The Dissertation Committee for Afonso Cesar Rezende Souza Certifies that this is the approved version of the following dissertation:

Activity and Kinetics of Microbial Extracellular Enzymes in Organic-Poor Sands of a South Texas Estuary

Committee:

Wayne Gardner – Supervisor

Tamara Pease – Co Supervisor

Tracy Villareal

Dong-Ha Min

Carol Arnosti

Activity and Kinetics of Microbial Extracellular Enzymes in Organic-Poor Sands of a South Texas Estuary

by

Afonso Cesar Rezende Souza, BS,; MS

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2009

Dedication

To my wife Claudia, my parents Cesar and Maria Luiza, brother Daniel, sister Cristiana, and grandmother Emilia.

Acknowledgements

I would first like to thank my supervisor Dr. Wayne Gardner for providing support and motivation during this program. I would also like to thank Dr. Tamara Pease for giving me the opportunity to join the University of Texas at Austin research team and the necessary means to conduct my experiments. Appreciation goes to Dr. Tracy Villareal, Dr. Carol Arnosti, Dr. Dong-Ha Min, and Dr. Henrietta Edmonds for their participation in my committee and their valuable input. In addition, I thank the graduate student advisor, Dr. Joan Holt, for her support and friendship.

I also appreciated the friendship of the Marine Science Institute students and personnel. Many thanks to the group in the administration, Katy Quade, Jamey Pelfrey, Patty Web, and Dana Jensen for keeping me up to date with school deadlines and other support and to the librarians Liz DeHart and Tommie Adams for keeping the literature available 24 hours. Special thanks to Mike Rasser, Terry Palmer, and Andy Evans who initiated in this program with me and helped make my experience at Port Aransas much more fun.

Finally, I would like to thank and express my gratitude to my parents, my sister and my brother, my grandmother, and to my wife Claudia. Their support, encouragement, and sacrifice made this experience abroad and everything else in my life possible.

Activity and Kinetics of Microbial Extracellular Enzymes in Organic-Poor Sands of a South Texas Estuary

Publication No._____

Afonso Cesar Rezende Souza, Ph.D.

The University of Texas at Austin, 2009

Supervisor: Wayne S. Gardner

Co-supervisor: Tamara K. Pease

The respective kinetics of bacterial leucine aminopeptidase and β-glucosidase activities were investigated to improve understanding of factors controlling activity and hydrolytic capacity in estuarine organic-poor sands. Depth distributions of enzyme activity and bulk organic matter content were determined in sediments of Aransas Bay and Copano Bay Texas, to investigate enzyme dynamics as related to the geochemical properties of the sediment. Vertical profiles of activity in sediment showed that the enzymes were more active at the surface and that the potential hydrolysis rate of leucine aminopeptidase was higher than that of β-glucosidase. Vertical patterns of enzyme activity correlated (weakly) with variations in sediment organic matter (TOC, TN, and carbohydrates) content. Enrichments of sediment samples with monomeric organic compounds and inorganic nutrients did not affect leucine aminopeptidase and β-glucosidase activities in short- and long-term incubations. Enzyme activity was independent of nutrient availability and suggested that microbial communities were not

nutrient-limited. Time-course assays of bacterial hydrolysis of TOC, TN, and carbohydrates provided information about how substrate limitation may affect enzyme activity. Positive correlations between bulk TOC and TN content and enzyme activity indicated enzyme dependence on polymeric substrate content. Induction of enzyme activity after sediment enrichments with specific labile compounds confirmed the importance of available organic substrate to enzyme hydrolysis efficiency.

A kinetic approach established the occurrence of enzyme inhibition and its effects on enzyme hydrolytic capacity. The addition of a specific-enzyme substrate to sediment samples modified enzyme parameters and indicated that a substrate-reversible type of inhibitor could reduce enzyme hydrolytic capacity. The addition of polyphenol, as a natural inhibitor of enzyme activity, to the sediment resulted in a concomitant reduction of leucine aminopeptidase activity and ammonium regeneration rate, and thus demonstrated a close coupling between enzyme activity and sediment ammonium regeneration. These research results demonstrate the dynamic nature of the hydrolytic enzymes, provide information about the mechanisms of induction and inhibition of activity, and demonstrate some implications of reducing the hydrolytic capacity to organic matter decomposition and nutrient regeneration rates.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

INTRODUCTION

Background

Prokaryotes have an important role in the global biogeochemical cycles due to their ubiquity (Whitman et al. 1998) and ability to mediate organic matter (OM) decomposition and nutrient remineralization processes (Azam et al. 1983). Extracellular enzymes are needed to initiate microbial OM decomposition because a large fraction of the total OM is composed of macromolecules, which must be broken down outside the microorganisms' cells prior to assimilation (Weiss et al. 1991; Amon and Benner 1996). Factors controlling enzyme activity and effects on OM hydrolysis are thus important to OM decomposition and nutrient regeneration.

Enzymes affect the biogeochemistry of coastal marine sediments. Coastal sediments constitute one of the largest organic carbon reservoirs on Earth (Seiter et al. 2004). About 80% of global OM is sequestered in nearshore and continental shelf sediments (Hedges et al. 1999). Therefore, the sediments are a major carbon sink and provide sites for OM remineralization; they play a crucial role in the biogeochemical cycling of elements (Premuzic et al. 1982). About 55% of global OM remineralization occurs in coastal sediments even though coastal marine regions constitute only about 7.5% of the total benthic marine area (Henrichs and Reeburgh 1987; Wollast 1991; Middleburg et al. 1997).

Permeable sediments are a major component of the various sediment types found in benthic coastal environments (Emery 1968). Sands are common in benthic near-shore zones (Riggs et al. 1996). This granular material accumulates in areas affected by high-

energy bottom currents and wave orbital motion (Jahnke et al. 2000; Marinelli et al. 1998) to form unconsolidated sedimentary deposits. This type of sediment has a high capacity to transmit fluids through the sediment matrix (Lerman 1979). The flow of porewater supplies bacteria with substrate and electron acceptors, stimulates the rate of OM decomposition, and increases the efflux of microbial metabolic byproducts to overlying waters (Huettel and Rusch 2000; Rasheed et al. 2003). Benthic community metabolism in carbon-poor sands often results almost entirely from microbial heterotrophic activity (Cammen 1991), and OM-decay rates can reach rates comparable to those in carbon-rich sediments (Kristensen et al. 1997). Most benthic bacteria are particle-bound (Lucas et al. 2003; Schallemberg et al. 1989), and as little as 1% of the enzymatic activity in estuarine intertidal sediments results from enzymes freely dissolved in the porewater (Mayer 1989).

The effects of environmental factors on extracellular enzyme activity vary according to the system considered (Nannipieri et al. 1982; Duddrige and Wainwright 1992; Hoppe et al. 1990; Chrost 1990; Nannipieri et al. 2002; Arnosti and Jorgensen 2003). The contrasting dynamics of potential enzymatic hydrolysis in various benthic marine environments reflect variable enzyme responses to environmental factors (Mayer 1986; Meyer-Reil 1986; Mayer 1989; 1990; King 1991; Poremba and Hoppe 1995; Boetius et al. 1996; Arnosti and Holmer 2003; Boer et al. 2008). For example, aminopeptidase activity increased with depth at one site but did not change with depth at another site (Poremba and Hoppe 1995). Variable enzyme behavior prevents indirect assessments of potential hydrolytic capacity of the benthic microbial community, based on standard sediment geochemical characteristics or other indicators of microbial activity.

ENZYMES AND ORGANIC MATTER DECOMPOSITION

Enzyme catalysis

Enzymes are proteins capable of increasing chemical reaction rates several-fold without being consumed in the reaction. They function as catalysts because they create alternative courses for chemical reactions, which are faster than the original reaction pathways (Bender et al. 1984). Microbial extracellular enzymes are produced by cytoplasmic membrane-bound ribosomes and are transported through the membrane to the exterior of the cell (Priest 1984). They can either be released into the environment or remain attached to the outer surface of the bacteria cell. Surface-bound enzymes may offer bacteria an advantage over the free-released enzymes, because surface-bound ones maintain the reactions they catalyze close to the bacterial cell, facilitating product uptake (Hoffman and Decho 1999). However, cell-free enzymes can offer foraging advantages since they can hydrolyse substrate in excess of microbial growth demands (Vetter et al. 1998)

Although enzymes are comprised of various sequences of 20 standard amino acids, only a few amino acids are involved in the actual catalytic process: serine, cysteine, aspartate, glutamate, lysine, histidine, and tyrosine (Smith and Wood 1991). They are referred to as side-chain amino acids and each of them has a specific reactive group that interacts with substrates. Serine, for example, interacts with substrates through the hydroxyl group (-OH), while aspartate interacts through the carboxyl group (-COOH) (Smith and Wood 1991, references therein).

