dissolved organic carbon in the lower Mississippi River. Geochimica et Cosmochimica Acta, 68: 959-967.

Billen, G., A. Fontigny, 1987, Dynamics of a *Phaeocystis*-dominated spring bloom in Belgian coastal waters, II. Bacterioplankton dynamics. Marine Ecology Progress Series, 37: 249-257.

Boyle, E., A.T. Collier, Dengler, A.T., Edmond, J.M., Ng, A.C., Stallard, R. F., 1974, On the chemical mass-balance in estuaries. Geochimica et Cosmochimica Acta, 38: 1719-1728.

Bronk, D.A., 2002. Dynamics of DON. In: C. Carlson, and D. Hansell, (Ed.), Biogeochemistry of Marine Dissolved Organic Matter. Academic, San Diego.

Bukaveckas, P.A., L.E. Barry, M.J. Beckwith, V. David, Lederer., 2011, Factors determining the location of the chlorophyll maximum and the fate of algal production within the tidal freshwater James River, Estuaries and Coasts, 34: 569-582.

Chen, J.F., Y. Li, K.D. Yin, H.Y. Jin, 2004, Amino acids in the Pearl River Estuary and adjacent waters: origins, transformation and degradation, Continental Shelf Research, 24: 1877-1894.

Chrost, R.J., 1991, Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: R.J. Chrost, (Ed.) Microbial Enzymes in Aquatic Environments, Springer-Verlag, New York.

Coffin, R. B., 1989. Bacterial uptake of dissolved free and combined amino-acids in the estuarine waters. Limnology and Oceanography, 34: 531-542.

Cowie, G.L., J.I. Hedges, 1994. Biochemical indicators of diagenetic alteration in organic-matter mixtures. Nature, 369: 304-307.

Davis, J., K. Kaiser, R. Benner, 2009. Amino acid and amino sugar yields and compositions as indicators of dissolved organic matter diagenesis. Organic Geochemistry, 40: 343-352.

Dauwe, B., J.J. Middelburg, 1998, Amino acids and hexosamines as indicators of organic matter degradation state in North Sea sediments, Limnology and oceanography, 43: 782-798.

Dzurica, S., C. Lee, E.M. Cosper, E.J. Carpenter, 1989. Role of environmental variables, specifically organic compounds and micronutrients, in the growth of the chrysophyte *Aureococcus anophagefferens*. In: E.M Cosper, V.M. Bricelj, E.J. Carpenter, (Ed.), Novel Phytoplankton Blooms: Causes and Impacts of Recurrent Brown. Tides and Other Unusual Blooms. Springer-Verlag, Berlin.

Fuhrman, J., 1987, Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach. Marine Ecology Progress Series, 37: 45-52.

Grace, B., T. Bianchi, 2010, Sorption and desoroption dynamics of bulk dissolved organic matter and amino acids in the Mississippi River plume – a microcosm study, Marine and Freshwater Research, 61: 1067-1081.

Goutx, M., S.G. Wakeham, C. Lee, M. Duflos, C. Guigue, Z. Liu, B. Moriceau, R. Sempere, M. Tedetti, J. Xue, 2007. Composition and degradation of marine particles with different settling velocities in the northwestern Mediterrannean sea. Limnology and Oceanography, 52: 1645-1664.

Grossart, H.P., K.W. Tang, T. Kiorboe, H. Ploug, 2007. Comparison of cell-specific activity between free-living and attached bacteria using isolates and natural assemblages. FEMS Microbiology Letters, 266: 194-200.

Guldberg, L.B., K. Finster, N.O.G. Jorgensen, M. Middelboe, B.A. Lomstein, 2002. Utilization of marine sedimentary dissolved organic nitrogen by native anaerobic bacteria. Limnology and Oceanography, 47: 1712-1722.

Hill, B.P., K.M. Wright, 1994, A new model for bacterial growth in heterogeneous systems, Journal of Theoretical Biology, 168: 31-41.

Hollibaugh, J.T., F. Azam, 1983, Microbial degradation of dissolved proteins in seawater. Limnology and Oceanography, 28: 1104-1116.

Hoppe, H.G., 1983. Significance of exoenzymatic activities of the ecology of brackish water – Measurements by means of methylumbelliferyl substrates. Marine Ecology-Progress Series, 11: 299-308.

Hoppe, H.G., H. Ducklow, B. Karrasch, 1993. Evidence for dependency of bacterial-growth on enzymatic-hydrolysis of particulate organic matter in the mesopelagic ocean. Marine Ecology-Progress Series, 93: 277-283.

Hoppe, H.G., C. Arnosti, G. Herndl, 2002. Ecological significance of bacterial enzymes in the marine environment. In: R. Burns, R. Dick, (eds.), Enzymes in the environment: Activity, ecology and applications, Marcel Dekker, 73-107.

Ingalls, A.E., C. Lee, S.G. Wakeham, J.I. Hedges, 2003. The role of biominerals in the sinking flux and preservation of amino acids in the Southern Ocean along 170 degrees W. Deep-Sea Research Part II, 50: 713-738.

Ingalls, A.E., Z.F. Liu, C. Lee, 2006, Seasonal trends in the pigment and amino acid compositions of sinking particles in biogenic $CaCO_3$ and SiO_2 dominated regions of the Pacific sector of the Southern Ocean along 170 degrees W. Deep-Sea Research Part I, 53:836-859.

Kaiser, K., R. Benner, 2009. Biochemical composition and size distribution of organic matter at the Pacific and Atlantic time-series stations. Marine Chemistry, 113: 63-77.

Keil, R.G., D.L. Kirchman, 1991. Dissolved combined amino acids in marine waters as determined by a vapor-phase hydrolysis method. Marine Chemistry, 33: 243-259.

Keil, R.G., D.L. Kirchman, 1993, Dissolved combined amino acids: chemical form and utilization by marine bacteria. Limnology and Oceanography, 38:1256-1270.

Keil, R.G., D.L. Kirchman, 1999. Utilization of dissolved protein and amino acids in the northern Sargasso Sea. Aquatic Microbial Ecology, 18: 293-300.

Kroer, N., N. O. G. Jorgensen, R. B. Coffin, 1994, Utilization of dissolved nitrogen by heterotrophic bacterioplankton: a comparison of three ecosystems, Applied and Environmental Microbiology, 60: 4116-4123.

Kuznetsova, M., C. Lee, 2001. Enhanced extracellular enzymatic peptide hydrolysis in the sea-surface microlayer. Marine Chemistry, 73: 319-332.

Kuznetsova, M., C. Lee, 2002. Dissolved free and combined amino acids in nearshore seawater, sea surface microlayers and foams: Influence of extracellular hydrolysis. Aquatic Sciences, 64: 252-268.

Kuznetsova, M., C. Lee, J. Aller, N. Frew, 2004, Enrichment of amino acids in the sea surface microlayer at coastal and open ocean sites in the North Atlantic Ocean. Limnology and Oceanography, 49: 1605-1619.

Lee, C., J.L. Bada, 1977. Dissolved amino acids in equatorial Pacific, Sargasso Sea, and Biscayne Bay. Limnology and Oceanography, 22: 502-510.

Lee, C., S.G. Wakeham, J.I. Hedges, 2000. Composition and flux of particulate amino acids and chloropigments in equatorial Pacific seawater and sediments. Deep-Sea Research Part I 47: 1535-1568.

Lindroth, P., K. Mopper, 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthaldialdehyde. Analytical Chemistry, 51:1667-1674.

Liu, Z., M. Kobiela, G.A. McKee, T. Tang, C. Lee, Mulholland, M.R., Hatcher, P.G., 2010. The effect of chemical structure on the hydrolysis of tetrapeptides along a river-to-ocean transect: AVFA and SWGA. Marine Chemistry, 119: 108-120.

Marshall, H. G., R. Lacouture, C. Buchanan, J. Johnson, 2006, Phytoplankton assemblages associated with water quality and salinity regions in Chesapeake Bay, USA, Estuarine, Coastal and Shelf Science, 69:10-18.

Moon, C., M. Dunstan, 1990, Hydrodynamic trapping in the formation of the chlorophyll *a* peak in turbid, very low salinity waters of estuaries, Journal of Plankton Research, 12:323-336.

Montoya, J.P., M. Voss, P. Kahler, D. Capone, 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Applied Environmental Microbiology, 62: 986-993.

Mulholland, M.R., C.J. Gobler, C. Lee, 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnology and Oceanography, 47: 1094-1108.

Mulholland, M.R., C. Lee, P.M. Glibert, 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Marine Ecology-Progress Series, 258: 3-17.

Mulholland, M.R., C. Lee, 2009. Peptide hydrolysis and the uptake of dipeptides by phytoplankton. Limnology and oceanography, 54: 856-868.

Mulholland, M.R., R. E. Morse, G. E. Boneillo, P. W. Bernhardt, k. C. Filippino, L.A. Procise, J. L. Blanco-Garcia, H. G. Marshall, T. A. Egerton, W., S. Hunley, K. A. Moore, D. L. Berry, C. J. Gobler, 2009, Understanding causes and impacts of the dinoflagellate, *Cochlodinium polykrikoides,* blooms in the Chesapeake Bay. Estuaries and Coasts, 32:734-747.

Orcutt, K.M., F. Lipschultz, K. Gundersen, R. Arimoto, A.F. Michaels, A.H. Knap, J.R. Gallon, 2001. A seasonal study of the significance of N_2 fixation by *Trichodesmium spp*. At Bermuda Atlantic Time-series Study (BATS) site. Deep Sea Research Part II, 48: 1583-1608

Pantoja, S., C. Lee, 1994, Cell-surface oxidation of amino acids in seawater, Limnology and Oceanography, 39: 1718-1726.

Pantoja, S., C. Lee, J.F. Marecek, 1997. Hydrolysis of peptides in seawater and sediment. Marine Chemistry, 57:25-40.

Pantoja, S. C. Lee, 1999. Peptide decomposition by extracellular hydrolysis in coastal seawater and salt marsh sediment. Marine Chemistry, 63: 273-291.

Pantoja, S., P. Rossel, R. Castro, L.A. Cuevas, G. Daneri, C. Cordova, 2009. Microbial degradation rates of small peptides and amino acids in the oxygen minimum zone of Chilean coastal waters. Deep-Sea Research Part II – Topical Studies in Oceanography, 56: 1019-1026.

Payne J.W., 1980. Transport and utilization of peptides by bacterial, pp211-256. In: J.W. Payne, (Ed.), Microorganisms and Nitrogen Sources. Wiley, Chichester,.

Reinthaler, T., E. Sintes, G.J. Herndl, 2008. Dissolved organic matter and bacterial production and respiration in the sea-surface microlayer of the open Atlantic and the western Mediterranean Sea. Limnology and Oceanography, 53: 122-136.

Repeta, D.J., T.M. Quan, L.I. Aluwihare, and A.M. Accardi, 2002. Chemical characterization of high molecular weight dissolved organic matter in fresh and marine waters. Geochimica et Cosmochimica Acta, 66: 955-962.

Rosenstock, B., W. Zwisler, M. Simon, 2005. Bacterial comsumption of humic and non-humic low and high molecular weight DOM and the effect of solar irradiation on the turnover of labile DOM in the Southern Ocean. Microbial Ecology, 50: 90-101.

Pritchard, D.W., 1952. Salinity distribution and circulation in the Chesapeake Bay estuarine system. Journal of Marine Research, 11: 106-123.

Sellner, K.G., E.W. Nealley, 1997. Diel fluctuations in dissolved free amino acids and monosaccharides in Chesapeake Bay dinoflagellate blooms. Marine Chemistry, 56: 193-200.

Somville, M., G. Billen, 1983. A method for determining exoproteolytic activity in natural waters. Limnology and Oceanography, 28: 190-193.

Weiss, M.S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, G.E. Schulz, 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science, 254: 1627-1630.

Welschmeyer, N.A., 1994. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. Limnology and Oceanography, 39: 1985-1992.

Williams, P.J.L., T. Berman, O. Holm-Hansen, 1976, Amino acid uptake and respiration by marine heterotrophs. Marine Biology, 35: 41-47.

Wong, G.T.F., 1979. Alkalinity and pH in the southern Chesapeake Bay and the James River estuary. Limnology and Oceanography, 24: 970-977.

Xue, J., C. Lee, S.G. Wakeham, R.A. Armstrong, 2011. Using principal components analysis (PCA) with cluster analysis to study the organic geochemistry of sinking particles in the ocean. Organic Geochemistry, 42:356-367.

Yamashita, Y., E. Tanoue, 2003. Distribution and alteration of amino acids in bulk DOM along a transect from bay to oceanic waters. Marine Chemistry, 82: 145-160.

Yunker, M.B., R.W. Macdonald, D.J. Veltkamp, W.J. Cretney, 1995, Terrestrial and marine biomarkers in a seasonally ice-covered Arctic estuary – integration of multivariate and biomarker approaches. Marine Chemistry, 49: 1-50.

Tables:

	Salinity	DO mgL^{-1}	Chl*a* mgL^{-1}	TPC μMC	TPN μMN	DFAA μM	DCAA μM	DFAA /THAA
Jul 2008								
J1	1.25	4.78	9.59	65.9	10.9	0.05	0.62	0.08
J5	5.24	4.54	11.4	116	19.8	0.04	1.07	0.04
J10	9.82	4.69	8.40	75.7	15.7	0.08	0.49	0.17
J16	15.7	6.08	13.3	76.1	12.2	0.03	0.68	0.05
J20	19.5	5.34	5.61	42.3	8.8	0.06	0.90	0.06
J22	22.4	4.59	4.61	67.1	10.5	0.07	0.83	0.08
Aug 2008								
A1	0.94	4.75	17.4	90.7	15.1	0.06	1.11	0.05
A5	4.57	4.07	10.2	68.2	12.2	0.09	0.94	0.08
A10	9.80	4.83	7.91	64.5	12.0	0.12	1.54	0.07
A16	16.0	4.05	2.95	36.7	6.5	0.10	0.99	0.09
Bloom	20.8	7.20	31.6	119	16.8	0.20	1.58	0.11
A22	22.6	5.25	6.91	108	18.1	0.55	1.19	0.31
A23	22.9	6.34	10.0	175	28.1	0.25	0.69	0.24
A29	29.4	5.02	0.26	10.4	1.8	0.11	0.46	0.19

Table 4.1. Environmental parameters during the cruises of July and August, 2008. DFAA/THAA: Molecular ratio of DFAA to THAA.