Side-chain amino acids are found primarily in crevices on the enzymes' molecular structures called active binding sites, where the transformation of substrate into product takes place. It was believed previously that the binding site had a rigid shape complementary to that of a specific substrate. While the idea of enzyme specificity for a

substrate is still maintained, a more recent model proposes that both the enzyme and its substrate can alter their shapes so that they become complementary on binding (Smith and Wood 1991).

A key factor in determining the ability of an enzyme to recognize and bind to the substrate is the electrical complementarity between the substrate and the side chain amino acids (Page and Williams 1987; Dugas 1996). As the enzyme molecule approaches the substrate to initiate the binding process, a loss in entropy occurs, due to the reduction in space available for enzyme and substrate molecules (Page and Williams 1987). This reduction in entropy makes the reaction between the two molecules unfavorable thermodynamically. To overcome this problem, the enzyme binds to the substrate (Page and Williams 1987) forming an enzyme-substrate complex (Hames and Hooper 2000). The bound substrate moves into a transitional state that has a lower free energy of activation than that of a non-enzyme-catalyzed reaction. This movement allows the chemical reaction to occur at a faster rate, without altering its change in free energy (Page and Williams 1987; Hames and Hooper 2000), as shown in Figure 1.1. Finally, the substrate is transformed into product and released into the environment, while the enzyme is regenerated, regaining the capacity to bind to another macromolecule.

Enzyme-mediated organic matter decomposition

Organic matter degradation is a process of consecutive breakdown of polymeric high molecular weight compounds into their monomeric constituents. This breakdown process consists of a series of enzyme-mediated reactions of depolymerization, through which labile polymeric OM fractions are transformed into smaller and lower molecular weight compounds (Colberg 1988; Amon and Benner 1996; Arnosti 1996). Extracellular

hydrolysis of macromolecular constituents of OM is involved directly in the depolymerization process (Christian and Karl 1995; Arnosti 1996).

Bacteria are unable to assimilate large molecules due to physical limitations of the cell wall (Fabiano and Donovaro 1998, Hoppe 1991, and Meyer-Reil 1991), which makes the cell membrane impermeable to substrates larger than 600 daltons (Weiss et al. 1991). Bacteria therefore release extracellular enzymes to break down organic polymers into simpler forms (Hoppe 1983; Munster and Chrost 1990; Hoppe 1991; Meyer-Reil 1991; Deming and Barros 1993; Boshker and Cappengerg 1998) (Figure 1.2).

Microorganisms possess a suite of extracellular enzymes to convert polymeric substrate into monomers (Arnosti and Repeta 1994; Vetter and Demming 1994; Gajewski et al. 1997; Podgorska and Mudryk 2003). Bacterial enzymatic hydrolysis of polymers has been identified in a wide range of environments from polar to hydrothermal vents (Andrade et al. 1999; Cummins and Black 1999) and the hydrolysis step is important for both aerobic (Chrost 1991) and anaerobic decomposition (Kristensen et al. 1995). Inefficient enzyme hydrolysis can hinder anaerobic processes (Kristensen et al. 1995). The process of anaerobic microbial-mediated decomposition of OM proceeds through a consortium of metabolic pathways in which the byproduct of one group of microorganisms becomes the substrate for the next group (Fenchel et al. 1998). Labile low molecular weight compounds are the source of reduced organic substrate essential to fermentation, a critical intermediate microbial pathway in the complete oxidation of organic carbon in anaerobic systems (Burdige 2001). The fermentative pathway is coupled to major metabolic guilds involved in the terminal decomposition of organic substrates, such as dissimilatory reduction of nitrate, iron (III), and sulfate. Major byproducts of fermentation include acetate, and lactate, small organic compounds, which are available to microorganisms and can be oxidized completely to carbon dioxide.

Source, quality, and quantity of organic matter

The composition and quality of OM influence its decomposition rate in sediments. Carbohydrates, proteins, and lipids are the three major compound classes of OM. Proteins are derived from intracellular components (Suárez and Marañón 2003; Helland et al. 2003) while lipids are part of the microbial cell wall and can also serve as energy storage compounds in eukaryotes (Albers et al. 1996). Humic poloymers are detrital materials usually rich in aliphatic substances that were subjected to significant condensation (Rashid 1985). Polymers of humics are typically large molecular weight molecules high C:N ratio (Hedges 1988) operationally defiined by their insolubility at acidic pH (Rashid 1985).

OM components are often consumed at different rates. The N is lost through preferential use of amino acid nitrogen (Tanoue and Handa 1980). The nitrogen fraction of plankton-derived organic matter is often used about 50% faster than the carbon fraction (Tyson 1995). Phytoplankton rich in nitrogen and labile carbon compounds tend to decay faster than terrestrial plants, which typically have a high carbon and low nitrogen content. Plants produce compounds such cutans and lignin, which increase resistance to degradation (de Leeuw and Largeau 1993). For example, seagrasses, which are rich in nitrogen and labile compounds, decay at a faster rate than mangrove leaves (Fourqurean and Schrlau 2003). The quantity and rate of input also influences OM decomposition rates. Areas with high primary productivity can yield sediments with high OM content, as in the sediments of Cape Lookout Bight, where about 70% of the local primary production is preserved in due to high sediment accumulation rates (Martens and Klump 1984).

Oxygen exposure

Oxygen affects the nature and extent of OM decomposition in sediments. Relative decomposition rates of labile material under oxic and anoxic conditions are similar (Cowie and Hedges 1992; Calvert and Pedersen 1992; Kristensen et al. 1995). Oxygen *per se* is not involved in the extracellular enzymatic reactions (Ganesh et al. 1999). However, decomposition of recalcitrant materials is often more efficient under oxic than anoxic conditions (Lee 1992). For example, degradation rates of relic OM increased when physical disturbances exposed anoxic sediments to oxygen. Under oxic conditions, strong oxidants such as hydrogen peroxide, superoxides, and hydroxyl radical, can breakdown C-C molecular bonds (Canfield 1994; Emerson and Hedges 2003). An example of hydroxyl radicals breaking down C-C bonds is the aerobic decomposition of aromatic compounds such as lignins, which are usually resistant to degradation under anoxic conditions (Sun et al. 2002).

Humification

Geopolymerization also known as humification is the abiotic condensation of dissolved low molecular weight organic compounds to form high molecular weight humic compounds. The condensation process modifies the structure of OM and decreases its reactivity (Tissot and Welte 1978; Hedges 1988). A typical process of humification is the abiotic combination of carbohydrates and proteins to form structurally complex polymers (Thurman 1985; Hedges 1988). These two compounds loose their original characteristics after complexation and become unrecognizable to the active binding sites of enzymes. The inability of enzymes to hydrolyze these materials leads to selective preservation of humic materials in sediments. Geopolymerization in sediments occurs at different rates (Henrichs 1992). The protein and carbohydrate reactants undergoing

humification are initially labile compounds, which bacteria can consume quickly (Alperin et al. 1994). Humification is likely a prolonged process of OM sequestration (Burdige and Gardner 1998).

Adsorption to the sediment matrix

The sorption of dissolved organic matter to sediment surface can protect a small fraction of OM from degradation (Mayer 1994; Hedges and Keil 1995). The proportion of OM to mineral surface area is remarkably constant, around 1 mg of organic carbon per square meter (Keil et al. 1994). This observation helps explain the pattern of high OM content found in sediment with high surface-area to grain-volume ratio (silt) relative to coarse materials (sand). However, the distribution of OM adsorbed on sediment surfaces is not homogeneous (Mayer 1999). Mesopores (small cavities, ranging from 5 to 50 nm) in minerals can also protect sediment OM from degradation, by preventing access of bacteria and enzymes to organic substrates in the pores (Mayer 1994). Later work showed that OM seems to be physically preserved as blebs between sediment grains (Ranson et al. 1998).

An interesting feature common to all mechanisms of OM preservation described above is that they are hypothesized to prevent or hinder extracellular enzymes accessibility and degradation of the substrate. For example, geopolymerization reactions change the molecular structure of OM so that enzymes no longer recognize the organic compounds as hydrolysable substrates. Adsorption and mesopore protection create physical limitations for enzymes to access and degrade OM. These observations combined point to the central role of microbial enzymes in the OM decomposition process.

ENZYME ACTIVITY IN MARINE ENVIRONMENTS

Microorganisms synthesize different extracellular enzymes. This study focuses on two microbial enzymes, leucine aminopeptidase and β-glucosidase. Leucine aminopeptidase is a family of proteases among many families of proteases (Matsui et al. 2006) and is characterized as a metallo-enzyme of broad specificity that releases N-terminal amino acids from proteins and peptides (Gonzales and Baudouy 1996; Matsui et al. 2006). Beta-glucosidase, conversely, is a group of enzymes specialized in cleaving glycosidic bonds that belong to two of the 82 families of enzymes grouped as glycosyl-hydrolases (Bhatia et al. 2002).

Substrate induction is an important mechanism controlling the activity of enzymes (Boschker and Cappenberg 1998; Witte et al. 2003). The amount and composition of OM are cues for the synthesis of enzymes. Specific enzymes are produced for the decomposition of specific components of OM. For example, the composition of macrophyte biomass, which contains lignin, cellulose and hemicellulose (Bacic et al. 1988), induces the production of glucosidase preferentially, while algae material, with high protein content and lower polysaccharide content of different composition, tends to favor the synthesis of proteases (Boschker and Cappenberg 1998).

The relative amount of specific substrates can determine the proportion of each enzyme produced. For example, in the Arabian Sea, bacterial enzymatic activities were proportional to the chloroplastic pigments content, a proxy for phytodetritus, proteins, and dissolved amino acids (Lochte et al. 1999). Bacterial enzymatic activity may follow these changes in molecular structure and relate to the usable fraction of organic material. OM composition changes during its descent through the water column and bacteria typically respond to the available substrate by releasing the necessary extracellular enzymes (Lochte et al. 1999). The proportion of various extracellular enzyme activities in

sewater changed vertically at one station in the Arabian Sea in relation to changes in detrital-matter composition (Lochte et al. 1999). In the same location, aminopeptidase activity remained relatively constant throughout the water column, while β-glucosidase activity became more active in depths of up to 60 m. On the other hand, the activity of chitobiase, responsible for the degradation of chitin, was dominant at 1200 m or deeper (Lochte et al. 1999).

Enzymes in sediments have a similar specificity-mechanism in the hydrolysis of OM (Mayer 1986; Meyer-Reil 1986; Arnosti and Holmer 2003). The activity of enzymes, typically, co-varies with changes in substrate content. For example, proteolytic enzyme activity decreased with depth and correlated with sediment protein content in an intertidal mudflat. Although the relationship was weak, the variation of activity could be explained by a correlation between enzyme activity and substrate concentrations in the sediment (Mayer 1989). The complex nature of OM in sediments may be responsible for weak correlations between microbial enzyme activities and sediment total organic carbon (TOC), total nitrogen (TN), and for the decrease of enzymatic hydrolysis with depth (Meyer-Reil 1986; King 1991). This lack of a strong correlation suggests that factors, other than substrate content, may contribute to the control of OM hydrolysis (Meyer-Reil 1986). Changes in enzyme activity with depth may depend on the characteristics of the enzyme, the sediment OM, or both (Poremba and Hoppe 1995). For example, the degree of hydrolysis of carbohydrate compounds was constant with depth in sediments with different POM content (Arnosti and Holmer 2003). These inconsistencies, which have been observed between enzyme activity and the organic substrates, indicate that other factors may control the activity of extracellular enzymes in sediments.

RESEARCH QUESTIONS ADDRESSED IN THIS THESIS

1. Do nutrients limit microbial synthesis of extracellular enzymes?

Microorganisms require nutrients (C, N, and P) for metabolism and growth. For example, phosphorus is involved in nucleic acids and phospholipids synthesis (Madigan et al. 2002) and nitrogen is required for protein synthesis. Bacteria can assimilate inorganic minerals in the porewater to fulfill their metabolic demand. For example, ammonium can be assimilated and used for amino acid synthesis (Tyler 1978).

The hypothesis is: nutrient limitation affects the ability of microbes to synthesize of β-glucosidase and leucine aminopeptidase to break down substrates. The rationale is that the lack of available organic (e.g. glucose and/or amino acids) and inorganic (e.g. nitrate, phosphate, ammonium) nutrients to the bacteria may limit their enzyme synthesis. Although inorganic nutrients tend to increase with sediment depth, it may be possible, if not likely, that the available nutrients may not always fulfill metabolic demand. This limitation could explain the reduced enzymatic activity found typically in deeper layers of marine sediments. The approaches used to test the hypothesis were (1) to enrich sediments (with low leucine aminopeptidase and β-glucosidase activities) with glucose, amino acids, nitrate, phosphate, ammonium, and (2) measure the effects of the nutrient enrichments on enzyme activity. An increase in enzyme activity after the addition of nutrients may indicate that the benthic microbial community was nutrient-limited and hindered in synthesizing enzymes.

2. Does low content of labile compounds explain the reduction of enzyme activity in sediments?

Substrate availability induces the activity of bacterial extracellular enzymes (Meyer-Reil 1987; Mayer and Rice 1992; Boetius and Lotche 1996). Enzyme activities

return to low background levels with the consumption of the detrital material (Boetius and Lotche 1996). The lack of a correct enzyme-inducer however can lead to low rates of enzyme hydrolysis (Arnosti 2004) which may explain the poor correlations between enzyme activity and bulk measurements of OM content.

Most vertical profiles of extracellular activity in sediments show a decrease of activity with increasing depth (Mayer 1986; Meyer-Reil 1986; King 1991). The hypothesis examined is that enzymatic hydrolysis decreases after the enzyme-substrate fractions of the organic matter are consumed. To test this hypothesis, specific enzyme-substrates were added to sediments with low levels of enzyme activity to determine the enzymatic response to the availability of substrate. An increase in activity after substrate addition could indicate that the low levels of enzyme activity occurring in deep layers of marine sediments were due to lack of hydrolysable material.

3. Does enzyme inhibition reduce the hydrolytic capacity of the microbial enzymes?

Enzymes associate with non-specific compounds, which can modify the enzyme's structure and cause loss of affinity and inhibition of activity (Chrost 1991). Inhibitors can also compete with the substrate for binding sites and decrease the rate of hydrolysis. The hypothesis is that substrate-reversible enzyme inhibitions occur in the sediment and contribute to reduction of the enzyme's potential hydrolytic capacity. The addition of a specific enzyme-substrate should alleviate inhibition pressure by competing for, or removing inhibitors from, biding sites and lead to an increase in enzyme activities.

4. Is ammonium regeneration linked directly to aminopeptidase activity in sediments?

In coastal environments, most N remineralization occurs in sediments (Warnken et al. 2000) and the supply rate of OM to sediments is a major factor controlling the rates (Blackburn 1991). Organic nitrogen is largely bound in proteins, and thus is unavailable to microbial metabolism without the initial aminopeptidase hydrolysis of the proteins to amino acids (Cowie and Hedges 1992; Pantoja et al. 1997; Pantoja and Lee 1999).

The hypothesis is that ammonium production depends on the hydrolytic capacity of proteases to generate substrates that are metabolized into ammonium. Enzymes may control ammonium production because ammonium production depends on available substrates and enzymes provide a tool to obtain such substrates. The approach was to: (1) add proteins as an organic nitrogen source, which require depolymerization before microbial metabolism, (2) add an enzyme inhibitor, and (3) measure ammonium production. If enzyme activity is inhibited, slower ammonium production should occur.

Controlling mechanisms of marine sedimentary OM remineralization and preservation are still not well understood (Henrichs 1992; Canfield 1994; Mayer 1994; Hedges and Keil 1995). The processes involved in OM degradation are various (Lee and Wakeham 1989; Tissot and Welte 1984; Quay 1992; Ransom et al. 1998; Marinelli and Boudreau 1996; Hedges et al. 1999) and although a required step, enzyme hydrolysis is not included among the main mechanisms affecting OM decomposition and preservation.

The theme of this work is to examine factors constraining the activity of extracellular enzymes in shallow, sandy estuarine sediments of two bays in the Mission-Aransas estuary, Texas. The study focuses on examining the activities of leucine aminopeptidase and β-glucosidase extracellular enzymes, which mediate the transition of particular organic matter to dissolved forms, which are either available to

microorganisms or require further hydrolysis prior to intracellular use. Enzyme activity in estuarine sand, and how this activity is affected by (1) nutrient limitation, (2) labile substrate availability, and (3) inhibition, is examined with fluorescent enzyme substrate analogs. The dynamics of enzymatic hydrolysis in shallow coastal sediments are examined for natural microbial consortia in fresh sediment and in sediment enriched with specific organic and inorganic compounds. Enzyme activity and sediment biogeochemical characteristics are measured by fluorimetry and standard analytical methods, respectively. Results from this study will provide information about mechanisms controlling enzyme activity and advance understanding of the decomposition and fate of the OM in sandy coastal sediments.

Overview of the dissertation

The dissertation presents research results in several chapters. Chapter 2 describes the distribution of enzyme activity in sandy sediments of the Mission-Aransas Estuary. Chapter 3 examines how enriching the sediment with organic monomers and inorganic nutrients affects enzyme activity. Evidence for enzyme dependence on the availability of labile substrate in sediments is provided in Chapter 4. Chapter 5 describes how enzyme kinetics can determine the presence of enzyme inhibition and effects on enzyme hydrolytic capacity. Chapter 6 demonstrates the link between ammonium regeneration and aminopeptidase activity. Chapter 7 summarizes all of the experimental results and suggests future directions for more research.