	k_w^{AVFA}	k_{pw}^{AVFA}	K_f^{AVFA}	k_{pf}^{AVFA}	k_w^{ALA4}	k_{pw}^{ALA4}	k_f^{ALA4}	τ_w	τ_f	R_w^{AVFA}	R_w^{ALA4}	HCO_3^-	Urea	Glu	Diala	%
	h^{-1}	h^{-1}	h^{-1}	h^{-1}	h^{-1}	h^{-1}	h^{-1}	h	h	μMh^{-1}	μMh^{-1}	μMh^{-1}	μMh^{-1}	μMh^{-1}	μMh^{-1}	ON
Jul 2008																
J1	0.030	0.210	-0.002	ND	0.012	ND	0.003	12:36	24:00	0.02	0.01	4.79	0.16	0.01	0.03	228%
J5	0.019	0.410	0.002	ND	0.013	ND	N/A	8:36	24:00	0.02	0.01	10.61	0.32	0.03	0.08	550%
J10	0.030	0.170	0.003	ND	0.011	ND	-0.001	5:53	24:00	0.01	0.01	19.77	0.05	0.03	0.03	417%
J16	0.036	0.140	0.008	ND	0.017	ND	0.001	8:17	24:00	0.02	0.01	21.69	0.22	0.02	0.03	214%
J20	0.015	0.230	ND	0.140	0.015	ND	0.004	6:25	23:17	0.01	0.01	30.61	0.05	0.02	0.01	221%
J22	0.029	0.350	ND	0.330	0.016	ND	0.004	8:59	24:00	0.02	0.01	5.66	0.26	0.04	0.02	225%
Aug 2008																
A1	0.051	0.240	0.009	ND	0.019	ND	0.002	8:13	50:00	0.06	0.02	N/A	N/A	0.02	0.03	99%
A5	0.029	0.190	-0.002	ND	0.011	ND	0.001	6:04	50:00	0.03	0.01	6.03	0.36	0.02	0.06	299%
A10	0.017	0.140	-0.002	0.160	0.007	ND	0.000	15:40	28:41	0.03	0.01	6.56	0.06	0.01	0.05	254%
A16	0.022	0.130	-0.004	0.390	0.003	ND	0.002	5:48	12:19	0.02	0.00	7.39	0.03	0.01	0.03	197%
Bloom	0.155	0.190	0.011	0.210	0.028	0.18	-0.001	0:00	12:21	0.24	0.04	N/A	N/A	0.08	0.03	42%
A22	0.048	0.390	0.005	ND	0.013	ND	0.001	10:18	26:45	0.06	0.02	6.17	0.04	0.03	0.01	74%
A23	0.034	0.220	-0.002	0.550	0.014	ND	0.001	12:35	12:35	0.02	0.01	19.01	0.19	0.05	0.06	455%
A29	0.010	0.320	0.005	ND	-0.001	ND	0.000	11:43	31:43	0.00	0.00	0.40	0.02	0.00	0.01	296%

Table 4.2. Peptide hydrolysis and nutrient uptake during July and August 2008. Hydrolysis rate constants of LYA-AVFA are calculated during the exponential decrease stage. The lag time is the length of the slow hydrolysis phase. k_w^{AVFA} : rate constant of LYA-AVFA hydrolysis in whole water; k_{pw}^{AVFA} : rate potential of LYA-AVFA hydrolysis in whole water; k_f^{AVFA}: rate constant of LYA-AVFA hydrolysis in filtered water; k_{pf}^{AVFA} : rate potential of LYA-AVFA hydrolysis in filtered water; k_w^{ALA4} : rate constant of LYA-ALA_4 hydrolysis in whole water; k_{pw}^{ALA4} : rate potential of LYA-ALA4 hydrolysis in whole water; K_f^{ALA4} : rate constant of LYA-ALA_4 hydrolysis in filtered water; τ_w : lag time of LYA-AVFA in whole water sample; τ_f: lag time of LYA-AVFA in filtered sample; R_w^{AVFA}: hydrolysis rate of LYA-AVFA in whole water sample; R_w^{ALA4}: hydrolysis rate of LYA-ALA_4 in whole water sample; HCO_3^-, Urea, Glu, Diala: uptake rate of these compounds. %ON is the percentage of uptake of Glu and Diala to R_w^{AVFA}.

	SAL	k_w	k_f	k_{pw}	k_{pf}	τ_f	τ_w	[Chla]	[DFAA]	[DCAA]	[TPN]	[TPC]	UGLU	UA2	FC
PC1_DFAA	ns	ns	ns	-0.73	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.47
PC2_DFAA	0.54	-0.47	ns	ns	ns	-0.57	-0.46	ns	ns	ns	ns	ns	0.74	ns	ns
PC1_DCAA	0.85	ns	ns	ns	0.61	ns	ns	ns	ns	ns	ns	ns	ns	-0.63	ns
PC2_DCAA	ns	ns	ns	ns	0.60	-0.47	ns	ns	ns	ns	ns	ns	ns	ns	ns
SAL	-	ns	ns	ns	0.56	-0.52	ns	ns	ns	ns	ns	ns	ns	-0.58	0.56
k_w		-	0.67	ns	ns	ns	0.62	ns	ns	0.46	ns	ns	ns	ns	ns
k_f			-	ns	ns	ns	ns	ns	ns	0.48	ns	ns	ns	ns	ns
k_{pw}				-	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
k_{pf}					-	-0.65	ns	ns	ns	ns	ns	ns	ns	ns	ns
τ_f						-	ns	ns	ns	ns	ns	ns	-0.47	ns	ns
τ_w							-	-0.57	ns	ns	ns	ns	-0.53	ns	ns
[Chla]								-	ns	0.56	ns	0.53	0.73	ns	ns
[DFAA]									-	ns	ns	ns	ns	ns	0.89
[DCAA]										-	ns	ns	ns	ns	ns
[TPN]											-	0.98	0.67	0.51	ns
[TPC]												-	0.75	0.46	ns
UGLU													-	ns	ns
UA2														-	ns
FC															-

Table 4.3. Pearson correlation analysis of all environmental parameters at all stations. PC1 and PC2 are the first two principal components of DCAA and DFAA composition from all stations (see Fig. 8B). [DFAA] and [DCAA] here represent the concentration of DFAA and DCAA respectively. The correlation is signification with $r > 0.458$, $p < 0.05$, $n=14$. ns. = $p > 0.05$. See Figure 4.5 for abbreviations.

	SAL	k_w	k_f	k_{pw}	k_{pf}	τ_f	τ_w	[Chla]	[DFAA]	[DCAA]	[TPN]	[TPC]	UGLU	UA2	FC
PC1_DFAA	0.82	ns	ns	ns	0.65	ns	ns	ns	0.60	ns	ns	ns	ns	-0.72	0.50
PC2_DFAA	ns	ns	ns	ns	ns	ns	ns	ns	-0.61	ns	ns	ns	ns	ns	-0.58
PC1_DCAA	ns	ns	ns	-0.75	ns	ns	ns	ns	ns	ns	-0.49	ns	ns	ns	-0.56
PC2_DCAA	0.63	-0.46	ns	ns	ns	-0.50	-0.57	0.47	ns	ns	ns	ns	0.72	-0.60	ns
SAL	-	ns	ns	ns	0.58	-0.61	ns	ns	0.57	ns	ns	ns	0.47	-0.65	0.51
k_w		-	0.66	ns	ns	ns	0.73	ns	ns	ns	ns	ns	ns	ns	ns
k_f			-	0.62	ns	ns	0.56	ns	0.48	ns	0.50	ns	ns	ns	ns
k_{pw}				-	ns	ns	ns	ns	0.47	ns	0.70	0.63	ns	ns	ns
k_{pf}					-	-0.62	ns	ns	ns	ns	-0.47	ns	ns	ns	ns
τ_f						-	ns	ns	ns	ns	ns	ns	ns	ns	ns
τ_w							-	-0.54	ns	ns	ns	ns	-0.65	ns	ns
[Chla]								-	ns	0.46	0.47	0.66	0.82	ns	ns
[DFAA]									-	ns	ns	ns	ns	ns	0.95
[DCAA]										-	ns	ns	0.46	ns	ns
[TPN]											-	0.95	0.61	ns	ns
[TPC]												-	0.74	ns	ns
UGLU													-	ns	ns
UA2														-	-0.48
FC															-

Table 4.4. Pearson analysis of all environmental parameters at the estuarine stations (excluding J22, A23 and A29). PC1 and PC2 are the first two principal components of DFAA and DCAA composition at the estuarine stations (as in Fig. 8B excluding Bay stations). [DFAA] and [DCAA] here represent the concentration of DFAA and DCAA respectively. The correlation is signification with $r > 0.458$, $p < 0.05$, n=14. ns. = $p > 0.05$.

Figures:

Figure 4.1. Sampling stations during July (J) and August (A), 2008. The number following J (July) or A (August) is salinity of that station. Map is from Google Earth.

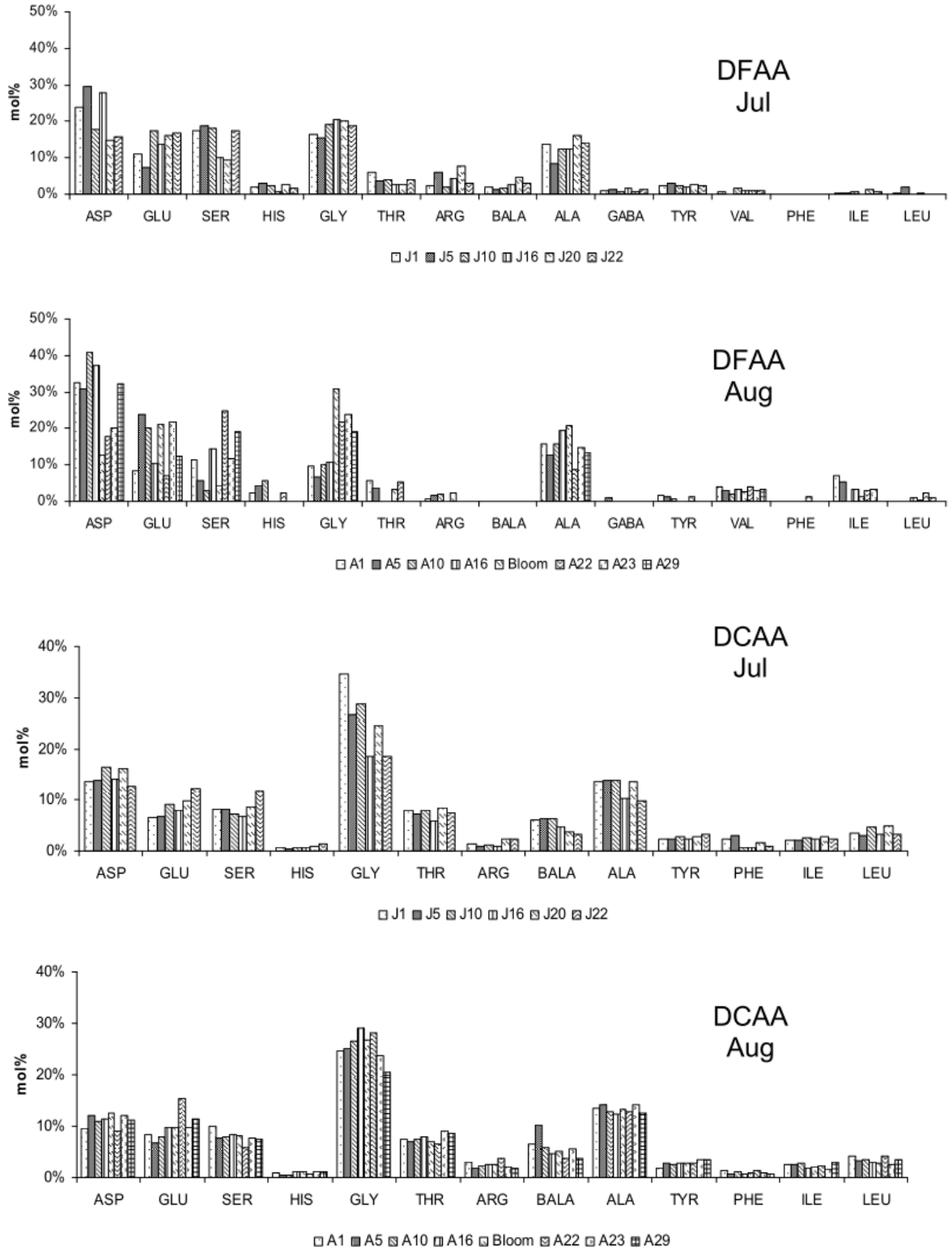

Figure 4.2. Mole percentages of DFAA and DCAA in July and August.

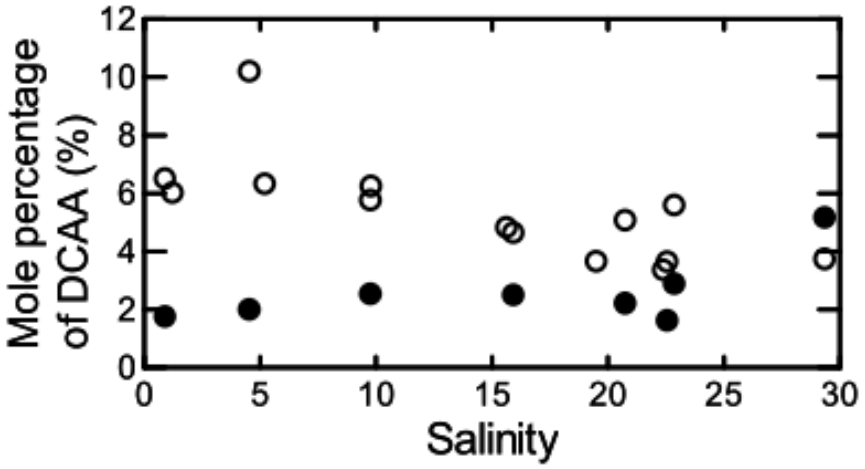

Figure 4.3. Change of mol% of BALA (open circle) in DCAA with salinity in both July and August; and change of mol% of GABA (solid circle) in DCAA only in August.

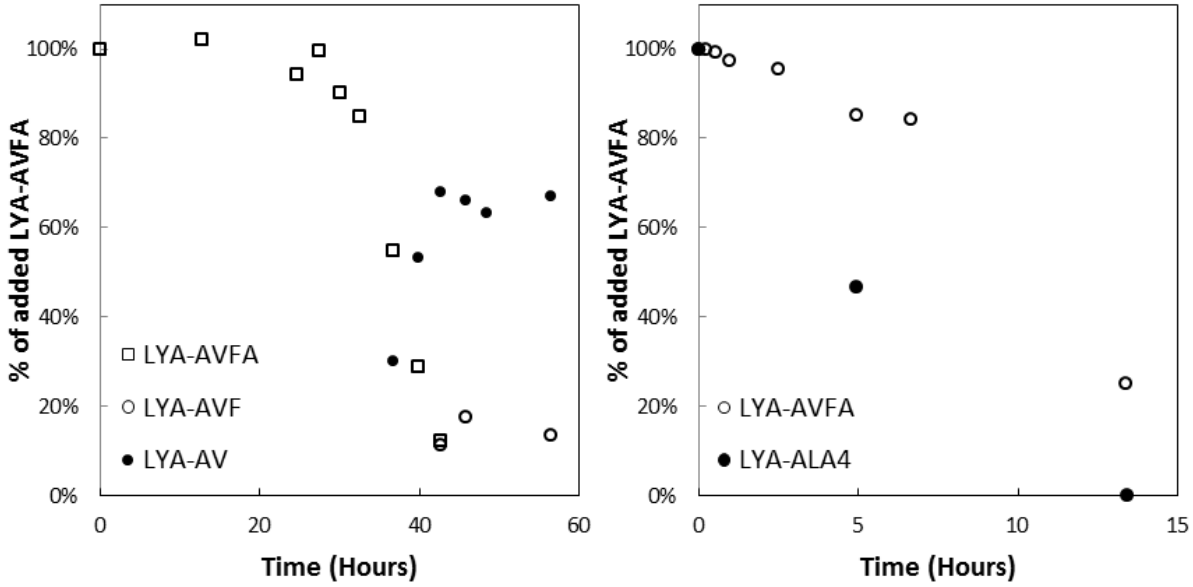

Figure 4.4. A) Time course of hydrolysis of LYA-AVFA and production of its degradation products in filtered water samples from Sta. A10; B) Loss of LYA-AVFA (open circles) and LYA-ALA_4 (solid circles) in whole water sample at Bloom station in August.