Main results of this study suggest that enzyme hydrolysis is promoted by unique conditions of the sediment. In the sediment studied, there was a distinct difference in enzyme kinetics between the surficial and deep sediment layers. Substrate induction had variable effects on enzyme activity and increasing porewater glucose, amino acid, nitrate,

phosphate, and ammonium concentrations did not affect activity. However, ammonium production in the sediment was a function of leucine aminopeptidase activity. The findings of this work should contribute to the establishing of enzyme hydrolysis role in OM remineralization and bring insight for future studies on the process of OM alteration and preservation.

Chapter 2: Vertical Profiles of extracellular enzymes in Sediments of Aransas and Copano Bays

INTRODUCTION

Estuarine benthic environments are characterized by rapid sediment accumulations (Nichols and Briggs 1985) and high supplies of organic material (OM) (Roden et al. 1985). Large quantities of riverine minerals, organic loads, and autochthonous OM produced in the estuary are deposited at the sediment surface. Despite intense primary productivity and supply of OM, coastal sandy sediments often contain only low amounts of OM (Shum and Sundby 1996), in part because sandy sediments enhance OM decomposition (Jahnke et al. 2005). Sands are permeable sediment matrices that allow significant porewater advection (Reimers et al. 2004). Porewater flow transports OM, oxygen, and nutrients to deeper sections of the sediment, which enhance microbial activity and OM remineralization (Huetter and Rusch 2000).

The OM remineralization is initiated by microbial extracellular enzymes (Rogers 1961), which have been studied extensively in marine environments (Mayer 1986; Meyer-Reil 1986; Mayer 1989; 1990; King 1991; Poremba and Hoppe 1995; Boetius et al. 1996; Arnosti and Holmer 2003; Boer et al. 2008). Vertical patterns of enzyme activity are variable and related to the specific characteristics of the benthic environment and the specific enzyme considered. Enzyme activities tend to co-vary with changes in substrate content and temperature (Meyer-Reil 1987; Mayer 1989; Boetius and Lotchte 1996; Boschker and Capenberg 1998; Boer et al. 2008). However, enzyme activity is sometimes quite constant with depth irrespective of substrate content (Poremba and Hoppe 1995; Arnosti and Holmer 2003). The relationship to sediment conditions is also specific for each enzyme. For example, in sediments of the continental margin, pullulan and chondroitin hydrolysis decreased with depth whereas arabinogalactan hydrolysis

showed no depth pattern in the same core (Arnosti and Holmer 2003), indicating that a confluence of factors influence the activity of extracellular enzymes in sediments.

Due to variable enzymatic responses found in natural samples, the work presented in this chapter provides information on vertical distributions of extracellular aminopeptidase and β-glucosidase activities in sandy estuarine sediments. Sediment depth profiles of leucine-aminopeptidase and β-glucosidase activities are determined and compared to concentration profiles of sediment total organic carbon (TOC), total nitrogen (TN), total carbohydrate, and total polyphenol content with 1-cm resolution. These parameters are examined to consider the hypothesis that leucine aminopeptidase activity relates to TN content and β-glucosidase activity relates to TOC in the sediments. The profiles were determined in sediment samples collected from Aransas Bay during five field surveys between 2005 and 2008 and from Copano Bay in September 2007.

Sediment biogeochemical characteristics were determined after each field trip. Sedimentary OM matter was quantified based on bulk TOC and TN content. Total carbohydrates were quantified by acid extraction. Methodology is described in detail in this chapter to avoid repetition in the following chapters.

MATERIAL AND METHODS

Sediment geochemical parameters

Sediment core samples were collected in Aransas Bay and Copano Bay (Figure 2.1) at bare stretches of sand distant from seagrass beds in about 1.5m of water. Copano Bay sediments were sampled only once in September 2007 whereas Aransas Bay sediments were collected in five discrete samplings trips undertaken during a four-year span. Water temperature and salinity were measured with a mercury thermometer and

hand-held refractometer, respectively. Sediment samples were collected with 50-mm inner diameter polycarbonate cores, which were pushed by hand into the sediment down to 20 cm. Six replicate cores were collected at a sampling site per sampling trip. The cores were maintained in the dark near *in situ* temperatures during transport to the University of Texas Marine Science Institute.

In the laboratory, each sediment core was placed over a core extruder and sectioned with a metal spatula at 1-cm intervals down to 10 cm. One-cm sections from the same depth interval of the respective cores were pooled in a pre-combusted (450 ^{o}C, 4 h) glass beaker. The pooled sub-samples were homogenized in a beaker and transferred to pre-combusted 20-ml glass scintillation vials. Geochemical characteristics were measured in triplicate for each depth interval (0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, and 9-10 cm). For clarity, each depth interval was depicted as 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, .5, 8.5, and 9.5 cm on the y-axis of graphs. I.e., to represent the depth interval, average values of triplicate samples were plotted half way between the marks on the y-axis scale. A portion of the homogenized sub-samples were frozen at -20 ^{o}C for analysis of sediment geochemical characteristics (TOC, TN, total carbohydrates, and polyphenols). The remaining homogenized "live" samples were processed immediately to quantify enzyme activities at each depth interval.

Scintillation vials with frozen sediment were placed inside a freeze-drier for 24 hours. The freeze-dried sediments samples were milled with a mortar and pestle. The mortar and pestle were cleaned and air-dried with three successive washes of methanol, acidic-methanol, and dichloromethane prior to adding the respective sediment layers to avoid cross-contamination. Powdered samples were stored at room temperature in capped pre-combusted glass scintillation vials until analysis.

Replicate samples were placed into 5 x 9 mm tin capsules and combusted in a Carlo-Erba 1108 CHN elemental analyzer, for which oven, reduction, and oxidation columns temperatures were set at 60, 650 and 1020 ^{o}C, respectively, for total organic carbon (TOC) and total nitrogen (TN) measurements (Hedges and Stern 1984). TOC was measured on samples pre-exposed to HCl vapors for 24 hours to remove inorganic carbon (Harris et al. 2001). Standard curves were prepared from different concentrations of acetanilide standards. Sediments collected in May 2005 were the only samples analyzed for stable carbon and nitrogen isotopic content.

Sediment total extractable carbohydrate content was measured using the phenol-sulfuric acid assay (Liu et al. 1973). Two ml of distilled water were transferred into each triplicate glass tube containing 100 mg of freeze-dried and homogenized sediment sample from a respective depth interval. Under the hood, 1 ml of 5% aqueous phenol (w/v) was added to each tube followed by 5 ml concentrated sulfuric acid. After 30 minutes, supernatant absorbance was measured at 485 nm. The least-square regression line, of absorbance versus concentration of glucose standard solutions (4.5 to 72 μg glucose ml^{-1}) provided a calibration equation with a coefficient of determination (r^2) of 0.99 and a coefficient of variation (CV) equal to or lower than 3%. Sediment carbohydrate content was estimated from the calibration regression line.

Total polyphenol content in the sediment was determined colorimetrically using the Folin-Denis method (Box 1983) adapted for analysis with microplates (Zang et al. 2006). Triplicate 500-mg freeze-dried-ground sediment samples were extracted with 2 ml of 50% methanol-water (v/v) for 2 hours at room temperature in the dark. After centrifugation, 50 μl of supernatant was transferred to individual microplate wells containing 250 μl of distilled water. Subsequently, 50 μl of Folin reagent (Fluka, 47742) and 40 μl of 7.5% Na_2CO_3 (Sigma, S-6139) were added to the polyphenol extract. The

solution was incubated for another 2 hours for color development in the dark at room temperature. Extract absorbance was measured at 760 nm and total polyphenol content was quantified using calibration equations ($r^2 = 0.99$, CV $\leq$ 4 %) determined with tannic acid standard solutions (Sigma, 403040). The calibration curve blanks were distilled water and the control for reagents was 50% distilled water and methanol (v/v) without Folin and Na_2CO_3 reagent solutions (Zang et al. 2006).

Potential extracellular enzyme activity

Enzyme assay

Enzyme-substrate stock solutions were prepared by dissolving 5 mg of 4-methylumbeliferil-β-D-glucopyranoside and L-leucine 7-amido-4-methylcoumarin (Sigma-Aldrich) in 3 ml Methylcellosolve (ethylene glycol monomethylether) to measure the activity of β-glucosidase and aminopeptidase, respectively. Methylcellosolve does not inhibit or excite enzyme activity (Hoppe 1983). Enzyme assays were conducted in triplicate for each depth interval (0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, and 9-10 cm). For clarity, each depth interval was depicted as 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5 cm on the y-axis of graphs. Each sediment-slurry consisted of 0.9 ml overlying core water, filtered through 0.2 μm syringe filters, mixed with 1.0 cm^3 of wet sediment. At the start of the time-course assay, fluorescence (time zero) was measured in triplicate slurries shortly after 0.1 ml of substrate analog stock solution was transferred into the slurry. The substrate analog final concentration in the slurry was 250 μM. The endpoint fluorescence intensity was measured after the slurries were incubated 4 hours in the dark at *in situ* temperature. Enzyme activity was calculated from the change in fluorescence over the incubation time as calibrated with standards. Fluorescence of heat-killed

sediments, boiled for 30 minutes, served as a control to correct for confounding fluorescence due to non-enzymatic reactions. The fluorescence values of filtered seawater were considered as blanks. The fluorescence values of killed-controls and blanks were subtracted from those of the sample to correct for background fluorescence. A calibration curve was created by fitting a straight line through fluorescence values against the fluorochrome concentrations of standard solutions in filtered seawater at concentrations of 1, 5, 20, 30, 40, to 50□ μM.

Statistical analysis

The 95% confidence interval, based on the variability of triplicate measurements, represents the margin of error of the parameter estimate. The degree of association between enzyme activity and sediment geochemical parameters was determined using the Pearson's correlation coefficient with the statistical software Minitab student version 12.

RESULTS AND DISCUSSION

Site Description

Aransas and Copano Bays are part of the Mission-Aransas estuary (Figure 2.1). The estuary is formed by sand barrier islands that run parallel to shore off the coastline. Gulf of Mexico seawater accesses the bay through the Aransas Pass channel. The mean water depth of the estuary is 1.5 m and the annual average salinity is about 20 (Engle et al. 2007; Bianchi et al. 1999). The irregular inflow influences salinity distribution throughout the estuary. This estuary receives freshwater from three main defined sources: gauged riverine, precipitation, and ungauged sources. The average monthly riverine inflow to the bay is about $6x10^5 m^3$/day (Engle et al. 2007) and comprises 15% of the

gauged input. Freshwater discharges are event-driven, and consist mainly of isolated pulses of freshwater after sporadic storms. The other two sources are 39% from ungauged sources and 46% from rainwater (Armstrong 1987). Aransas Bay region receives 800 mm of precipitation annually on average from sporadic rain events while the bay loses about 1500 mm of freshwater per year through evaporation (Armstrong 1987). The sum of these processes generates a net 0.2 km^3/yr net inflow of freshwater, which is small relative to most of Texas estuaries (Armstrong 1982). Based on such net inflow, water residence time for Aransas Bay averages about 4 years (Armstrong 1982). Long water-residence times combined with sporadic and ephemeral pulses of freshwater suggest that internal recycling of nutrient via the sediments or the water column could be a significant source of nutrients to organisms in Aransas Bay

Enzyme assay requirements

Background

The enzymes β-glucosidase and leucine-aminopeptidase are distributed widely in the environment and associated with bacterial heterotrophic metabolism. β-glucosidase releases sugars by cleaving β-linkages at the terminal ends of polysaccharides (Bhatia et al. 2002) and leucine-aminopeptidase releases amino acids by cleaving peptide bonds at the terminal nitrogen molecule of proteins and peptides (Gonzales and Baudouy 1996; Matsui et al. 2006). The potential activity of β-glucosidase relates to the rate at which the enzyme cleaves β-glycosidic bonds (Bhatia et al. 2002), thus hydrolyzes glucose from methylumbelliferyl molecules. Leucine-aminopeptidase activity is assessed by the rate it cleaves peptide bonds linking leucine and methylcoumarin molecules. Enzyme activity is determined by measuring the increase in fluorescence signal over a fixed time interval (Hoppe 1983; Hoppe et al. 1988; Chrost 1991; Boetius and Lochte 1996). Enzymes

cleave the linkages between the substrate and the fluorochrome molecules. The fluorochrome molecule, when free in solution, emits light at 445-nm wavelength at pH 10 after excitaion at 365 nm.

Pre-requisites

Valid assays to study enzyme activity require: (1) enzyme substrate-saturation information, (2) establishment of a linear relationship between enzyme activity and enzyme concentration, and (3) adequate controls (Gul et al. 1998). A lack of linearity can arise from inappropriate incubation time and substrate-analog concentration. The concentration of substrate analog and the incubation period were defined by kinetic analysis and linearity-tests, respectively. The kinetic curve defines the substrate concentration at which each enzyme becomes saturated with substrate. Substrate saturation concentrations were defined by measuring enzyme activity as a function of substrate-analog concentration. The linearity test defines the time of incubation over which enzymes operate at steady-state. Values of enzyme activity obtained at steady state are referred to as initial velocity, which is directly proportional to enzyme concentration.

The use of substrate analogs to estimate the potential activity of extracellular enzymes in natural environments requires that certain conditions for the assay be defined. The following tests were run to determine the correct substrate analog concentration, optimize the enzyme reactions, and minimize possible changes of the microbial community due to manipulation and bottle effects (Hoppe 1983, 1989; King 1986).

Linearity Test

The incubation time should be optimized to avoid changes in the microbial community and substrate content during the assay and provide a steady-state rate. Long incubation periods may allow substrate-induction or catabolite-repression to change the activity of the enzymes during the incubation period (Chrost 1989). The linearity test was conducted to determine the time interval in which the enzyme reaction rate was proportional to the enzyme concentration. Enzyme reaction rates were assessed by monitoring formation of the respective products. The results indicated that fluorescence intensity of the fluorochrome analogs, released due to leucine-aminopeptidase or β-glucosidase activity in the sediment, increased linearly with time (Figure 2.2). This linear association shows that fluorescence intensity increases with the amount of fluorochrome in solution and that enzyme activity was not induced or repressed within the 4h-incubation.

Saturation Test

Reactions that depend directly on substrate concentration are considered to be first-order reactions. Analog-substrate concentration for enzyme assays must yield zero-order kinetics to be usable. Reaction rates in this kinetic condition are proportional to the enzyme concentration and independent of the substrate concentration (Figure 2.3). The enzyme maximum velocity (V_{max}) defines substrate concentration for zero-order kinetics. The addition of excessive substrate analog can suppress enzyme activity by substrate or product inhibition. The zero-order region for both enzymes occurred with substrate analog concentrations of 250 μM.

Aransas Bay

Sediment Geochemical Characteristics

Aransas Bay water temperature ranged from 10^{o} C in the winter to 28^{o} C in the summer and the salinity was close to 23 in both seasons. The June 2008 sampling trip had the highest salinity of 34 in the overlying water. Sediment carbon and nitrogen isotopic values were determined in May 2005. Sediment TOC $\delta^{13}C$ values ranged from -19 to -17.5‰ (Figure 2.4), typical of OM derived from a mixture of seagrass and phytoplankton in the South Texas coast (Fry et al. 1977). The isotopic signature of terrestrial material ranges from -26 to -32‰ (Hedges et al. 1986); TOC isotope values, therefore, suggest that the contribution of terrigenous OM was small at this site. Delta ^{13}C was enriched slightly from -18.5‰ to -17.5‰ between the 1-2 cm and 5-6 cm intervals. Microbial remineralization of OM often does not change the carbon isotopic signature of sediment OM (Meyers 1994; Hodell and Schelske 1998), but some studies have observed isotopic enrichment of OM during decomposition (Freudenthal et al. 2001). The possibility that the observed isotopic change resulted from a temporal shift in the source of OM to this site, or from sedimentation of material transported in suspension during storm events, was not supported by the nitrogen isotopic signature. The ^{15}N values varied from +5 to 6.3 ‰ downcore to 9-10 cm, a signature of marine plankton (Nuwer and Keil 2005).

Sediment C/N (w/w) ratios were low down-core to the 9-10 cm interval with values ranging from 6 to 14 (Figure 2.5). The surface C/N ratios were lower than values reported for other Gulf of Mexico estuaries, in which C/N ratios ranged between 10 to 20 (Bianchi et al. 1993). Fresh phytoplankton C/N ratio ranges from 5 to 7 (Hedges et al. 1988) while seagrass, with low nitrogen content, has a C/N ratio of around 20 (Atkinson and Smith 1983) and terrigenous material has a C/N ratio larger than 20. The low C/N in Aransas Bay suggests that the OM could be of marine origin with phytoplankton and

benthic microalgae comprising dominant sources. The observed low C:N values may also have resulted in part from the consumption of carbon by organisms and incorporation of N during degradation of OM (Turley and Lochte 1990).

The C:N ratios peaked at around 7 cm depth during the months of September 2006 and April 2008. The increase was due to lower TN content relative to TOC at that depth interval, possibly from entrapped seagrass material. In fact, seagrass detritus was identified visually at 7 cm in April 2008 samples. A more detailed understanding of the sources of OM to this estuary would require the analysis of specific biomarkers (Cowie and Hedges 1984; Meyers and Ishiwatari 1993).

Total nitrogen and TOC content were measured with sediment depth in five field trips. Total nitrogen varied with depth, but the variations were characteristic of the sample-collection times. For example, TN showed different patterns with depth in May 2005, September 2006, and April 2008, respectively (Figure 2.6). TN values varied around a range of values between 0.04 to 0.14 μg/mg dry weight sediment (dws), except in May 2005 when the TN was close to 0.3 μg/mg dws. These values are lower than the average of 5 μg/mg found in most of Gulf of Mexico estuaries (Bianchi et al. 1993). However, TN contents lower than 1 μg/mg dws were found in sediments of Trinity-San Jacinto, Guadalupe, and Nueces estuaries (Bianchi et al. 1999).