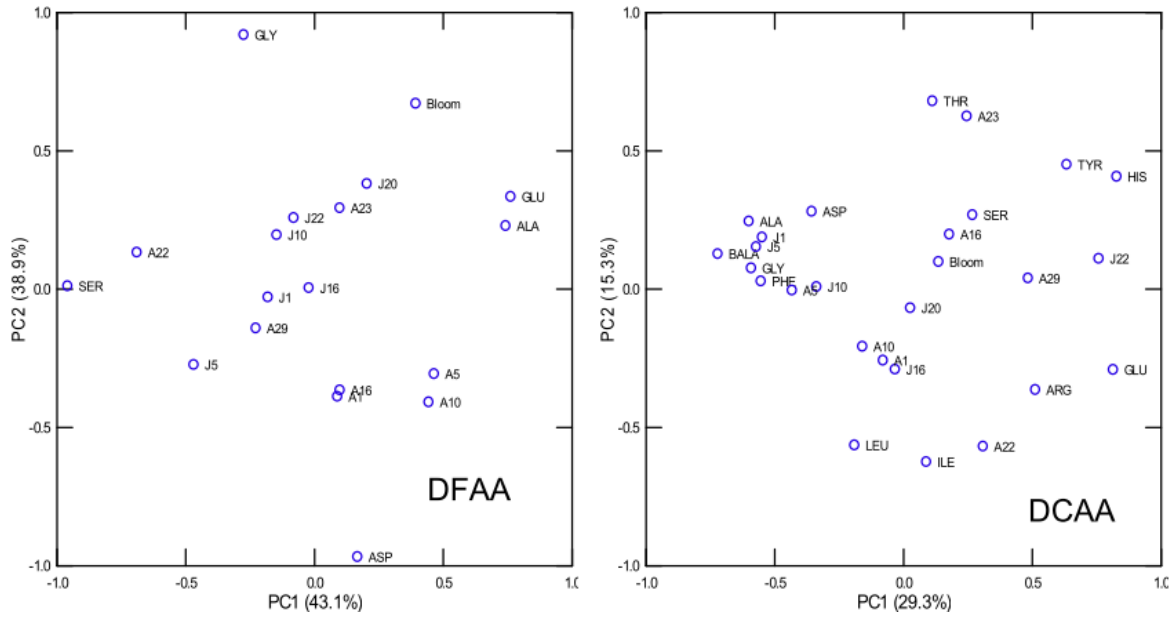

Figure 4.5. Plot of first two PCs for mole% of DFAA from July and August cruise; and plot of first two PCs for mole% of DCAA from July and August cruises. Abreviations are as follows: ASP, GLU, SER, HIS, GLY, THR, ARG, BALA, TYR, PHE, ILE, LEU: DCAA composition; DFAA and DCAA: DFAA and DCAA concentrations; FC: ratio of DFAA/DCAA; Kw and Ks: peptide hydrolysis rate constants in whole and filtered water samples; Lagw and Lags: lag times in both whole and filtered water samples, UGLU and UA2: uptake rates of amino acids and dipeptides, SAL: salinity; Chla: chlorophyll a; O2: oxygen, TPC and TPN: particulate carbon and nitrogen; NO2: nitrite; NO3: nitrate; PO4: phosphate; UREA: urea; SIO4: silicate; NH4: ammonium.

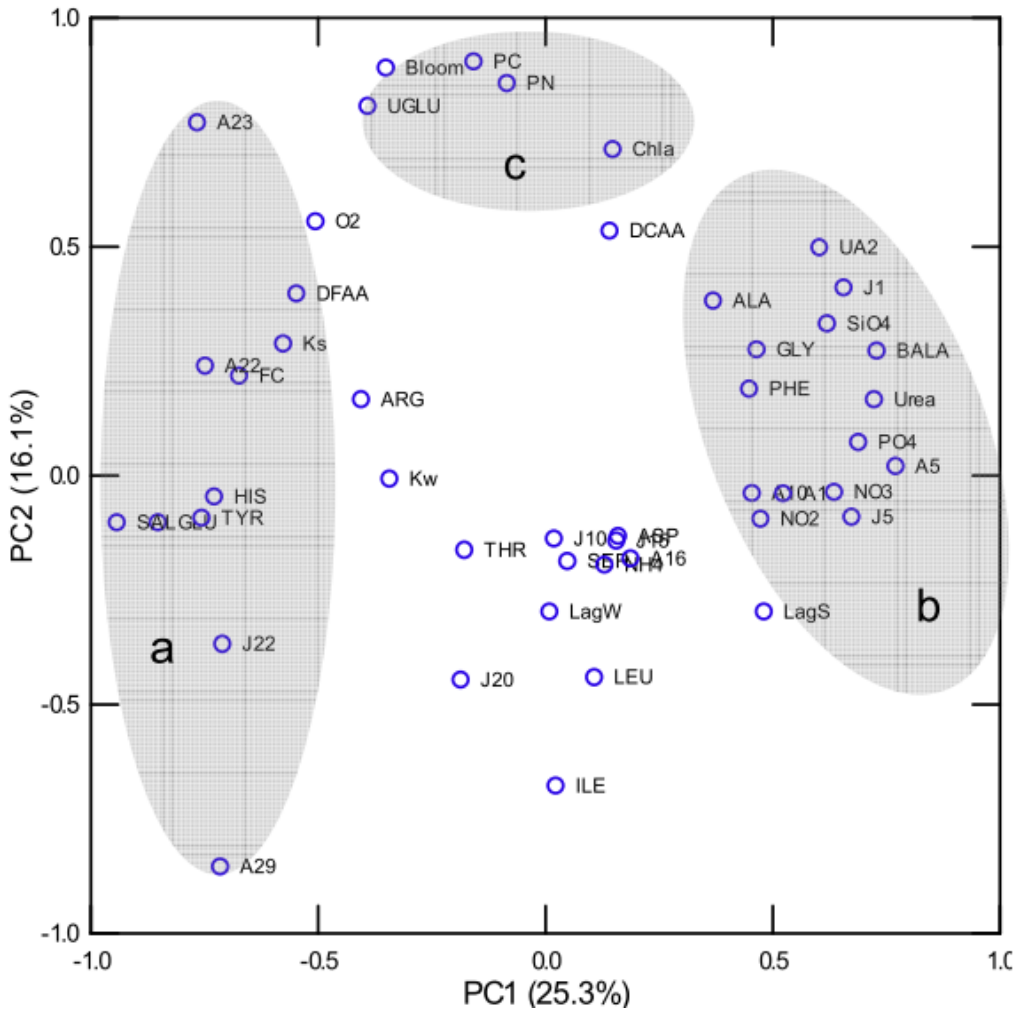

Figure 4.6. Plot of first two PCs from the PCA of all environmental parameters from all sites. Shaded areas represent clusters of freshwater stations with low salinity, high nutrient input and degraded organic matter on the left (a), open ocean stations with high salinity, low chlorophyll but fresh organic matter on the right (b), and the Bloom station with high chlorophyll and high particulate nitrogen (TPN) near the top of the graph (c). Other stations lie between these three groupings.

Chapter Five

Conclusions and Implications

5.1. Summary of major findings

Proteinaceous materials are major components of marine organic matter. The relatively little knowledge we have of source and fate of proteinaceous materials limits our understanding of the cycling of marine organic matter in marine environments. This thesis study focuses on the degradation and transport of proteinaceous materials in various marine environments with particular attention to the: 1) impact of cell membrane structure and post translational modification on protein degradation; 2) impact of mineral deposition on protein degradation; and 3) impact of diverse ecosystems on protein degradation.

In Chapter 2, the impact of cell membrane structure and post translational modification on protein degradation in seawater was studied using a model protein, Surface layer (S-layer) protein from the cyanobacterium *Synechococcus*. S layer protein is a specific membrane glycoprotein that is widely found in both Eubacteria and Archaea. Along with a polysaccharide sheath, this heavily glycosylated protein covers the outermost cell surface in a regularly ordered planar crystalline structure. With special attention to changes in S-layer protein and peptidoglycan, we studied the degradation of two species of marine cyanobacterium, *Synechococcus* CCMP2370 and CCMP1334, with and without S-layer structure, respectively. Based on evidence from both biochemical and chemical analysis as well as electron microscopy, we found that glycoproteins like S-Layer protein were as rapidly degraded as other non-glycosylated proteins. We also studied degradation of both *Synechococcus* species after they had been treated with buffers that strip the S-layer from the cell surface. Stripped cells of CCMP2370 lost their cell membrane structure and degraded more rapidly and became more

enriched in D-amino acids, while stripped CCMP1334 cells maintained their membrane structures and degradation was not enhanced. These results suggest that 1) post translational modifications like glycosylation may not preferentially preserve glycoproteins like S-layer protein during the degradation of *Synechococcus* in coastal seawater; and 2) the surface layer structure functions as a defensive barrier on the cell membrane, similar to peptidoglycan, to protect cyanobacterial cells from degradation. This is, to the best of our knowledge, the first time the role of prokaryotic membrane proteins like S layer have been investigated with regard to degradation of marine organic matter in the water column.

Marine particles transport organic matter across the oceanic water column to the sediment where the organic matter can be buried. As a net sink of CO_2 over geological time, this pathway is one of the few natural carbon removal mechanisms from the biosphere. Picoplankton like cyanobacteria have not until recently been thought important to particle transport, even though cyanobacteria are one of the major primary producers in the surface waters of the open ocean. In Chapter 3, we provide evidence using electron microscopy (EM) – dispersive X-ray spectroscopy (EDS) and bulk analysis of amino acids that Si can be deposited on the extracellular polymeric substances (EPS) produced by cyanobacteria, particularly when they begin to decompose. We also found that Si associated with organic micro-blebs collected from the deep ocean. Both nano-particle imaging analysis and bulk analysis show a surprisingly similar appearance between EPS-associated Si in cyanobacteria and micro-blebs in the open ocean. Accordingly, EPS-associated Si might be a precursor of the Si-enriched organic micro-blebs we observed in the ocean. This evidence supports the hypothesis that cyanobacterially derived extracellular polymeric substances can trap Mg and Si in the seawater. Meanwhile, this

previously unexplored source of particulate silicate mineral may be as important as siliceous diatoms in oceanic silicate cycling and further impact on organic matter export in the oceans.

In Chapter 4, the cycling of dissolved organic nitrogen was investigated along transects of the James River estuary and lower Chesapeake Bay salinity gradient during the summer of 2008. A new fluorescent analog, Lucifer Yellow Anhydride-alanine-valine-phenylalanine-alanine (LYA-AVFA), was developed to measure extracellular peptide hydrolysis. Compared with another previously developed analog, LYA-tetraalanine (LYA-ALA_4), LYA-AVFA has a consistent hydrolysis pattern in studied area. Hydrolysis rates of LYA-AVFA and LYA-ALA_4 were compared to the uptake of various organic nitrogen species (urea, glutamic acid and dialanine) along the salinity transect. Changes in the abundance and composition of individual dissolved amino acids were also examined. Results from the James River estuary suggest that peptide hydrolysis and organic nitrogen uptake are not always tightly coupled to each other along salinity transects as a response to the changing environmental conditions in the studied area. This is because diverse input and removal processes can influence both peptide hydrolysis and uptake, but not necessarily simultaneously. For example, peptide hydrolysis potentials and uptake of free amino acids were strongly associated with particulate nitrogen, suggesting a strong influence of particle-associated processes on organic nitrogen cycling in the estuary. Hydrolysis potentials appeared to be more associated with sediment resuspension, while uptake and initial hydrolysis rates were more associated with phytoplankton biomass. As a response to changing hydrolysis and uptake of organic nitrogen, a change in dissolved amino acid abundance and composition was observed from the fresh end of the estuary to the mouth of Chesapeake Bay, which most likely resulted from the mixing of multiple sources and their consequent impacts on hydrolysis and uptake, e.g. terrestrial input, sediment resuspension and local phytoplankton growth.

5.2. Directions for future work

This dissertation explores the complicated natures and diverse functions of proteinaceous materials with particular attention to their influence on the degradation of proteinaceous materials in marine environments. These structures and functions are species-specific and protein-specific, and greatly influence the decomposition of cellular organic matter from primary producers during their transport in various marine environments. Although our work sheds light on the importance of characteristics and functions of individual proteins on biogeochemical cycling, this could be even better understood using recently fast growing techniques like proteomics. The underlying assumption in proteomics is that organisms maintain specific proteomes as a response to their chemical and physical environments (Maron et al. 2007; Morris et al. 2010). However, marine biogeochemical systems can be particularly complex because non-living organic matter which can record its original proteome can be transported from their sources (e.g. surface water for phytoplankton) to other locations (e.g. the deep ocean). This causes an apparent uncoupling between the proteins and their corresponding environments (e.g., the finding of pico-phytoplankton proteins in the deep ocean). This uncoupling veils the direct linkage between proteomes and their corresponding biogeochemical processes. But, on the other hand, if the source can be unraveled, it provides information on biogeochemical cycling of proteins in the ocean. The effect of this uncoupling has been noticed in a few pioneering studies (Nunn et al. 2010; Moore et al. 2012), but the extent of this effect is poorly understood. Thus, I look forward to linking my studies on marine proteinaceous materials with the rapid expanding field of proteomics and using proteomes as biomarkers to trace the fate of proteins in marine environments.

Another important area for future study is how post translational modifications in marine picoplankton affect their degradation. These preliminary studies unveil the complexity of protein modifications, but much remains poorly understood. The application of various affinity chromatographic techniques can selectively purify glycoproteins from a mixture of proteins. These techniques would allow us to access and evaluate glycoproteins in natural samples, thus potentially helping to improve our understanding of various glycosylation processes in marine environments.

The interaction of extracellular polymeric substance with Si and Mg was addressed in our studies during the decomposition of cyanobacterial cells. To our best knowledge, this study was the first to unveil the close interaction between microbially derived extracellular polymeric substance with Si under the mild conditions found in the water column. This finding may substantially change our understanding of biogenic silicate cycling in marine environments, which was thought to be driven by large siliceous phytoplankton like diatom in the open ocean. For this reason, a detailed study on the relationship between biogenic silicate and pico-plankton is desired. And the importance of cyanobacteria in regulating the global cycling of organic matter and biogenic silicate needs to be further addressed.

The cycling of marine organic matter was investigated in an estuarine region using a new fluorescent analog, LYA-AVFA. Enzymatic peptide hydrolysis rates changed greatly between river and marine end members and illustrated how the sources, decomposition and transport of proteinaceous materials varied as a response to the changing estuarine environment. Further studies would be enhanced by the development and characterization of novel peptide analogs.

References:

Maron, P.-A., L. Ranjard, C. Mougel, and P. Lemanceau. 2007. Metaproteomics: a new approach for studying functional microbial ecology. Microbial ecology 53: 486-93, doi:10.1007/s00248-006-9196-8

Moore, E. K., B. L. Nunn, D. R. Goodlett, and H. R. Harvey. 2012. Identifying and tracking proteins through the marine water column: Insights into the inputs and preservation mechanisms of protein in sediments. Geochimica et Cosmochimica Acta 83: 324-359, doi:10.1016/j.gca.2012.01.002

Morris, R. M., B. L. Nunn, C. Frazar, D. R. Goodlett, Y. S. Ting, and G. Rocap. 2010. Comparative metaproteomics reveals ocean-scale shifts in microbial nutrient utilization and energy transduction. The ISME journal 4: 673-85, doi:10.1038/ismej.2010.4

Nunn, B. L., Y. S. Ting, L. Malmström, Y. S. Tsai, A. Squier, D. R. Goodlett, and H. R. Harvey. 2010. The path to preservation: Using proteomics to decipher the fate of diatom proteins during microbial degradation. Limnology and Oceanography 55: 1790-1804, doi:10.4319/lo.2010.55.4.1790

Bibliography

Abramson, L., S. Wirick, C. Lee, C. Jacobsen, and J. A. Brandes. 2009. The use of soft X-ray spectromicroscopy to investigate the distribution and composition of organic matter in a diatom frustule and a biomimetic analog. Deep Sea Research Part II 56: 1369-1380, doi:10.1016/j.dsr2.2008.11.031

Admiraal, W., C. Riaux-Gobin, and R. W. P. M. Laane. 1987. Interactions of ammonium , nitrate , and D- and L- amino acids in the nitrogen assimilation of two species of estuarine benthic diatoms. 40: 267-273.

Aplin, J. D., and R. C. Hughes. 1982. Complex carbohydrates of the extracellular matrix structures, interactions and biological roles. Biochimica et biophysica acta 694: 375-418.