The TOC content in sediment depth profiles ranged from 0.2 to 1.5 μg/mg dws, but depth patterns were not consistent (Figure 2.7). For example, TOC content decreased with depth in May 2005 but increased below 5 cm in April 2008. The loss of TOC in the first 1-cm interval was about 50%, except for May 2005 when the TOC decreased about 64%. Sediment TOC values were lower than average values reported for Gulf of Mexico estuaries, which ranged from 1 to 4 μg/mg dws for (Bianchi et al. 1999). Typical TOC content in productive systems is about 2 μg/mg dws (Henrichs and Farrington 1986).

However, OM content in sandy sediments is often lower than 0.5% by weight, even in productive areas such as the Middle Atlantic Bight continental shelf (Rusch et al. 2003). Low OM content of sands can be attributed to hydrodynamic characteristics, which favor physical removal of fine particles and support heterotrophic decomposition (Huetter 1992).

Bioturbation or sediment resuspension can introduce fresh organic material into sub-surface sediment layers (Reimers et al. 2004). Although bioturbation is a significant mechanism mixing sediment in estuaries, benthic fauna were scarce in Aransas Bay. Visual inspection of the bottom surface during sample collection showed consistent smooth sediment surfaces without mounds. Sieving about 5 kg sediment from 17 core samples through a 2 mm mesh did not expose or collect any benthic macrofauna. Polychaetes were observed visually in about 16% of all cores collected during this project. The number of individuals was never higher than two per core. This observation supports the idea that sediment resuspension from physical forces, rather than bioturbation, was the main mechanism mixing these sediments.

Sediment total carbohydrate distribution showed a concentration minimum at depths of about 1-2 and 3-4 cm deep, except for the sediments collected in May 2005, when error bars for replicate measurements were often large (Figure 2.8). Observed visual changes in sediment coloration from clear to dark gray sands in this depth interval was consistent with the concept of relatively high oxygen depletion rates and potential reduction of advective exchange of solutes. Intensive microbial activity is not surprising at this depth interval, the likely transition zone between oxic and reduced conditions. The availability of different electron acceptors in the transition zone may have allowed a variety of terminal microbial pathways to coexist, which could increase the use of labile organic matter as an energy source for different biochemical reactions (Deming and

Baross 1993; Canfield 1993). This explanation, in turn, is consistent with the small proportion of 0.01% by weight of total carbohydrates to total sediment weight and may suggest that labile carbohydrates were consumed to fuel bacterial metabolism.

The total carbohydrate content in May 2005 was almost double that observed in the other sampling trips to Aransas Bay (P=0.01) (Figure 2.8). The difference may be due to high vernal phytoplankton production, which can occur in subtropical estuaries (Cabecadas et al. 2004). Although phytoplankton cell density in Mission-Aransas estuaries does not exhibit strong seasonality, cell numbers tend to peak between the months of November and May (Texas Department of Water Resources 1981; Armstrong 1987). However, the sediment-surface carbohydrate contents observed in September 2006 and April 2007, respectively, were about one-half of the amounts observed in May 2005.

The fraction of total carbohydrate to total sediment organic carbon in the sediment was about 50%. This proportion is much higher than the 5 to 7% reported for other coastal sediments (Henrichs 1992). This high proportion does not mean that carbohydrates fractions were labile; it shows however that a significant portion of the OM in the sediment was soluble in strong acid. Productivity of South Texas estuaries is high; daily rates of suspended phytoplankton production in the adjoining San Antonio Bay and Corpus Christi Bay reached 2.5 g C m^{-2} d^{-1} (McIntyre and Cullen 1996) and 0.5 g C m^{-2} d^{-1} (Flint 1984), respectively. On an annual basis, such production is comparable to that of the Louisiana-Texas continental shelf, where annual carbon production averages 159 g C m^{-2} y^{-1} (Chen et al. 2000). Ratios of carbohydrate to total organic carbon of up to 40% were found in high TOC anoxi muds (Henrichs and Farrington 1987; Martens and Klump 1984; Klok et al. 1984). Carbohydrates comprise up to 40% of phytoplankton and 20% of microbial biomass (Parsons et al. 1984; Benner and Kaiser

2003). Structural material from terrestrial plants could account for a portion of the low TOC content of Aransas Bay sediments. Another possibility is that incomplete extraction of refractory compounds, or effects of different methodological protocols and lack of intercalibration, could account for lack of agreement in estimates of total sediment carbohydrate content in the different studies (Panagiotopolus and Sempere 2005; Underwood et al. 1995).

Total polyphenol content in May 2005 samples was 0.012 mg/mg dws at the top 1 cm interval. It decreased to about one-half of that value within the first 3 cm, peaked at 3-4 cm, and decreased down to about 0.002 mg/mg dw at 9-10 cm (Figure 2.9). In contrast, profiles of 2006 sediments showed no significant change in polyphenol content with depth (H_o: slope not different from zero, regression, t=1.5, P=0.12) while troughs occurred at 4-5 and 8-9 cm in 2008. The amount of polyphenols measured at the top 1cm interval of 2006 and 2008 were about 2.5 fold lower than that of May 2005. The difference between 2005 and the other years could be due to the presence of polyphenol-enriched detrital material deposited on the sediments before May 2005. Macrophytes, for example, synthesize phenolic compounds such as phlorotannins and phlorotannin-like materials (van Heemst et al. 1999). However, the C/N ratio of OM for most of the sediment was about 4 for the month of May 2005, which may indicate that the sources were microbial or planktonic rather than terrestrial or macroalgal-materials. The constant concentration with depth in 2006 and 2008 samples suggest that polyphenols were non-reactive or not degraded. Terrestrial and marine phenolic compounds undergo microbial degradation under oxic conditions (Ferreira et al. 1992). Bacteria can remove hydrolysable fractions of polyphenols while recalcitrant compounds, such as condensed polyphenols, accumulate in the sediments. The constant concentration may be due to either aerobic consumption of hydrolysable fractions of polyphenols or photobleaching,

which may have occurred in the water column prior to sedimentation. Alternative possibilities are that the organic material was already recalcitrant before deposition on the sediment surface or that the compounds were sorbed to sediment particles in the sediments, and unavailable to microbes. More knowledge of local sedimentation rates and sediment resuspension events is needed to explain the low concentrations of polyphenols in the 2005 sediment profiles.

Enzyme Activity

Extracellular enzymatic activities of both aminopeptidase and glucosidase were highest at the surface and decreased with depth in Aransas Bay sediments (Figures 2.10 and 2.11). Aminopeptidase activities were up to 8 fold higher at the surface than at 9-10 cm depth while glucosidase rates were up to 15 fold faster at the surface, a pattern common in benthic environments (Mayer 1986; Meyer-Reil 1991; King 1991). Surface peaks in activity may be a response to labile substrate that accumulates at the top of the sediment, due to deposition of fresh organic material, release of eukaryotic extracellular enzymes (Vrba et al. 2004), and production of polymeric exudates by benthic microalgae (Underwood et al. 1995). Algal exudates contain bioavailable polysaccharides and low concentrations of proteins (Staats et al. 2000), which drive the activities of different extracellular enzymes as demonstrated by positive correlations (Boer et al. 2008).

The lowest surficial aminopeptidase activity occurred in December 2005. Slurries incubated at in situ temperature showed activity for the first 1-cm interval of 7 μM/h was 3 to 4 fold lower than those at that interval for the other months. The low activity in December may have been due to temperature effects. Glucosidase activity, in contrast, was not influenced by temperature, as the surface glucosidase activity of December 2005 was not different from that measured in other months.

Depth profiles of enzyme activity showed irregular small peaks at various depths. These increases in activity may relate in part to the amount of labile OM at that specific depth. For example, peak glucosidase activity in April 2008 coincided with high TOC and TN content at the 5-6 cm depth interval (Figures 2.6-2.8). However, the relationship between enzyme activity and substrate was inconsistent. For example, enzyme activities did not follow the increase in total carbohydrate content in sediment layers observed between 2-3 and 5-6 cm intervals in May 2005 (Figure 2.8). The lack of correlation with substrate suggests that the organic material may not be suitable for enzymatic hydrolysis and illustrates the importance of knowing the composition of measured organic matter and possible inhibitors to interpret enzymatic results.

Aminopeptidase activity was higher than β-glucosidase activity consistently, regardless of sediment depth, a pattern common in aquatic environments (Poremba and Hoppe 1995; Lotche et al. 1999). The largest differences occurred in the top 1-cm interval samples. Aminopeptidase activities were about 4 fold higher than those of β-glucosidase in May 2005, December 2005, and April 2008, while the difference was about 1.6 fold in December 2005 samples (Figure 2.10). This pattern is interesting because, as expected, TOC content was always higher than TN content in the sediment. If most of the carbon were in the form of "hydrolysable carbohydrate," carbon content should favor the activity of β-glucosidase. The low activity of β-glucosidase suggests that this enzyme is not relevant for this TOC and that most of the total carbohydrate was likely not suitable for hydrolytic degradation by β-glucosidase. Despite sands low surface area, a portion of the OM could be adsorbed onto sediment particles (Hedges and Keil 1995), which may have limited its degradation to a certain degree (Keil et al. 1994). In addition, some aminopeptidase are constitutive and microorganisms tend to maintain a regular synthesis of this enzyme because of high cellular demand for nitrogen (Foreman

et al. 1998). Although labile C can serve as both an energy and food source, N is a crucial component of cell structural and metabolic apparatus; therefore, it is remineralized preferentially to carbon and becomes a factor controlling bacterial activity (Jorgensen et al. 1999). Total nitrogen content is less than 0.05% of the total sediment weight in the sands of Aransas Bay; therefore maintaining high aminopeptidase activity for the immediate breakdown nitrogenous compounds can be an efficient strategy to obtain nitrogen.

Depth profiles of glucosidase activity also showed a distinct asymptotic pattern down-core after the 4-cm depth interval (Figures 2.10 and 2.11), perhaps due to compositional and physico-chemical differences between the top first 1-cm and the sediment layers below 4-cm interval. Changes in redox conditions and the type of electron acceptors available for enzyme synthesis may also be important (Goel et al. 1997).

Copano Bay

Sediment Geochemical Characteristics

Sediment samples were collected in Copano Bay in September 2007 when the water temperature was 24^{o} C and the salinity was 9. The lower salinity value compared to Aransas Bay was due to a high freshwater inflow event in July 2009 (Personal Communication, Dr. Dong-Ha Min, University of Texas Marine Science Institute). This site is located much closer to the Aransas River than the site in Aransas Bay (Figure 2.1). Sediment was comprised of sand (~0.5mm) with low organic carbon content. Total nitrogen content varied from 0.06 to 0.12 μg/mg sediment dws (Figure 2.12). This range of values is similar to those measured in Aransas Bay sediments during both May 2005

and April 2008 sampling trips (Figure 2.6). Vertical profiles of TN content showed a mid-depth minimum after a 50% decrease in TN content within the first 4 cm. TN content increased from 0.06 to 0.12 μg/mg dws between 3-4 and 9-10 cm depths, a vertical distribution pattern similar to that of Aransas Bay sediments sampled in April 2008. However, the total nitrogen content of Copano Bay sediments was about one-half that of that observed in Aransas Bay sediments during May 2005.

The vertical distribution of sediment TOC had a mid-depth minimum at the 4-5 cm interval (Figure 2.12). However, TOC decrease was more accentuated than the TN change. The TOC content decreased by 79% within the first 2 cm and then increased by 73% over the 4-5- to 9-10-cm intervals. TOC ranged from 0.19 to 0.89 μg/mg dws. This range of values resembled those in Aransas Bay sediments. The exception was a 1.2 fold higher TOC content in the top 1 cm of sediments at Aransas Bay in May 2005.

The large change in TOC content change with depth, compared to TN results, caused in a sharp decrease in sediment C/N ratio from 7.3 in the top first 1-cm interval to 3.0 at the 4-5 cm interval (Figure 2.12). This decrease suggests that the carbon fractions of OM deposited in the sediment were respired while nitrogen fractions were immobilized and perhaps converted to microbial biomass. This shift in TOC signature has been documented experimentally during long-term OM decay experiments, where different loss-rates of C and N shifted the OM C:N ratios to lower values than occurred in the original matter (Fourqurean et al. 2003).

Vertical profiles of total carbohydrate content in Copano Bay sediments included a mid-depth concentration minimum between 1-2 and 3-4 cm intervals (Figure 2.13). A 3-fold decrease in carbohydrate content occurred from the top 1-cm interval (0.72 μg/mg dws) to the 1-2 cm section (0.24 μg/mg dws). Below the 3-4 cm depth interval, the content increased linearly (r^2=0.99) back to 0.72 μg/mg dws. The carbohydrate levels

then decreased down to the 7-8-cm interval and remained constant to the 9-10 cm depth. The vertical changes in carbohydrate content correlated with changes in TOC content ($r = 0.83$, $P<0.01$).

Total polyphenol content was quite constant down to the 9-10 cm depth in the sediment core (Figure 2.13). No significant difference in polyphenol content was observed among the ten sediment sections (ANOVA, $P>0.05$), even though values varied between 0.002 and 0.005 μg/mg dws. The amounts measured in Copano Bay sediments varied within the same range as those in Aransas Bay. This result was unexpected since the sampling site in Copano Bay is closer to the river and receives significant freshwater input, as indicated by a salinity of 9 measured at the time of sampling. As mentioned before, polyphenolic compounds are common components of terrestrial plants and are transferred to estuaries by river discharge.

Enzyme Activity

Aminopeptidase activity was highest at the top 1-cm interval and lowest at mid-depth (Figure 2.14). Activity decreased sharply from 9.2 μM/h in the 0-1cm interval to 5.4 μM/h in the 2 cm interval. After the 1-2-cm interval, the activity reduced by 25% at 4 cm interval, followed by a straight ($r^2=0.84$) increase with depth at the 9-10-cm interval. Aminopeptidase activity at 0-1 cm was lower than observed in Aransas Bay except for samples collected in December 2005, which had activities about 20% lower than in Copano Bay. Variations of aminopeptidase activity followed those of TOC, TN, and total carbohydrate content closely ($r = 0.90$, 0.88, and 0.56, respectively).

The vertical profile of β-glucosidase activity was similar to that of aminopeptidase. An activity peak occurred at the top 1-cm interval and a mid-depth trough at the 1-2 cm interval (Figure 2.14). Activity between the 1-2 cm interval and 4-5

cm interval were about 7 fold slower than that in the 1-cm interval. The drop in activity may relate to the 5-fold decrease in TOC content and the 3-fold decrease in total carbohydrate content between the 0-1 and 1-2 cm layers. The linear (r^2=0.91) increase in activity with depth below the 4-5 cm interval may be due to glucosidase response to the higher TOC and total carbohydrates was observed after the 4-5 cm interval. The magnitude of glucosidase activities were related significantly to those of TOC and carbohydrate content (r = 0.97 and 0.90), respectively.

Correlations

Correlation coefficients were obtained from average values of enzyme activities, TOC, TN, total carbohydrates, and total polyphenols measured in sediments from 1 cm depth intervals in Aransas and Copano Bays. In Aransas Bay, activities of both aminopeptidase and glucosidase correlated negatively with sediment depth (r = -0.86 and -0.61, respectively, Table 2.1). The weaker coefficients for the change in glucosidase activity with depth may have been caused in part by the insignificant change in activity in sediments below 4 cm (Figures 2.10 and 2.11).

In Aransas Bay, correlations between enzyme activities and geochemical characteristics were variable (Table 2.1). As hypothesized, aminopeptidase varied positively with changes in TN, and polyphenol content (r=0.86, P<0.01 and r=0.72, P=0.02), but did not correlate significantly with total carbohydrate and TOC content. The stronger association of aminopeptidase with TN is reasonable, due to the proteolytic nature of this enzyme. Although TOC broad substrate category for specific type of enzyme, β-glucosidase correlated with TOC, as hypothesized, and to TN, and polyphenol contents respectively (r=0.71, P=0.02, r=0.84, P<0.01, r= -0.71 P=0.02). Interestingly, it did not correlate with total carbohydrate content (r= 0.47, P=0.17). The lack of strong

association between enzyme activity and substrate content has also been observed in intertidal sediments (Mayer 1986).

The direct relationship between enzyme activity and OM content was strong in Copano Bay where correlation coefficients of aminopeptidase and TOC and TN content were 0.90 and 0.88, respectively (Table 2.2). However, aminopeptidase activity did not correlate significantly with carbohydrate content ($r=0.60$, $P=0.07$). Variations of glucosidase activity, on the other hand, related positively to TOC, TN, and total carbohydrate content ($r= 0.97$, 0.97, and 0.90 respectively).

CONCLUSIONS

Considerable information is available on enzyme activity in marine environments, but minimal information is available for sandy sediments. Sands cover a significant portion of coastal benthic environments (Emery 1968) and are place of intensive OM remineralization (Huettel and Rusch 2000; D'Andrea et al. 2002). In contrast to silt, where microbial metabolism is diffusion-controlled (Boudreau 1997; Rocha 1998 and 2000; Foster et al. 1996), sands allow porewater advection and the exchange of solutes between sediments and water column (Foster et al. 1996; Ziebis et al. 1996; Rocha 1998). Because of the low TOC and TN content of sands, the importance of biogeochemical processes in sandy marine environments have likely been underestimated (Shum and Sundby 1996).