Armstrong, R. A., C. Lee, J. I. Hedges, S. Honjo, and S. G. Wakeham. 2002. A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. Deep-Sea Research Part II 49: 219-236.

Armstrong, R. A., M. L. Peterson, C. Lee, and S. G. Wakeham. 2009. Settling velocity spectra and the ballast ratio hypothesis. Deep Sea Research Part II 56: 1470-1478, doi:10.1016/j.dsr2.2008.11.032

Arnosti, C. 2004. Speed bumps and barricades in the carbon cycle: substrate structural effects on carbon cycling. Marine Chemistry 92: 263-273, doi:10.1016/j.marchem.2004.06.030

Arnosti, C., 2011, Microbial extracellular enzymes and the marine carbon cycle, Annual Review of Marine Science, 3:401-425

Asper, V. L., W. G. Deuser, G. A. Knauer, and S. E. Lohrenz. 1992. Rapid coupling of sinking particle fluxes between surface and deep ocean waters. Nature 357: 670-672.

Azam, F., T. Fenchel, J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The Ecological Role of Water-Column Microbes in the Sea. Marine Ecology Progress Series 10: 257-263.

Baines, S. B., B. S. Twining, M. A. Brzezinski, J. W. Krause, S. Vogt, D. Assael, and H. McDaniel. A surprising role for picocyanobacteria in the marine silicon cycle. PNAS, Submitted.

Benner, R., 2002. Chemical composition and reactivity, pp. 59-90. In: C. Carlson and D. Hansell (Ed.), Biogeochemistry of Marine Dissolved Organic Matter. Academic, San Diego.

Bennett, R. H., B. Ransom, M. Kastner, R. J. Baerwald, M. H. Hulbert, W. B. Sawyer, H. Olsen, and M. W. Lambert. 1999. Early diagenesis: impact of organic matter on mass physical properties and processes, California continental margin. Marine Geology 159: 7-34.

Berges, J. A., and M. R. Mulholland. 2008. Enzymes and cellular N cycling, p. 1385-1444. In D.G. Capone, D.A. Bronk, and M.R. Mulholland [eds.], Nitrogen in the Marine Environment. Elsevier/Academic.

Berman, T., and D. Bronk. 2003. Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. Aquatic Microbial Ecology 31: 279-305, doi:10.3354/ame031279

Bianchi, T. S., T. Filley, K. Dria, and P. G. Hatcher, 2004. Temporal variability in sources of dissolved organic carbon in the lower Mississippi River. Geochimica et Cosmochimica Acta, 68: 959-967.

Billen, G., and A. Fontigny, 1987, Dynamics of a *Phaeocystis*-dominated spring bloom in Belgian coastal waters, II. Bacterioplankton dynamics. Marine Ecology Progress Series, 37: 249-257.

Borch, N. H., and D. L. Kirchman. 1999. Protection of protein from bacterial degradation by submicron particles. Aquatic Microbial Ecology 16: 265-272.

Boyle, E., A.T. Collier, Dengler, A.T., Edmond, J.M., Ng, A.C., Stallard, R. F., 1974, On the chemical mass-balance in estuaries. Geochimica et Cosmochimica Acta, 38: 1719-1728.

Brahamsha, B. 1996. An abundant cell-surface polypeptide is required for swimming by the nonflagellated marine cyanobacterium *Synechococcus*. Proceedings of the National Academy of Sciences of the United States of America 93: 6504-6509.

Bronk, D.A., 2002. Dynamics of DON. In: C. Carlson, and D. Hansell, [Ed.], Biogeochemistry of Marine Dissolved Organic Matter. Academic, San Diego.

Bronk, D. A., Q. N. Roberts, M. P. Sanderson, E. A. Canuel, P. G. Hatcher, R. Mesfioui, K. C. Filippino, M. R. Mulholland, and N. G. Love. 2010. Effluent organic nitrogen (EON): bioavailability and photochemical and salinity-mediated release. Environmental Science & Technology 44: 5830-5, doi:10.1021/es101115g

Bukaveckas, P.A., L.E. Barry, M.J. Beckwith, V. David, Lederer., 2011, Factors determining the location of the chlorophyll maximum and the fate of algal production within the tidal freshwater James River, Estuaries and Coasts, 34: 569-582.

Chen, J.F., Y. Li, K.D. Yin, H.Y. Jin, 2004, Amino acids in the Pearl River Estuary and adjacent waters: origins, transformation and degradation, Continental Shelf Research, 24: 1877-1894.

Cho, W., and R. V. Stahelin. 2005. Membrane-protein interactions in cell signaling and membrane trafficking. Annual review of Biophysics and Biomolecular Structure 34: 119-51, doi:10.1146/annurev.biophys.33.110502.133337

Chrost, R.J., 1991, Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: R.J. Chrost, [Ed.] Microbial Enzymes in Aquatic Environments, Springer-Verlag, New York.

Coffin, R. B., 1989. Bacterial uptake of dissolved free and combined amino-acids in the estuarine waters. Limnology and Oceanography, 34: 531-542.

Collier, J. L., and A. R. Grossman. 1992. Chlorosis induced by nutrient deprivation in *Synechococcus sp*. strain PCC 7942: not all bleaching is the same. Journal of bacteriology 174: 4718-26.

Cowie, G. L., and J. I. Hedges. 1992. Sources and reactivities of amino acids in a coastal marine environment. Limnology and Oceanography 37: 703-724, doi:10.4319/lo.1992.37.4.0703

Cowie, G. L., and J. I. Hedges, 1994. Biochemical indicators of diagenetic alteration in organic-matter mixtures. Nature, 369: 304-307.

Dauwe, B., and J. J. Middelburg, 1998, Amino acids and hexosamines as indicators of organic matter degradation state in North Sea sediments, Limnology and oceanography, 43: 782-798.

Dauwe, B., J. J. Middelburg, P. M. J. Herman, and C. H. R. Heip. 1999. Linking diagenetic alteration of amino acids and bulk organic matter reactivity. Limnology and Oceanography 44: 1809-1814.

Davis, J., K. Kaiser, and R. Benner, 2009. Amino acid and amino sugar yields and compositions as indicators of dissolved organic matter diagenesis. Organic Geochemistry, 40: 343-352.

Dean, R. T., S. Fu, R. Stocker, and M. J. Davies. 1997. Biochemistry and pathology of radical-mediated protein oxidation. The Biochemical Journal 324: 1-18.

Dittmar, T., H. P. Fitznar, and G. Kattner. 2001. Origin and biogeochemical cycling of organic nitrogen in the eastern Arctic Ocean as evident from D- and L-amino acids. Geochimica et Cosmochimica Acta 65: 4103-4114.

Dong, H.-P., D.-Z. Wang, M. Dai, and H.-S. Hong. 2010. Characterization of particulate organic matter in the water column of the South China Sea using a shotgun proteomic approach. Limnology and Oceanography 55: 1565-1578, doi:10.4319/lo.2010.55.4.1565

Dzurica, S., C. Lee, E.M. Cosper, E.J. Carpenter, 1989. Role of environmental variables, specifically organic compounds and micronutrients, in the growth of the chrysophyte *Aureococcus anophagefferens*. In: E.M Cosper, V.M. Bricelj, E.J. Carpenter, (Ed.), Novel Phytoplankton Blooms: Causes and Impacts of Recurrent Brown. Tides and Other Unusual Blooms. Springer-Verlag, Berlin.

Engel, A., and H. E. Gaub. 2008. Structure and mechanics of membrane proteins. Annual Review of Biochemistry 77: 127-48, doi:10.1146/annurev.biochem.77.062706.154450

Engelhardt, H., and J. Peters. 1998. Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer-cell wall interactions. Journal of Structural Biology 124: 276-302, doi:10.1006/jsbi.1998.4070

Fitznar, H. P., J. M. Lobbes, and G. Kattner. 1999. Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with o-phthaldialdehyde and N-isobutyrylcysteine in seawater and fossil samples (mollusks). Journal of Chromatography A 832: 123-132, doi:10.1016/S0021-9673(98)01000-0

Fu, S., M.-X. Fu, J. W. Baynes, S. R. Thorpe, and R. T. Dean. 1998. Presence of dopa and amino acid hydroproxides in proteins modified with advanced glycation end products (AGEs): amino acid oxidation products as a possible source of oxidative stress induced by AGE proteins. Biochemical Journal 330: 233-239.

Fuhrman, J. A., and T. M. Bell. 1985. Biological considerations in the measurement of dissolved free amino acids in seawater and implications for chemical and microbiological studies. Marine Ecology Progress Series 25: 13-21.

Fuhrman, J., 1987, Close coupling between release and uptake of dissolved free amino acids in seawater studied by an isotope dilution approach. Marine Ecology Progress Series, 37: 45-52.

Giessing, A. M. B., and L. M. Mayer. 2004. Oxidative coupling during gut passage in marine deposit-feeding invertebrates. Limnology and Oceanography 49: 716-726, doi:10.4319/lo.2004.49.3.0716

Goutx, M., S.G. Wakeham, C. Lee, M. Duflos, C. Guigue, Z. Liu, B. Moriceau, R. Sempere, M. Tedetti, J. Xue, 2007. Composition and degradation of marine particles with different settling velocities in the northwestern Mediterrannean sea. Limnology and Oceanography, 52: 1645-1664.

Grace, B., T. Bianchi, 2010, Sorption and desoroption dynamics of bulk dissolved organic matter and amino acids in the Mississippi River plume – a microcosm study, Marine and Freshwater Research, 61: 1067-1081.

Grossart, H.P., K.W. Tang, T. Kiorboe, H. Ploug, 2007. Comparison of cell-specific activity between free-living and attached bacteria using isolates and natural assemblages. FEMS Microbiology Letters, 266: 194-200.

Guldberg, L.B., K. Finster, N.O.G. Jorgensen, M. Middelboe, B.A. Lomstein, 2002. Utilization of marine sedimentary dissolved organic nitrogen by native anaerobic bacteria. Limnology and Oceanography, 47: 1712-1722.

Hedges, J. I., G. Eglinton, P. G. Hatcher, D. L. Kirchman, C. Arnosti, S. Derenne, R. P. Evershed, and I. Ko. 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Organic Geochemistry 31: 945-958.

Hill, C., and C. D. Brest. 1988. Interspecific and intraspecific composition and variation of free amino acids in marine phytoplankton. 44: 303-313.

Hill, B.P., K.M. Wright, 1994, A new model for bacterial growth in heterogeneous systems, Journal of Theoretical Biology, 168: 31-41.

Hitchen, P. G., and A. Dell. 2006. Bacterial glycoproteomics. Microbiology 152: 1575-80, doi:10.1099/mic.0.28859-0

Hollibaugh, J. T., and F. Azam. 1983. Microbial degradation of dissolved proteins in seawater. Limnology and Oceanography 28: 1104-1116.

Honjo, S., R. Francois, S. Manganini, J. Dymond, and R. Collier. 2000. Particle fluxes to the interior of the Southern Ocean in the Western Pacific sector along 170W. Deep Sea Research Part I 47: 3521-3548.

Hoppe, H.G., 1983. Significance of exoenzymatic activities of the ecology of brackish water – Measurements by means of methylumbelliferyl substrates. Marine Ecology-Progress Series, 11: 299-308.

Hoppe, H.-G. G., H. Ducklow, and B. Karrasch. 1993. Evidence for dependency of bacterial growth on enzymatic hydrolysis of particulate organic matter in the mesopelagic ocean. Marine Ecology Progress Series 93: 277-283, doi:10.3354/meps093277

Hoppe, H.G., C. Arnosti, G. Herndl, 2002. Ecological significance of bacterial enzymes in the marine environment. In: R. Burns, R. Dick, (eds.), Enzymes in the environment: Activity, ecology and applications, Marcel Dekker, 73-107.

Ingalls, A. E., C. Lee, and E. R. M. Druffel. 2003. Preservation of organic matter in mound-forming coral skeletons. Geochimica et Cosmochimica Acta 67: 2827-2841, doi:10.1016/S0016-7037(03)00079-6

Ingalls, A. E., C. Lee, S. G. Wakeham, and J. I. Hedges. 2003. The role of biominerals in the sinking flux and preservation of amino acids in the Southern Ocean along 170°W. Deep-Sea Research Part I 50: 713-738.

Ingalls, A. E., Z. F. Liu, and C. Lee, 2006, Seasonal trends in the pigment and amino acid compositions of sinking particles in biogenic $CaCO_3$ and SiO_2 dominated regions of the Pacific sector of the Southern Ocean along 170 degrees W. Deep-Sea Research Part I, 53:836-859.

Jiao, N., G. J. Herndl, D. a. Hansell, R. Benner, G. Kattner, S. W. Wilhelm, D. L. Kirchman, M. G. Weinbauer, T. Luo, F. Chen, and F. Azam. 2011. The microbial carbon pump and the oceanic recalcitrant dissolved organic matter pool. Nature Reviews Microbiology 9: 555-555, doi:10.1038/nrmicro2386-c5

Kaiser, K., and R. Benner. 2005. Hydrolysis-induced racemization of amino acids. Limnology and Oceanography: Method 3: 318-325.

Kaiser, K., and R. Benner. 2008. Major bacterial contribution to the ocean reservoir of detrital organic carbon and nitrogen. Limnology and Oceanography 53: 99-112.

Kaiser, K., and R. Benner. 2009. Biochemical composition and size distribution of organic matter at the Pacific and Atlantic time-series stations. Marine Chemistry 113: 63-77, doi:10.1016/j.marchem.2008.12.004

Kawasaki, N., and R. Benner. 2006. Bacterial release of dissolved organic matter during cell growth and decline : Molecular origin and composition. Limnology and Oceanography 51: 2170-2180.

Keil, R.G., D.L. Kirchman, 1991. Dissolved combined amino acids in marine waters as determined by a vapor-phase hydrolysis method. Marine Chemistry, 33: 243-259.

Keil, R. G., and D. L. Kirchman. 1993. Dissolved combined amino acids: Chemical form and utilization by marine bacteria. Limnology and Oceanography 38: 1256-1270, doi:10.4319/lo.1993.38.6.1256

Keil, R.G., D.L. Kirchman, 1999. Utilization of dissolved protein and amino acids in the northern Sargasso Sea. Aquatic Microbial Ecology, 18: 293-300.

King, K. 1974. Preserved amino acids from silicified protein in fossil Radiolaria. Nature 252: 690-692.

Klaas, C., and D. E. Archer. 2002. Association of sinking organic matter with various types of mineral ballast in the deep sea : Implications for the rain ratio. Global Biogeochemical Cycles 16: 1116-1129, doi:10.1029/2001GB001765

Knicker, H., A. W. Scaroni, and P. G. Hatcher. 1996. 13C and 15N NMR spectroscopic investigation on the formation of fossil algal residues. Organic Geochemistry 24: 661-669.

Konhauser, K. O., and M. M. Urrutia. 1999. Bacterial clay authigenesis: a common biogeochemical process. Chemical Geology 161: 399-413, doi:10.1016/S0009-2541(99)00118-7

Koval, S. F., and R. G. Murray. 1984. The isolation of surface array proteins from bacteria. Canadian Journal of Biochemistry and Cell Biology 62: 1181-9.

Kroer, N., N. O. G. Jorgensen, R. B. Coffin, 1994, Utilization of dissolved nitrogen by heterotrophic bacterioplankton: a comparison of three ecosystems, Applied and Environmental Microbiology, 60: 4116-4123.

Kuznetsova, M., and C. Lee. 2001. Enhanced extracellular enzymatic peptide hydrolysis in the sea-surface microlayer. Marine Chemistry 73: 319-332, doi:10.1016/S0304-4203(00)00116-X

Kuznetsova, M., C. Lee, 2002. Dissolved free and combined amino acids in nearshore seawater, sea surface microlayers and foams: Influence of extracellular hydrolysis. Aquatic Sciences, 64: 252-268.

Kuznetsova, M., C. Lee, J. Aller, N. Frew, 2004, Enrichment of amino acids in the sea surface microlayer at coastal and open ocean sites in the North Atlantic Ocean. Limnology and Oceanography, 49: 1605-1619.

Laursen, A. K., L. M. Mayer, and D. W. Townsend. 1996. Lability of proteinaceous material in estuarine seston and subcellular fractions of phytoplankton. Marine Ecology Progress Series 136: 227-234.

Lee, C., and J. L. Bada. 1977. Dissolved amino acids in the equatorial Pacific, the Sargasso Sea, and Biscayne Bay. Limnology and Oceanography 22: 502-510.

Lee, C., and C. Cronin. 1984. Particulate amino acids in the sea: Effects of primary productivity and biological decomposition. Journal of Marine Research 42: 1075-1097.

Lee, C., S. G. Wakeham, and J. I. Hedges. 2000. Composition and Flux of particulate amino acids and chloropigments in equatorial Pacific seawater and sediments. Deep-Sea Research I 47: 1535-1568.

Lindroth, P., and K. Mopper, 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthaldialdehyde. Analytical Chemistry, 51:1667-1674.

Liu, Z., M. E. Kobiela, G. A. McKee, T. Tang, C. Lee, M. R. Mulholland, and P. G. Hatcher. 2010. The effect of chemical structure on the hydrolysis of tetrapeptides along a river-to-ocean transect: AVFA and SWGA. Marine Chemistry 119: 108-120, doi:10.1016/j.marchem.2010.01.005

Lomas, M. W., and S. B. Moran. 2011. Evidence for aggregation and export of cyanobacteria and nano-eukaryotes from the Sargasso Sea euphotic zone. Biogeosciences 8: 203-216, doi:10.5194/bg-8-203-2011

Lourenc, S. O., E. Barbarino, U. M. L. Marquez, and E. Aidar. 1998. Distribution of interacellular nitrogen in marine microalgae: basis for the calculation of specific nitrogen-to-protein conversion factors. Journal of Phycology 811: 798-811.

Madigan, M. T., and J. M. Martinko. 2006. Brock Biology of Microorganisms, 11th ed. Pearson Education.

Marshall, H. G., R. Lacouture, C. Buchanan, and J. Johnson, 2006, Phytoplankton assemblages associated with water quality and salinity regions in Chesapeake Bay, USA, Estuarine, Coastal and Shelf Science, 69:10-18.

Maron, P.-A., L. Ranjard, C. Mougel, and P. Lemanceau. 2007. Metaproteomics: a new approach for studying functional microbial ecology. Microbial ecology 53: 486-93, doi:10.1007/s00248-006-9196-8

Mayer, L. M. 1994. Relationships between mineral surfaces and organic carbon concentrations in soils and sediments. Chemical Geology 114: 347-363.

Mayer, L., and D. Townsend. 1996. Lability of proteinaceous material in estuarine seston and subcellular fractions of phytoplankton. Marine Ecology Progress Series 136: 227-234.

McCarthy, M. D., J. I. Hedges, and R. Benner. 1998. Major bacterial contribution to marine dissolved organic nitrogen. Science 281: 231-234, doi:10.1126/science.281.5374.231

McCarren, J., J. Heuser, R. Roth, N. Yamada, M. Martone, and B. Brahamsha. 2005. Inactivation of swmA Results in the loss of an outer cell layer in a swimming *synechococcus* strain. Journal of Bacteriology 187: 224-230, doi:10.1128/JB.187.1.224

McCarren, J., and B. Brahamsha. 2009. Swimming motility mutants of marine *Synechococcus* affected in production and localization of the S-layer protein SwmA. Journal of Bacteriology 191: 1111-4, doi:10.1128/JB.01401-08

Messner, P. 1997. Bacterial glycoproteins. Glycoconjugate Journal 14: 3-11.

Michaels, A. F., and M. W. Silver. 1988. Primary production, sinking fluxes and the microbial food web. Deep Sea Research Part I: Oceanographic Research Papers 35: 473-490.

Michalopoulos, P., and R. C. Aller. 2004. Early diagenesis of biogenic silica in the Amazon delta: alteration, authigenic clay formation, and storage. Geochimica et Cosmochimica Acta 68: 1061-1085, doi:10.1016/j.gca.2003.07.018

Miyoshi, T., and S. Suzuki. 2004. Degradation of outer membrane proteins of *Synechococcus sp* . in vitro and in situ. Journal of Oceanography 60: 825-833.

Montoya, J.P., M. Voss, P. Kahler, and D. Capone, 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Applied Environmental Microbiology, 62: 986-993.

Moon, C., and M. Dunstan, 1990, Hydrodynamic trapping in the formation of the chlorophyll *a* peak in turbid, very low salinity waters of estuaries, Journal of Plankton Research, 12:323-336.

Moore, E. K., B. L. Nunn, D. R. Goodlett, and H. R. Harvey. 2012. Identifying and tracking proteins through the marine water column: Insights into the inputs and preservation mechanisms of protein in sediments. Geochimica et Cosmochimica Acta 83: 324-359, doi:10.1016/j.gca.2012.01.002

Moriceau, B., M. Goutx, C. Guigue, C. Lee, R. Armstrong, M. Duflos, C. Tamburini, B. Charrière, and O. Ragueneau. 2009. Si–C interactions during degradation of the diatom Skeletonema marinoi. Deep Sea Research Part II 56: 1381-1395, doi:10.1016/j.dsr2.2008.11.026

Morris, R. M., B. L. Nunn, C. Frazar, D. R. Goodlett, Y. S. Ting, and G. Rocap. 2010. Comparative metaproteomics reveals ocean-scale shifts in microbial nutrient utilization and energy transduction. The ISME journal 4: 673-85, doi:10.1038/ismej.2010.4

Mullin, J. B., and J. P. Riley. 1965. The spectrophotometric determination of silicate-silicon in natural waters with special reference to seawater. Analytical Chimica Acta 46: 491-501.

Mulholland, M.R., C.J. Gobler, and C. Lee, 2002. Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. Limnology and Oceanography, 47: 1094-1108.

Mulholland, M. R., C. Lee, and P. M. Glibert. 2003. Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Marine Ecology Progress Series 258: 3-17.

Mulholland, M.R.,and C. Lee, 2009. Peptide hydrolysis and the uptake of dipeptides by phytoplankton. Limnology and oceanography, 54: 856-868.

Mulholland, M.R., R. E. Morse, G. E. Boneillo, P. W. Bernhardt, k. C. Filippino, L.A. Procise, J. L. Blanco-Garcia, H. G. Marshall, T. A. Egerton, W., S. Hunley, K. A. Moore, D. L. Berry, and C. J. Gobler, 2009, Understanding causes and impacts of the dinoflagellate, *Cochlodinium polykrikoides*, blooms in the Chesapeake Bay. Estuaries and Coasts, 32:734-747.

Nagata, T., R. Fukudal, I. Koikel, K. Kogurel, and D. L. Kirchman. 1998. Degradation by bacteria of membrane and soluble protein in seawater. Aquatic Microbial Ecology 14: 29-37.

Nagata, T., B. Meon, and D. L. Kirchman. 2003. Microbial degradation of peptidoglycan in seawater. Limnology and Oceanography 48: 745-754, doi:10.4319/lo.2003.48.2.0745

Nguyen, Reno T, and R. H. Harvey. 2001. Preservation of protein in marine systems : Hydrophobic and other noncovalent associations as major stabilizing forces. Geochimica et Cosmochimica Acta 65: 1467-1480.

Nguyen, R., and R. H. Harvey. 2003. Preservation via macromolecular associations during Botryococcus braunii decay: proteins in the Pula Kerogen. Organic Geochemistry 34: 1391-1403, doi:10.1016/S0146-6380(03)00154-2

Nunn, B. L., Y. S. Ting, L. Malmström, Y. S. Tsai, A. Squier, D. R. Goodlett, and H. R. Harvey. 2010. The path to preservation: Using proteomics to decipher the fate of diatom proteins during microbial degradation. Limnology and Oceanography 55: 1790-1804, doi:10.4319/lo.2010.55.4.1790

Orcutt, K.M., F. Lipschultz, K. Gundersen, R. Arimoto, A. F. Michaels, A. H. Knap, and J. R. Gallon, 2001. A seasonal study of the significance of N_2 fixation by *Trichodesmium spp*. At Bermuda Atlantic Time-series Study (BATS) site. Deep Sea Research Part II, 48: 1583-1608

Palenik, B., and J. A. Koke. 1995. Characterization of a nitrogen-regulated protein identified by cell surface biotinylation of a marine phytoplankton. Applied and Environmental Microbiology 61: 3311-5.

Pantoja, S., and C. Lee, 1994, Cell-surface oxidation of amino acids in seawater, Limnology and Oceanography, 39: 1718-1726.

Pantoja, S., C. Lee, and J. F. Marecek. 1997. Hydrolysis of peptides in seawater and sediment. Marine Chemistry 57: 25-40.

Pantoja, S., and C. Lee. 1999. Peptide decomposition by extracellular hydrolysis in coastal seawater and salt marsh sediment. Marine Chemistry 63: 273-291, doi:10.1016/S0304-4203(98)00067-X

Pantoja, S., P. Rossel, R. Castro, L. A. Cuevas, G. Daneri, and C. Córdova. 2009. Microbial degradation rates of small peptides and amino acids in the oxygen minimum zone of Chilean coastal waters. Deep Sea Research Part II 56: 1055-1062, doi:10.1016/j.dsr2.2008.09.007

Parson, R. T., M. Takahashi, and B. Hargrave. 1977. Biological Oceanographic Processes, 2nd ed. Pergamon.

Payne J.W., 1980. Transport and utilization of peptides by bacterial, pp211-256. In: J.W. Payne, [Ed.], Microorganisms and Nitrogen Sources. Wiley, Chichester,.

Pereira, S., A. Zille, E. Micheletti, P. Moradas-Ferreira, R. De Philippis, and P. Tamagnini. 2009. Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. FEMS microbiology reviews 33: 917-41, doi:10.1111/j.1574-6976.2009.00183.x

Pogozelski, W. K., and T. D. Tullius. 1998. Oxidative strand scission of nucleic acids: routes initiated by hydrogen abstraction from the sugar moiety. Chemical Reviews 98: 1089-1108.

Pritchard, D.W., 1952. Salinity distribution and circulation in the Chesapeake Bay estuarine system. Journal of Marine Research, 11: 106-123.

Ransom, B., R. H. Bennett, R. Baerwald, and K. Shea. 1997. TEM study of in situ organic matter on continental margins: occurrence and the "monolayer " hypothesis. Marine Geology 138: 1-9.

Reinthaler, T., E. Sintes, and G. J. Herndl, 2008. Dissolved organic matter and bacterial production and respiration in the sea-surface microlayer of the open Atlantic and the western Mediterranean Sea. Limnology and Oceanography, 53: 122-136.

Repeta, D. J., T. M. Quan, L. I. Aluwihare, and A.M. Accardi, 2002. Chemical characterization of high molecular weight dissolved organic matter in fresh and marine waters. Geochimica et Cosmochimica Acta, 66: 955-962.

Resch, C. M., and J. Gibson. 1983. Isolation of the carotenoid-containing cell wall of three unicellular cyanobacteria. Journal of Bacteriology 155: 345-50.

Richardson, T. L., and G. A. Jackson. 2007. Small phytoplankton and carbon export from the surface ocean. Science 315: 838-40, doi:10.1126/science.1133471

Riethman, H. C., T. P. Mawhinney, and L. a Sherman. 1988. Characterization of phycobilisome glycoproteins in the cyanobacterium Anacystis nidulans R2. Journal of Bacteriology 170: 2433-40.

Robbins, L. L., and K. Brew. 1990. Proteins from the organic matrix of core-top and fossil planktonic foraminifera. Geochimica et Cosmochimica Acta 54: 2285-2292.

Rosenstock, B., W. Zwisler, M. Simon, 2005. Bacterial comsumption of humic and non-humic low and high molecular weight DOM and the effect of solar irradiation on the turnover of labile DOM in the Southern Ocean. Microbial Ecology, 50: 90-101.

Roth, L. C., and H. R. Harvey. 2006. Intact protein modification and degradation in estuarine environments. Marine Chemistry 102: 33-45, doi:10.1016/j.marchem.2005.10.025

Saijo, S., and E. Tanoue. 2005. Chemical forms and dynamics of amino acid-containing particulate organic matter in Pacific surface waters. Deep Sea Research Part I 52: 1865-1884, doi:10.1016/j.dsr.2005.05.001

Santschi, P. H., E. Balnois, K. J. Wilkinson, J. Zhang, J. Buffle, and L. Guo. 1998. Fibrillar polysaccharides in marine macromolecular organic matter as imaged by atomic force microscopy and transmission electron microscopy. Limnology and Oceanography 43: 896-908.

Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews 36: 407-77.

Schultze-Lam, S., G. Harauz, and T. J. Beveridge. 1992. Participation of a cyanobacterial S layer in fine-grain mineral formation. Journal of Bacteriology 174: 7971-81.

Schäffer, C., M. Graninger, and L. Boltzmann-institut. 2001. Review Prokaryotic glycosylation. Archaea 248-261.

Schäffer, C., and P. Messner. 2001. Glycobiology of surface layer proteins. Biochimie 83: 591-599.

Schäffer, C., M. Graninger, and P. Messner. 2001. Prokaryotic glycosylation. Archaea 1: 248-261.

Schäffer, C., and P. Messner. 2004. Surface-layer glycoproteins: an example for the diversity of bacterial glycosylation with promising impacts on nanobiotechnology. Glycobiology 14: 31R-42R, doi:10.1093/glycob/cwh064

Sellner, K.G., E.W. Nealley, 1997. Diel fluctuations in dissolved free amino acids and monosaccharides in Chesapeake Bay dinoflagellate blooms. Marine Chemistry, 56: 193-200.

Shacter, E. 2000. Quantification and significance of protein oxidation in biological samples. Drug Metabolism Reviews 32: 307-26, doi:10.1081/DMR-100102336

Singh, R., A. Barden, T. Mori, and L. Beilin. 2001. Advanced glycation end-products: a review. Diabetologia 44: 129-46, doi:10.1007/s001250051591

Sleytr, U., P. Messner, D. Pum, and M. Sara. 1996. Crystalline Bacterial Cell Surface Proteins, Academic Press.

Sleytr, U. B., and T. J. Beveridge. 1999. Bacterial S-layers. Trends in microbiology 7: 253-60.

Smarda, J., D. Smajs, J. Komrska, and V. Krzyzanek. 2002. S-layers on cell walls of cyanobacteria. Micron 33: 257-277.

Somville, M., and G. Billen. 1983. A method for determining exoproteolytic activity in natural waters. Limnology and Oceanography 28: 190-193.

Steinberg, D. K., C. A. Carlson, N. R. Bates, R. J. Johnson, A. F. Michaels, and A. H. Knap. 2001. Overview of the US JGOFS Bermuda Atlantic Time-series Study (BATS): a decade-scale look at ocean biology and biogeochemistry. Deep Sea Research Part II 48: 1405-1447, doi:10.1016/S0967-0645(00)00148-X

Strycek, T., J. Acreman, A. Kerry, G. G. Leppard, M. V. Nermut, D. J. Kushner, and I. J. Acreman. 1992. Extracellular fibril production by freshwater algae and cyanobacteria. Microbial Ecology 23: 53-74.

Tanoue, E., S. Nishiyama, M. Kamo, and A. Tsugita. 1995. Bacterial membranes : Possible source of a major dissolved protein in seawater. Geochimica et Cosmochimica Acta 59: 2643-2648.

Taylor, M. T., and K. Drickamer. 2002. Introduction to Glycobiology, Oxford University Press.

Tesson, B., C. Gaillard, and V. Martin-Jézéquel. 2008. Brucite formation mediated by the diatom Phaeodactylum tricornutum. Marine Chemistry 109: 60-76, doi:10.1016/j.marchem.2007.12.005

Tsukasaki, A., and E. Tanoue. 2010. Chemical qualification of electrophoretically detectable peptides and sugar chains in oceanic surface particulate organic matter. Marine Chemistry 119: 33-43, doi:10.1016/j.marchem.2009.12.004

Varki, A. 1993. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3: 97-130.

Voet, D., and J. G. Voet. 2005. Biochemistry, 3rd ed. John Wiley and Co.

Wakeham, S. G., C. Lee, J. I. Hedges, P. J. Hernes, and M. L. J. Peterson. 1997. Molecular indicators of diagenetic status in marine organic matter. Geochimica et Cosmochimica Acta 61: 5363-5369, doi:10.1016/S0016-7037(97)00312-8

Walter, M. R., J. Bauld, and T. D. Brock. 1972. Siliceous algal and bacterial stromatolites in hot spring and geyser effluents of Yellowstone national park. Science 178: 402-405.

Weiss, M. S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz. 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science 254: 1627-1630.

Welschmeyer, N. A., 1994. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. Limnology and Oceanography, 39: 1985-1992.

West, C. M. 1986. Current ideas on the significance of protein glycosylation. Molecular and Cellular Bochemistry 72: 3-20.

Williams, P. J. L., T. Berman, and O. Holm-Hansen, 1976, Amino acid uptake and respiration by marine heterotrophs. Marine Biology, 35: 41-47.

Wong, G.T.F., 1979. Alkalinity and pH in the southern Chesapeake Bay and the James River estuary. Limnology and Oceanography, 24: 970-977.

Xue, J., C. Lee, and S. G. Wakeham, R.A. Armstrong, 2011. Using principal components analysis (PCA) with cluster analysis to study the organic geochemistry of sinking particles in the ocean. Organic Geochemistry, 42:356-367.

Yamada, N., and E. Tanoue. 2003. Detection and partial characterization of dissolved glycoproteins in oceanic waters. Limnology and Oceanography 48: 1037-1048.

Yamashita, Y., and E. Tanoue, 2003. Distribution and alteration of amino acids in bulk DOM along a transect from bay to oceanic waters. Marine Chemistry, 82: 145-160.

Yunker, M. B., R. W. Macdonald, D. J. Veltkamp, and W. J. Cretney, 1995, Terrestrial and marine biomarkers in a seasonally ice-covered Arctic estuary – integration of multivariate and biomarker approaches. Marine Chemistry, 49: 1-50.

	Stripped CCMP2370					Intact CCMP2370					Stripped CCMP1334					Intact CCMP1334				
Incu. Time (Day)	0	2	7	15	30	0	2	7	15	30	0	2	7	15	30	0	2	7	15	30
DHAA ($\mu molL^{-1}$)																				
AVG_L-AA	1.42	0.66	1.30	1.45	1.12	8.64	2.53	8.82	3.78	3.28	1.23	2.29	1.27	2.87	0.64	2.84	0.32	3.64	3.52	0.90
STD_L-AA	1.23	0.39	0.79	1.24	0.73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AVG_D-AA	0.03	0.08	0.15	0.08	0.05	1.81	0.09	0.24	0.18	0.12	0.05	0.12	0.14	0.64	0.06	0.15	0.02	0.45	0.24	0.06
STD_D-AA	0.02	0.07	0.04	0.01	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total DHAA	1.46	0.74	1.45	1.53	1.17	10.45	2.62	9.06	3.96	3.39	1.27	2.41	1.41	3.51	0.70	2.99	0.34	4.09	3.76	0.96
DFAA ($\mu molL^{-1}$)																				
L-AA	0.07	0.36	0.12	0.02	0.02	5.93	0.18	1.50	0.49	0.18	0.02	0.09	0.04	0.00	0.00	2.10	0.42	0.03	0.67	0.03
D-AA	0.01	0.03	0.03	0.00	0.00	0.75	0.02	0.08	0.08	0.04	0.00	0.01	0.01	0.00	0.00	0.17	0.04	0.04	0.00	0.00
Total DFAA	0.08	0.39	0.15	0.02	0.02	6.68	0.21	1.58	0.57	0.21	0.02	0.11	0.04	0.00	0.00	2.26	0.45	0.07	0.67	0.03
PAA ($\mu molL^{-1}$)																				
L-AA	55.86	78.95	38.11	9.56	11.84	436.26	281.14	56.98	39.53	17.63	-	-	-	-	-	-	-	-	-	-
D-AA	1.24	1.31	0.84	0.32	0.44	7.40	5.65	0.94	0.76	0.38	-	-	-	-	-	-	-	-	-	-
Total PAA	57.10	80.27	38.95	9.88	12.27	443.66	286.80	57.92	40.29	18.01	-	-	-	-	-	-	-	-	-	-

Table. S2.1. Concentrations of amino acids during the incubation of *Synechococcus*.

L-AA(μM)		Days	ASP	GLU	SER	THR	HIS	GLY	BALA	ARG	ALA	GABA	TYR	VAL	MET	PHE	ILE	LEU
Stripped CCMP 2370	DHAA	0	0.043	0.050	0.033	0.027	0.003	0.071	0.003	0.028	0.054	0.002	0.012	0.025	0.003	0.017	0.020	0.036
		2	0.087	0.101	0.084	0.071	0.009	0.272	0.016	0.051	0.119	0.001	0.025	0.052	0.013	0.039	0.034	0.067
		7	0.240	0.248	0.233	0.243	0.009	0.452	0.034	0.075	0.250	0.002	0.043	0.110	0.025	0.061	0.062	0.113
		15	0.078	0.070	0.076	0.095	0.004	0.205	0.029	0.019	0.095	0.001	0.009	0.032	0.008	0.000	-	0.031
		30	0.043	0.046	0.045	0.039	0.007	0.726	0.051	0.008	0.046	0.003	0.011	0.016	0.010	0.012	0.041	0.041
Intact CCMP 2370	DHAA	0	0.475	3.422	0.539	0.337	0.018	1.040	0.018	0.144	1.939	0.012	0.082	0.216	0.063	0.295	-	0.043
		2	0.324	0.261	0.213	0.266	0.022	0.354	0.011	0.087	0.288	0.005	0.072	0.165	0.032	0.101	0.137	0.195
		7	0.828	2.360	0.637	0.775	0.070	1.204	0.100	0.355	0.834	0.015	0.173	0.389	0.101	0.250	0.299	0.432
		15	0.399	0.727	0.298	0.411	0.027	0.757	0.130	0.047	0.439	0.001	0.060	0.155	0.041	0.066	0.077	0.142
		30	0.189	0.240	0.143	0.114	0.025	1.880	0.124	0.024	0.211	0.006	0.020	0.097	0.022	0.044	0.060	0.075
Stripped CCMP 1334	DHAA	0	0.140	0.156	0.054	0.070	0.026	0.106	0.019	0.102	0.147	0.002	0.084	0.096	0.009	0.073	0.004	0.137
		2	0.280	0.291	0.090	0.151	0.031	0.149	0.064	0.144	0.207	0.003	0.162	0.200	0.036	0.125	0.128	0.229
		7	0.131	0.116	0.097	0.136	0.010	0.199	0.038	0.052	0.175	0.013	0.055	0.076	0.028	0.042	0.038	0.062
		15	0.345	0.275	0.184	0.205	0.034	0.688	0.118	0.076	0.422	0.000	0.071	0.151	0.038	0.056	0.069	0.137
		30	0.069	0.069	0.036	0.040	0.011	0.183	0.049	0.014	0.062	0.000	0.003	0.036	0.007	-	0.033	0.032
Intact CCMP 1334	DHAA	0	0.145	1.637	0.063	0.008	0.147	0.094	0.043	0.121	0.159	0.001	0.105	0.116	0.014	-	0.048	0.142
		2	0.035	0.040	0.012	0.022	0.010	0.014	0.004	0.026	0.028	0.001	0.025	0.027	0.002	0.017	0.019	0.040
		7	0.795	0.469	0.517	0.341	-	1.282	0.000	0.890	0.894	0.029	0.147	0.230	0.069	0.125	0.137	0.369
		15	0.427	0.406	0.197	0.182	-	0.489	0.058	0.184	0.373	0.018	0.156	0.264	0.079	0.185	0.182	0.316
		30	0.171	0.184	0.085	0.076	-	0.126	0.007	0.118	0.164	0.000	0.118	0.128	0.018	0.083	0.092	0.165
Stripped CCMP 2370	DFAA	0	0.003	0.031	0.002	0.003	0.001	0.005	0.000	0.002	0.006	-	0.001	0.003	0.009	0.001	0.001	0.001
		2	0.019	0.061	0.010	0.030	-	0.147	0.034	0.036	0.003	0.001	0.004	0.006	0.004	0.000	-	0.006
		7	0.010	0.084	0.002	0.003	-	0.005	0.004	0.002	0.005	-	0.001	0.001	-	-	-	0.001
		15	0.002	0.009	-	0.002	-	0.001	-	-	0.003	-	0.002		-	-	-	0.000
		30	0.002	0.012	-	0.002	-	0.000	-	0.002	0.002	-	-		-	-	-	-
Intact CCMP 2370	DFAA	0	0.184	3.973	0.413	0.115	0.003	0.054	-	0.115	0.861	-	0.004	0.078	0.013	0.006	0.035	0.075
		2	0.003	0.075	0.039	0.008	0.016	0.004	0.009	0.006	0.004	-	0.000		0.017	-	-	-
		7	0.031	0.951	0.040	0.084	0.011	0.095	0.033	0.033	0.113	0.001	0.023	0.043	0.013	-	-	0.029
		15	0.047	0.421	-	0.002	-	0.004	0.002	0.002	0.010	-	-	0.003	0.002	-	-	0.001
		30	0.021	0.135	-	0.002	-	0.005	0.002	0.004	0.002	-	-		0.006	-	-	0.001
Stripped CCMP 1334	DFAA	0	0.001	0.005	0.001	-	-	0.001	-	0.001	0.001	0.000	0.001	0.001	0.001	0.000	0.000	0.002
		2	0.003	0.031	0.001	0.010	-	0.021	0.003	0.005	0.004	-	0.003	0.002	0.005	0.002	0.001	0.000
		7	0.004	0.005	0.000	0.001	-	0.018	0.001	0.001	0.001	-	0.001	0.001	0.001	-	-	0.001
Intact CCMP 1334	DFAA	0	0.018	1.807	0.067	0.014	0.000	0.027	0.001	0.017	0.062	0.000	0.017	0.020	0.005	0.009	0.013	0.019
		2	0.027	0.044	0.007	-	0.018	0.149	0.059	0.030	0.027	0.001	0.012	0.011	0.009	0.007	0.007	0.010
		7	0.003	0.006	-	0.001	0.007	0.002	0.002	0.001	0.002	-	0.001	0.000	0.002	-	0.000	0.003
		15	0.003	-	0.002	-	0.003	0.012	0.002	-	0.004	-	0.002	0.001	0.003	-	0.632	0.005
		30	0.004	0.012	0.000	-	-	0.007	-	0.004	0.000	-	-	0.001	0.001	-	-	0.001

Stripped CCMP 2370	PAA	0	3.645	3.265	2.852	3.067	0.531	3.246	0.062	2.628	3.860	0.102	1.113	2.408	0.414	2.101	1.873	3.030
		2	0.524	0.457	0.348	0.333	0.070	0.640	0.002	0.312	0.659	0.005	0.115	0.322	0.043	0.246	0.220	0.538
		7	0.318	0.224	0.182	0.196	0.033	0.355	0.002	0.108	0.287	0.005	0.062	0.157	0.025	0.136	-	0.242
		15	0.193	0.152	0.111	0.115	0.017	0.216	-	0.063	0.181	0.004	0.036	0.093	0.014	0.073	0.067	0.127
		30	0.389	0.335	0.228	0.242	0.036	0.423	0.004	0.111	0.378	0.007	0.084	0.211	0.021	0.146	0.145	0.261
Intact CCMP 2370	PAA	0	2.778	2.888	2.070	2.367	0.325	2.532	0.025	1.588	3.245	0.070	0.866	2.104	0.227	1.339	1.633	2.652
		2	4.018	3.386	3.094	3.138	0.591	3.665	0.024	2.207	3.877	0.043	1.101	2.353	0.335	1.749	1.904	2.939
		7	2.088	1.768	1.392	1.335	0.302	2.729	0.021	0.709	2.242	0.007	0.460	0.981	0.145	0.858	0.778	1.627
		15	1.663	1.490	0.923	0.900	0.231	1.775	0.008	0.588	1.335	0.016	0.340	0.669	0.080	0.554	0.541	0.989
		30	1.213	1.118	0.657	0.703	0.173	1.259	0.007	0.425	1.005	0.013	0.258	0.549	0.050	0.407	0.413	0.746

Table S2.3. L-amino acid concentration in the incubation of *Synechococcus*

D-AA (μM)		Days	ASP	GLU	SER	THR
Stripped CCMP 2370	DHAA	0	0.005	0.008	0.002	0.004
		2	0.010	0.010	0.005	0.012
		7	0.028	0.048	0.013	0.010
		15	0.018	0.013	0.012	0.024
		30	0.017	0.010	0.008	0.015
Intact CCMP 2370	DHAA	0	0.033	1.348	0.029	0.395
		2	0.024	0.045	0.010	0.010
		7	0.052	0.088	0.035	0.061
		15	0.048	0.054	0.018	0.061
		30	0.034	0.037	0.014	0.034
Stripped CCMP 1334	DHAA	0	0.025	0.008	0.001	0.012
		2	0.050	0.023	0.015	0.029
		7	0.033	0.036	0.007	0.062
		15	0.155	0.213	0.014	0.257
		30	0.028	0.012	0.003	0.016
Intact CCMP 1334	DHAA	0	0.022	0.048	0.031	0.045
		2	0.006	0.003	0.002	0.005
		7	0.140	0.170	0.027	0.464
		15	0.097	0.040	0.048	0.059
		30	0.035	0.015	0.025	0.018
Stripped CCMP 2370	DFAA	0	0.001	0.002	0.006	0.001
		2	0.005	0.002	0.006	0.017
		7	0.009	0.016	0.001	0.003
		15	-	0.004	-	-
		30	-	0.002	-	0.001
Intact CCMP 2370	DFAA	0	0.005	0.096	0.025	0.621
		2	0.003	0.015	0.005	0.002
		7	0.030	0.019	0.003	0.029
		15	0.032	0.029	-	0.020
		30	0.031	0.002	0.002	-
Stripped CCMP 1334	DFAA	0	0.001	0.001	0.000	0.001
		2	0.001	0.009	0.001	0.004
		7	0.003	0.003		0.001
Intact CCMP 1334	DFAA	0	0.131	0.003	0.000	0.033
		2	0.009	0.002	0.005	0.020
		7	0.001	0.037	-	0.001
		15	-	0.000	-	0.003
		30	-	-	-	0.001
Stripped CCMP 2370	PAA	0	0.278	0.189	0.115	0.180
		2	0.034	0.017	0.008	0.021
		7	0.021	0.008	0.006	0.016
		15	0.013	0.008	0.005	0.023
		30	0.031	0.029	0.010	0.042
Intact CCMP 2370	PAA	0	0.185	0.114	0.080	0.074
		2	0.329	0.160	0.064	0.139
		7	0.151	0.048	0.018	0.070
		15	0.094	0.038	0.017	0.083
		30	0.081	0.035	0.014	0.062

Table S2.3. D-amino acid concentration in the incubation of *Synechococcus*

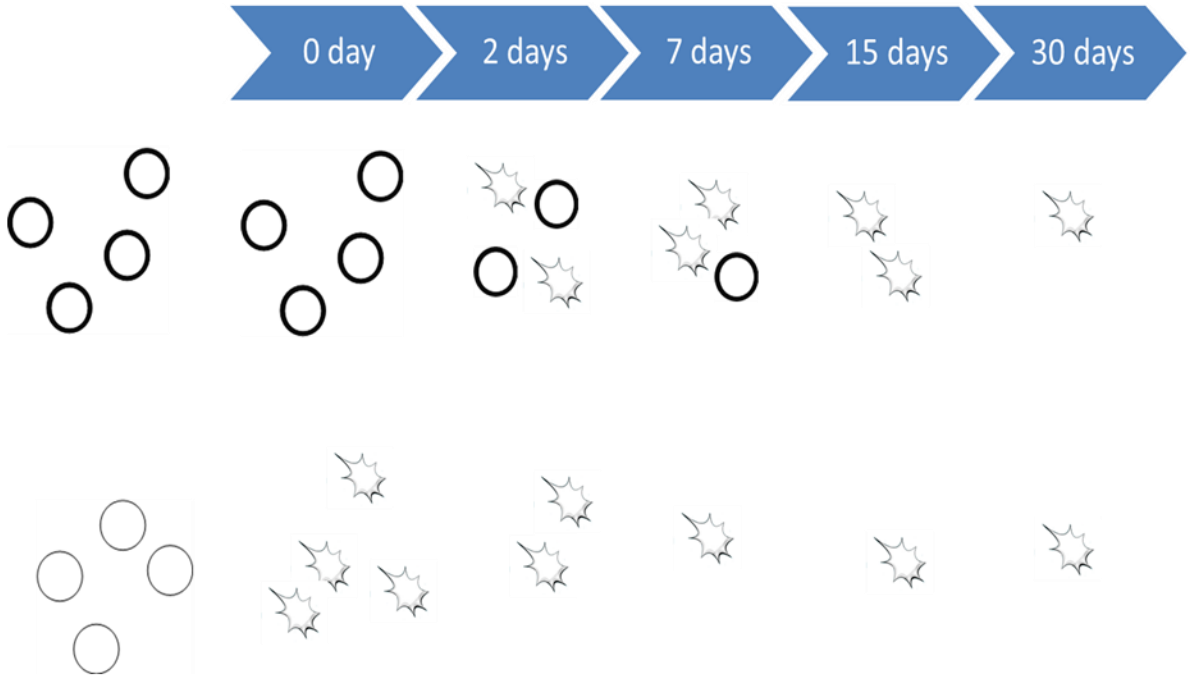

Figure S2. 1. Degradation mechanism of intact and stripped *Synechococcus* CCMP2370 cells during the dark incubations. Bold open circles represent intact cells and open circles represent stripped cells. When stripped cells were released into coastal seawater at day-0, they were subject to immediate cell lysis, followed by more rapid degradation in the following 30-day incubation.

Appendix of Chapter 3:

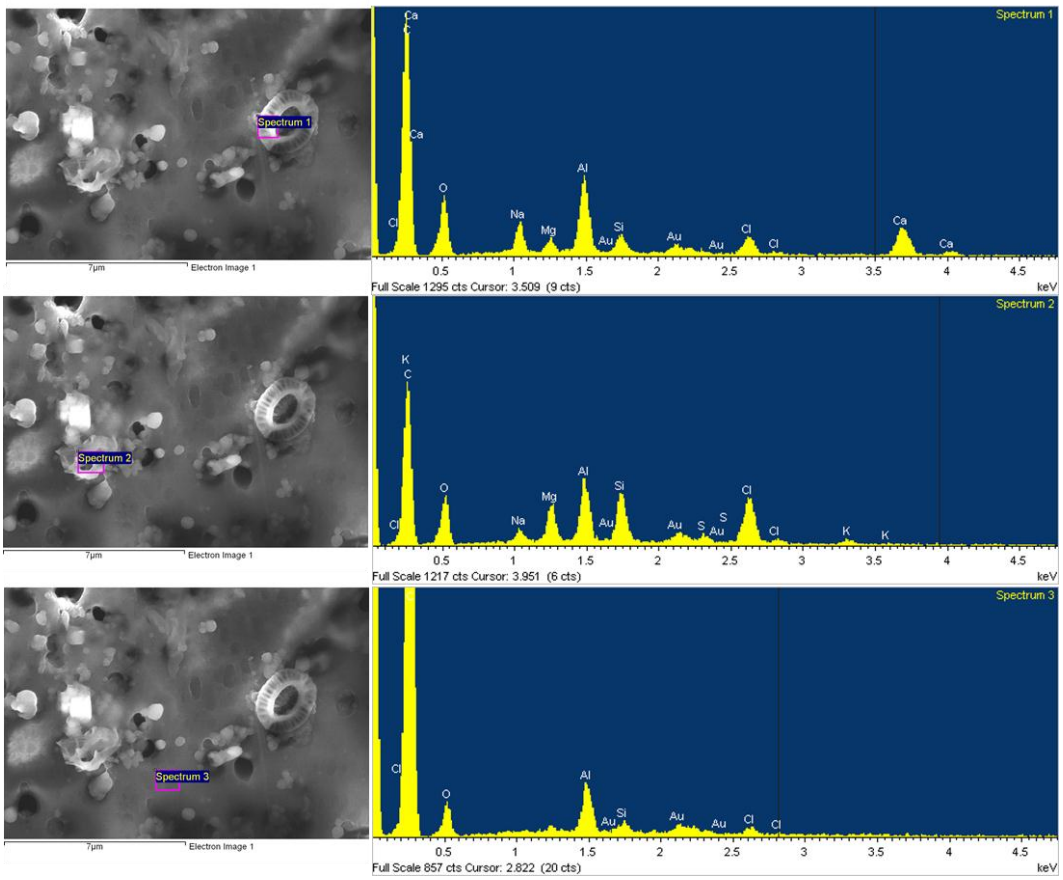

Figure. S3.1. Other EDX spectra of marine particles from Bermuda Rise that were collected at 50m depth (Enrichment of Al is from aluminum holder).

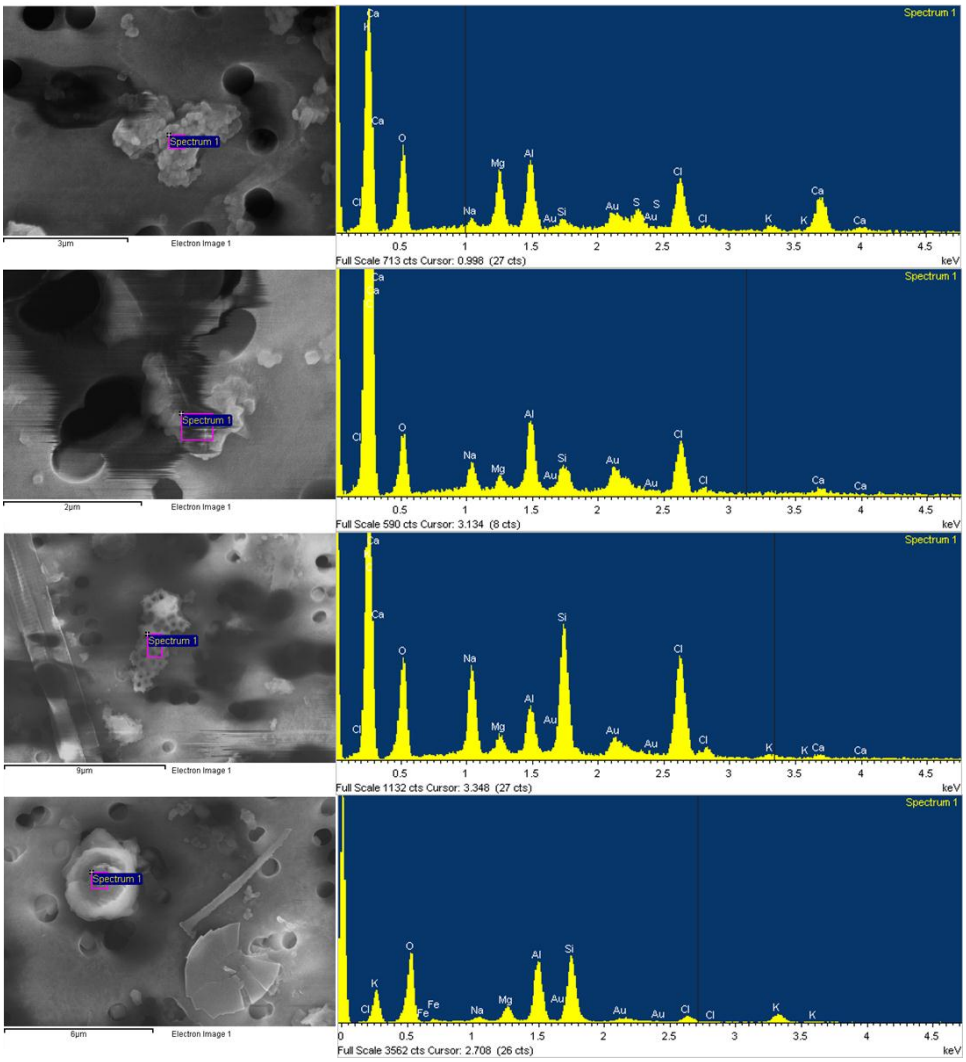

Figure S3.2. Other EDX spectra of marine particles from Bermuda Rise that were collected at 2000m depth (Enrichment of Al is from aluminum holder).

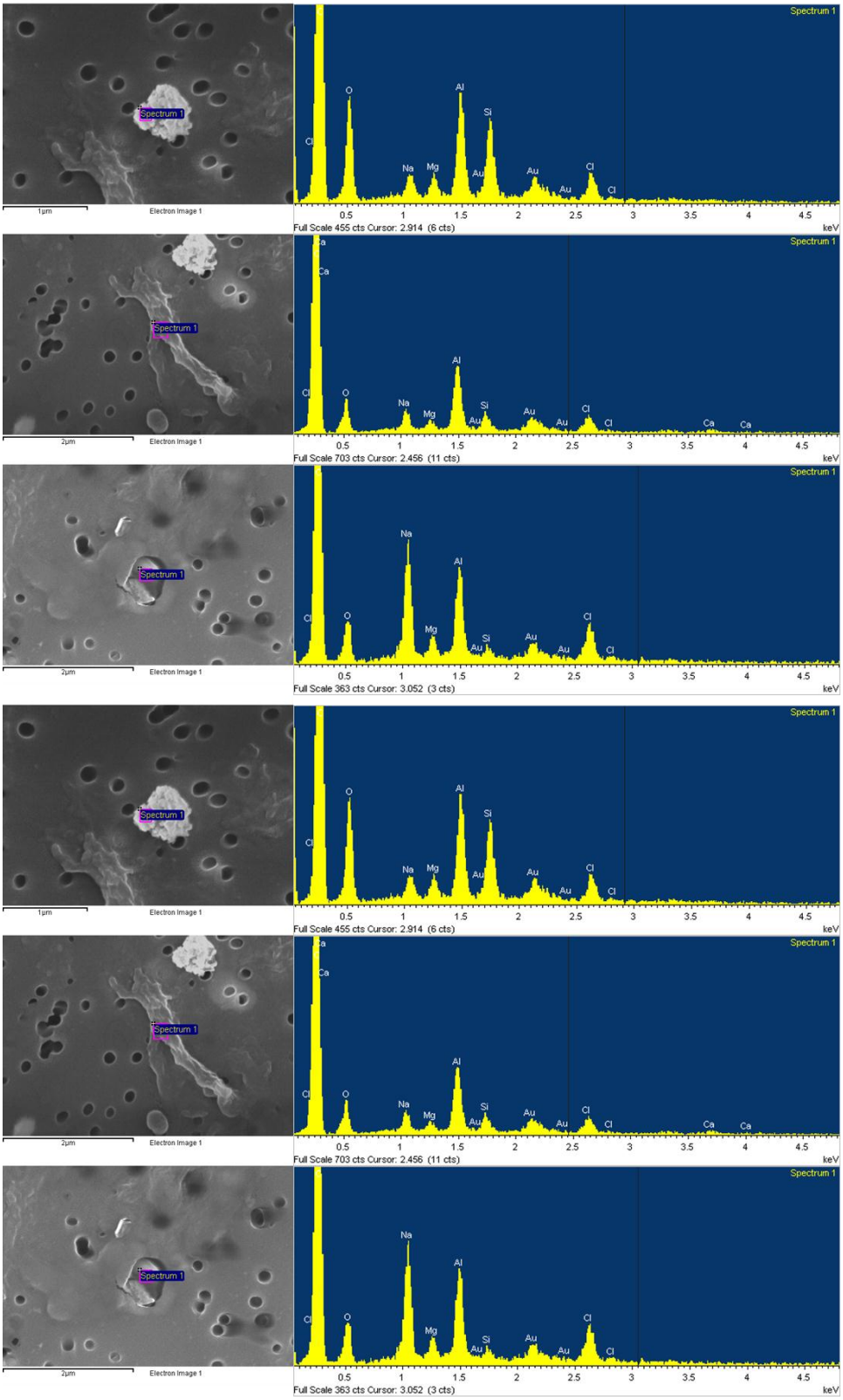

Figure S3.2. Continued: Other EDX spectra of marine particles from Bermuda Rise that were collected at 2000m depth (Enrichment of Al is from aluminum holder).

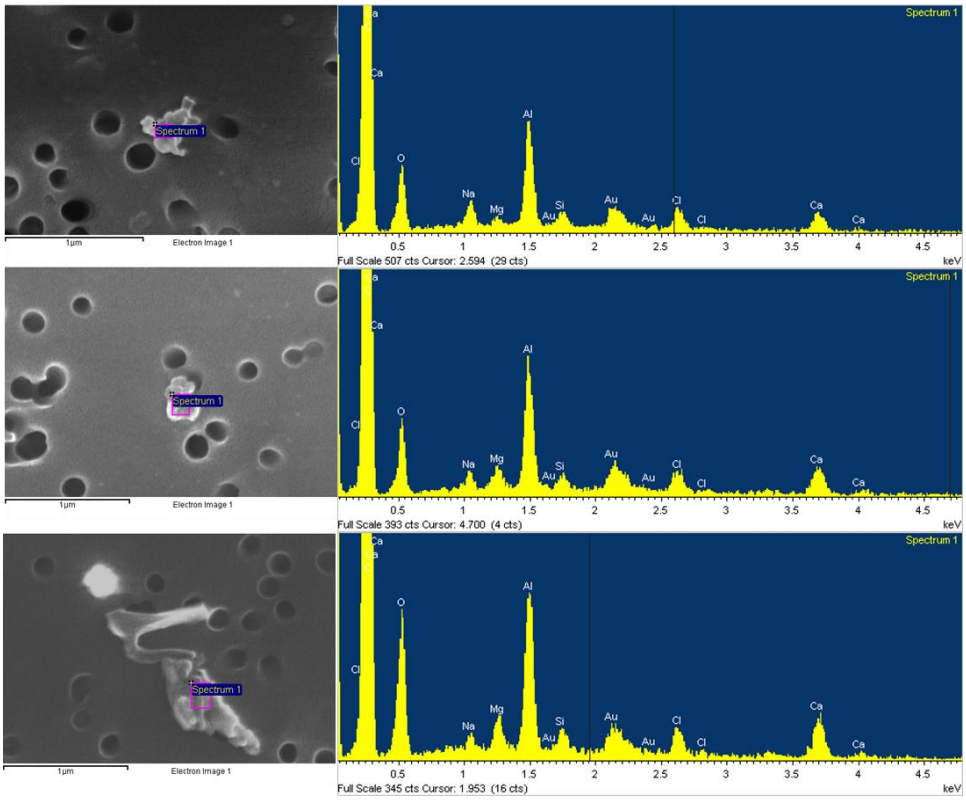

Figure S3.2. Continued: Other EDX spectra of marine particles from Bermuda Rise that were collected at 2000m depth (Enrichment of Al is from aluminum holder).

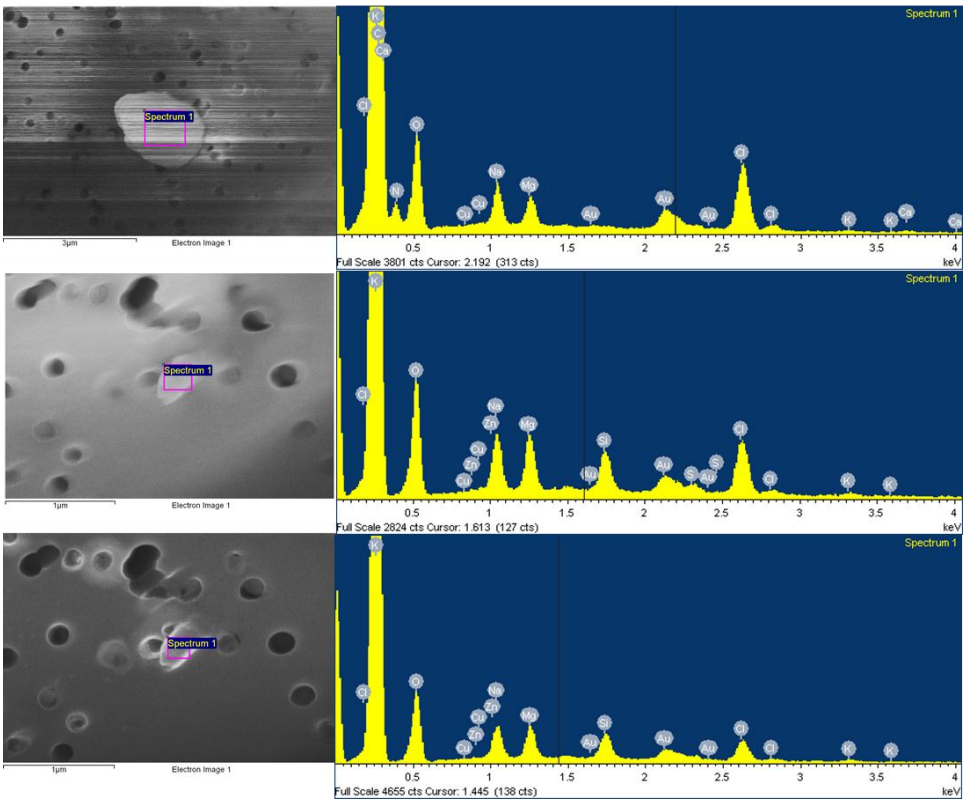

Figure. S3.3. Other EDX spectra of marine particles from Bermuda Rise that were collected at 4200m depth (no aluminum holder used on this filter).

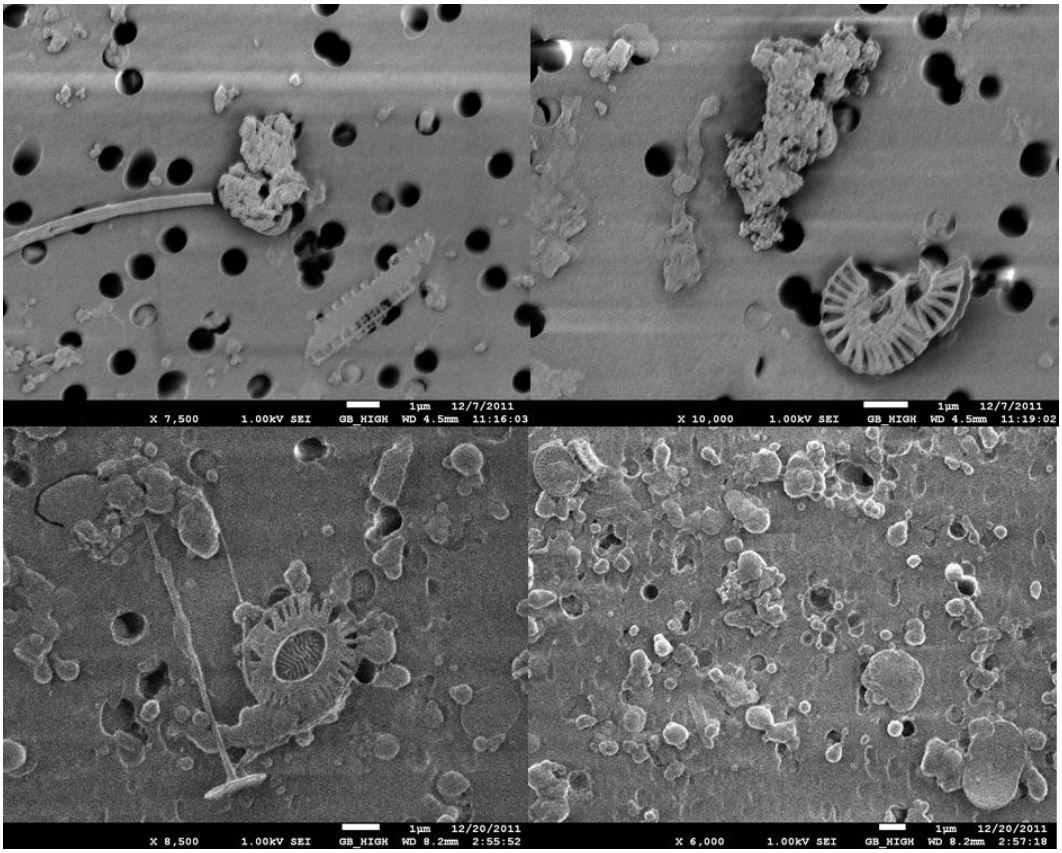

Figure S3.4. SEM of marine particles from Bermuda Rise that were collected at 200m (upper graphs) and 50m (bottom graphs).

#	%Mg	%Si	%Ca
1	63%	38%	0%
2	63%	38%	0%
3	11%	0%	89%
4	25%	0%	75%
5	16%	84%	0%
6	15%	0%	85%
7	17%	0%	83%
8	36%	6%	58%
9	24%	55%	21%
10	11%	85%	5%
11	12%	19%	69%
12	40%	60%	0%
13	0%	100%	0%
14	6%	94%	0%
15	-	-	-
16	100%	0%	0%
17	0%	100%	0%
18	20%	80%	0%
19	52%	48%	0%
20	24%	17%	60%
21	6%	16%	78%
22	51%	15%	34%
23	-	-	-
24	100%	0%	0%
25	100%	0%	0%
26	21%	79%	0%
27	27%	55%	18%
28	66%	34%	0%
29	10%	23%	66%
30	17%	16%	68%
31	21%	15%	64%
32	61%	39%	0%
33	75%	25%	0%
34	50%	50%	0%

Table. S3.1. Relative mass abundance of Ca, Mg, and Si in particulate matter that was collected from 50m and 2000m in the water column. 8 out of 34 particles were enriched in both Mg and Si.

Appendix of Chapter 4.

DFAA Concentrations	ASP (nM)	GLU (nM)	SER (nM)	HIS (nM)	GLY (nM)	THR (nM)	ARG (nM)	BALA (nM)	ALA (nM)	GABA (nM)	TYR (nM)	VAL (nM)	PHE (nM)	ILE (nM)	LEU (nM)	Total (nM)
Jul 2008																
J1	10.2	4.7	7.5	0.8	7.0	2.6	1.0	0.8	5.8	0.5	1.0	0.3	-	0.2	0.2	42.6
J5	8.8	2.2	5.6	0.9	4.6	1.1	1.8	0.4	2.5	0.4	0.9	-	-	0.1	0.6	30.0
J10	14.5	14.2	14.7	1.9	15.5	3.3	1.6	1.4	10.1	0.7	1.8	1.3	0.1	0.5	-	81.5
J16	8.5	4.2	3.1	0.2	6.3	0.9	1.4	0.8	3.8	0.5	0.6	0.3	-	-	0.1	30.6
J20	8.4	9.1	5.4	1.5	11.6	1.5	4.3	2.6	9.3	0.5	1.6	0.6	-	0.7	-	57.2
J22	10.0	10.6	11.0	1.0	11.9	2.6	2.0	2.0	8.9	0.9	1.5	0.6	-	0.3	-	63.2
Aug 2008																
A1	19.7	5.1	6.8	1.5	5.9	3.5	0.5	-	9.5	-	1.0	2.5	-	4.3	-	60.3
A5	25.7	19.7	4.7	3.7	5.5	3.1	1.5	-	10.6	0.8	1.0	2.5	-	4.4	-	83.2
A10	58.7	28.8	4.4	8.2	14.4	-	3.1	-	22.5	-	1.2	2.8	-	-	-	144.0
A16	37.2	10.3	14.4	-	10.9	-	0.0	-	19.5	-	-	3.3	-	3.4	1.0	100.1
Bloom	24.6	40.4	8.4	-	59.0	6.3	4.7	-	39.7	-	-	4.8	-	2.8	0.7	191.5
A22	95.2	37.0	132.0	12.4	115.6	28.5	0.0	-	45.8	-	7.3	21.7	7.2	16.4	12.6	531.8
A23	48.5	52.0	28.4	-	57.1	-	0.0	-	35.8	-	-	7.0	-	8.5	2.7	240.1
A29	35.0	13.6	20.7	-	20.6	-	0.0	-	14.6	-	-	3.7	-	-	-	108.3

Figure. S4.1. DFAA concentrations during July and Augest, 2008.

DCAA Concentrations	ASP (nM)	GLU (nM)	SER (nM)	HIS (nM)	GLY (nM)	THR (nM)	ARG (nM)	BALA (nM)	ALA (nM)	GABA (nM)	TYR (nM)	VAL (nM)	PHE (nM)	ILE (nM)	LEU (nM)	Total (nM)
Jul 2008																
J1	84.1	40.3	51.1	4.5	213.9	49.5	8.2	37.0	83.2	-	14.1	-	14.1	12.8	22.3	635.1
J5	146.7	71.8	87.0	5.9	285.3	78.1	11.1	67.3	147.3	30.4	25.8	62.3	32.5	22.6	33.5	1107.5
J10	80.3	44.1	35.0	3.2	140.3	39.1	5.7	30.4	68.0	-	13.5	-	3.7	12.2	22.4	497.8
J16	95.8	54.6	45.5	5.1	126.0	39.1	6.8	32.6	70.8	76.3	15.4	71.2	5.1	16.4	23.0	683.8
J20	146.0	89.4	77.4	8.7	222.1	75.4	20.9	32.7	121.4	-	25.4	-	14.0	24.9	43.6	901.9
J22	105.1	101.7	96.6	12.0	154.2	62.0	18.7	27.7	80.8	108.3	26.6	47.8	8.3	20.2	26.5	896.4
Aug 2008																
A1	116.1	102.0	121.6	11.9	297.9	90.8	35.3	78.3	163.1	21.9	23.1	47.8	18.2	32.4	50.5	1211.0
A5	123.7	68.7	78.5	5.7	255.1	71.8	18.3	103.6	145.4	21.0	27.6	34.4	8.2	25.9	33.9	1021.7
A10	170.3	124.5	123.9	7.6	414.9	117.3	36.5	89.7	201.8	42.2	41.4	52.6	19.4	44.4	54.4	1540.7
A16	118.8	103.7	87.9	11.2	305.1	83.4	26.1	48.4	130.1	28.5	28.2	27.0	6.4	19.0	30.9	1054.7
Bloom	206.4	162.4	134.0	19.2	440.2	115.0	41.9	83.2	216.8	40.2	44.2	49.8	15.2	34.7	45.6	1648.9
A22	110.3	188.5	72.3	9.5	347.8	80.0	45.8	44.5	157.9	28.3	34.7	34.8	16.0	30.0	51.4	1251.7
A23	91.8	75.2	58.6	9.5	181.2	69.1	16.5	42.6	108.6	28.9	27.0	24.4	6.9	11.8	20.4	772.5
A29	51.3	52.6	34.2	5.4	94.1	39.4	8.1	17.0	57.7	29.2	16.1	22.1	3.1	14.3	15.6	460.2

Figure. S4.2. DCAA concentrations during July and Augest, 2008.

DFAA Mol%	ASP	GLU	SER	HIS	GLY	THR	ARG	BALA	ALA	GABA	TYR	VAL	PHE	ILE	LEU
Jul 2008															
J1	24%	11%	18%	2%	16%	6%	2%	2%	14%	1%	2%	1%	0%	0%	0%
J5	29%	7%	19%	3%	15%	4%	6%	1%	8%	1%	3%	0%	0%	0%	2%
J10	18%	17%	18%	2%	19%	4%	2%	2%	12%	1%	2%	2%	0%	1%	0%
J16	28%	14%	10%	1%	21%	3%	4%	3%	12%	2%	2%	1%	0%	0%	0%
J20	15%	16%	9%	3%	20%	3%	8%	5%	16%	1%	3%	1%	0%	1%	0%
J22	16%	17%	17%	2%	19%	4%	3%	3%	14%	1%	2%	1%	0%	1%	0%
Aug 2008															
A1	33%	9%	11%	2%	10%	6%	1%	0%	16%	0%	2%	4%	0%	7%	0%
A5	31%	24%	6%	4%	7%	4%	2%	0%	13%	1%	1%	3%	0%	5%	0%
A10	41%	20%	3%	6%	10%	0%	2%	0%	16%	0%	1%	2%	0%	0%	0%
A16	37%	10%	14%	0%	11%	0%	0%	0%	19%	0%	0%	3%	0%	3%	1%
Bloom	13%	21%	4%	0%	31%	3%	2%	0%	21%	0%	0%	3%	0%	1%	0%
A22	18%	7%	25%	2%	22%	5%	0%	0%	9%	0%	1%	4%	1%	3%	2%
A23	20%	22%	12%	0%	24%	0%	0%	0%	15%	0%	0%	3%	0%	4%	1%
A29	32%	13%	19%	0%	19%	0%	0%	0%	14%	0%	0%	3%	0%	0%	0%

Figure. S4.3. DFAA composition during July and Augest, 2008.

DCAA Mol%	ASP	GLU	SER	HIS	GLY	THR	ARG	BALA	ALA	GABA	TYR	VAL	PHE	ILE	LEU
J1	14%	7%	8%	1%	35%	8%	1%	6%	13%	-	2%	-	2%	2%	4%
J5	14%	7%	8%	1%	27%	7%	1%	6%	14%	3%	2%	6%	3%	2%	3%
J10	16%	9%	7%	1%	29%	8%	1%	6%	14%	-	3%	-	1%	2%	5%
J16	14%	8%	7%	1%	18%	6%	1%	5%	10%	11%	2%	10%	1%	2%	3%
J20	16%	10%	9%	1%	25%	8%	2%	4%	13%	-	3%	-	2%	3%	5%
J22	13%	12%	12%	1%	19%	7%	2%	3%	10%	13%	3%	6%	1%	2%	3%
A1	10%	8%	10%	1%	25%	8%	3%	6%	13%	2%	2%	4%	2%	3%	4%
A5	12%	7%	8%	1%	25%	7%	2%	10%	14%	2%	3%	3%	1%	3%	3%
A10	11%	8%	8%	0%	27%	7%	2%	6%	13%	3%	3%	3%	1%	3%	3%
A16	11%	10%	8%	1%	29%	8%	2%	5%	12%	3%	3%	3%	1%	2%	3%
Bloom	13%	10%	8%	1%	27%	7%	3%	5%	13%	2%	3%	3%	1%	2%	3%
A22	9%	15%	6%	1%	28%	6%	4%	4%	13%	2%	3%	3%	1%	2%	4%
A23	12%	10%	8%	1%	24%	9%	2%	6%	14%	4%	4%	3%	1%	2%	3%
A29	11%	11%	7%	1%	20%	9%	2%	4%	13%	6%	3%	5%	1%	3%	3%

Figure. S4.4. DCAA composition during July and Augest, 2008.