The results of this study show that the sands of Aransas and Copano Bays are organic-poor, with TOC and TN content as low as 0.1 and 0.01% by weight respectively. According to carbon and nitrogen isotopic signature, the OM deposited in these sands may have originated from marine phytoplankton and benthic microalgae detritus, as well as a fraction from seagrass. Vertical distributions of TOC, TN, and carbohydrates

demonstrate that the contents varied with location and time of sampling. These parameters were highest at surface and decreased with sediment depth in Aransas Bay during May 2005. On some occasions, TOC and TN content showed a concentration minimum at mid-depths, such as those detected in the April 2008 sediment samples of Copano Bay and Aransas Bay, respectively.

Despite the relatively low sediment TOC and TN content, aminopeptidase and β-glucosidase activities measured in Aransas and Copano bays sediments were up to an order of magnitude higher than those of non-benthic aquatic environments. Activities were however comparable to activities of other benthic marine environments (King 1986; Mayer 1989 and 1992; Poremba and Hoppe 1995; Tholosan et al. 1999; Williams and Jochem 2006; Boer et al. 2008).

The vertical distribution patterns of aminopeptidase and β-glucosidase activity agree with the hypothesis that aminopeptidase activity should follow the changes in sediment TN content while β-glucosidase those of TOC content. Activity of both enzymes decreased with depth in Aransas Bay. However, the mechanism that may tie the respective enzymes to bulk OM parameters are still not clear. The β-glucosidase activity was systematically lower that aminopeptidase, and β-glucosidase vertical distribution showed an asymptotic pattern. In Copano Bay, however, vertical profiles of enzymes showed that there was a mid-depth activity minimum. The reduction of activity may reflect a response to substrate content at that depth. However, the relationships between enzyme activity and bulk substrate characteristics present inconsistencies. In some samples, enzyme activity decreased irrespective to TOC and TN content. This observation suggests the need for more information about the composition and activity of the complex compounds comprising the TOC and TN components. In addition, these inconsistencies suggest that variations of activity with depth may depend on the enzyme

considered and the time or location of sample collection. Further characterization of these high-molecular weight organic compounds and of the microbial populations producing extracellular enzymes would help elucidate our understanding of the mechanisms causing the breakdown of these complex high molecular weight organic compounds.

Chapter 3: Nutrient limitation on extracellular enzyme activity in Aransas Bay sediments

INTRODUCTION

River runoff is the major source of nutrients to most estuaries (Froelich et al. 1982; Pearl et al. 2002). However, south Texas estuaries have low freshwater inflow volumes compared to other Gulf of Mexico estuaries (Bianchi 1999). Microbial remineralization is an important source of nutrients to organisms in Texas estuaries (Warnken et al. 2008; Twilley et al. 1999). The degree to which N and P are released through microbial decomposition depends on the quality of organic matter (OM) (Valiela 1984; Wetzel 1999; Kleeberg 2002). As OM is transported deeper into the sediment, nitrogen-rich compounds are used preferentially to carbon fractions during bacterial decomposition of OM. The microbial community can become nutrient-limited when the nutritional value of the OM becomes insufficient to meet bacterial needs for growth and metabolism (Seitzinger 1988). For example, reduced nutrient inputs lead to low microbial production rates (Flaten et al.2003), whereas nutrient-rich eutrophic environments result in more intense benthic microbial metabolism (Teague et al. 1988).

Availability of inorganic nutrients to benthic microorganisms depends on physical and chemical characteristics of the sediment and on interspecific competition for the nutrient resources. In shallow estuaries, waves and tides create horizontal pressure gradients and generate bottom currents that promote advection of porewater through the sediment. Intense porewater advection occurs in sand, where permeability exceeding 10^{-12} m^2 (Reimers et al. 2004) may allow up 300 $l.m^{-2}.d^{-1}$ of water to flow through the sediment (Rusch and Huettel 2000) with a potential loss of nutrients (Vorosmarty and Loder 1994). Despite being a good resource for microbial growth, through production of DOC, the microphytobenthic community also removes nutrients from the sediment pool.

In shallow estuaries, sunlight may reach the sediment surface and allow benthic photoautotrophic metabolism to occur (McIntire et al. 1996). Microalgae inhabit surficial illuminated sediments at densities reaching $1x10^7$ cells/cm^3. Algal demand for inorganic nutrients can be substantial at high organism abundances (Sundback and Enoksson 1991). Microalgae therefore can compete with benthic microorganisms for nutrients (Rysgaard et al. 1995). For example, benthic algae reduced denitrifier nitrate uptake by 50% in a Danish estuary (Dalsgaard 2003). This competition for nutrients may result in reduced nutrient availability for the microbial community.

Nutrient limitation can hinder microbial activity. For example, the biomass production of phosphorus-limited heterotrophic bacteria in Florida Bay increased after sediment enrichments with two amino acids (products of aminopeptidase hydrolysis) and phosphate (Cotner et al. 2000). Production rates of carbon-limited bacteria increased after additions of glucose (a product of polysaccharide hydrolysis) in oligotrophic South Pacific waters (Kirchman 1990). If OM has a high C:N ratio, microorganisms in nutrient-poor environments could be subjected to low supply rates of N and P from OM decomposition and have limited hydrolytic capacity. For example, nutrient depletion decreased microbial metabolic activity and led to slow decomposition rates of OM (Enríquez et al. 1993), while nutrient-enrichments increased microbial activity, and degradation of organic matter, in sediment (Lopez et al. 1998).

The processes controlling aminopeptidase and β-glucosidase activities in sediments are not well understood, but enzyme synthesis is an energetically expensive process with a high nitrogen demand (Vetter et al. 1998). Nutrient availability affects enzyme synthesis (Gottschalk 1985). However, microbial communities that have been deprived of carbon and phosphate tend to produce enzymes to increase OM hydrolysis and the flow of metabolizable substrate to bacterial cells (Espeland and Wetzel 2001;

Romani and Sabater 2000; Huang et al. 1998). Although sediment porewater ammonium and phosphate are available for direct microbial uptake, (Goldman et al. 1987; Coveney and Wetzel, 1992; Kovarova-Kovar and Egli 1998) the benthic microbial community may still have limited hydrolytic capacity due to an insufficient hydrolysable carbon source (Lopez et al. 1995).

The objective of this study was to evaluate whether extracellular aminopeptidase and β-glucosidase activities in organic-poor sands of Aransas Bay depend on the nutritional status of the microbial community. Aransas Bay sands are organic-poor (Figure 2.6 and 2.7) and enzymatic activities decrease with sediment depth (Figures 2.10 and 2.11). The hypothesis was that sediment enrichments with glucose (organic C source) or amino acids (organic N and C source), and added inorganic nutrients (nitrate, phosphate, and ammonium), would increase the extracellular enzymatic activities of bacteria.

MATERIAL AND METHODS

Short-term incubation

Sediment for a 5-hour incubation experiment was collected in Aransas Bay in December 2005. Six sediment cores were collected manually in 1.5 m deep water by inserting Plexiglas cores of 7 cm inner diameter into the sediment down to 15 cm. The cores were capped with rubber stoppers and transported in coolers to the laboratory. In the laboratory, sediment cores were uncapped, and the overlying water was siphoned, filter-sterilized, and transferred into a pre-combusted glass bottle. The six sediment cores were extruded individually, sectioned at 1-cm intervals down to 10 cm with a metal spatula pre-sterilized with 95% ethanol. For every depth interval, 1-cm thick sediment

layers from the six cores were pooled and homogenized in respective pre-combusted glass beakers. For each depth interval, 1 ml wet sediment was combined with 1 ml of filtered (0.2-μm pore size) overlying seawater in 50-ml serum bottles.

The experiment included two controls and five treatments of nutrient-enriched samples. The control treatments were a killed control (KC), in which the sediment was first boiled for 30 minutes, and an unamended sediment control (Control). The enrichment treatments were triplicate sediment slurries amended with: (a) 100 μM glucose (GLU), (b) 4 μM dissolved free amino acids (DFAA), (c) 100 μM nitrate (NO_3^-), (d) 300 μM phosphate (PO_4^{-3}), or (e) 4 mM ammonium (NH_4^+). Sediment slurry bottles of sediments deeper than 1 cm were assumed to be anoxic, thus they were capped, crimp-sealed, flushed with N_2, and incubated for 5 hours before the enzyme assay. After the 5 hours, slurry bottles were opened and the contents transferred to 15-ml falcon tubes for enzyme activity analysis. Enzyme assays were conducted as described in Chapter 2.

Long-term incubation

Sediment samples used in the 48-hour incubation experiment were collected from Aransas Bay in March 2007. In this experiment, six sediment cores were sectioned at depth intervals 0-1, 4-5, and 9-10 cm representing respective mean depths of 0.5 cm, 4.5 cm, and 9.5 cm. The six 1-cm thick sediment sub-samples pooled from each depth interval were homogenized in respective beakers. Three replicate sediment slurries per depth interval were allocated to each of the seven treatments (a-f) described in the previous section. Slurries of the top 1-cm of sediments were enriched and maintained under oxic conditions, by leaving the bottle cap loose to allow gas exchange. For sediment sections of 4-5 and 9-10 cm intervals, three replicate slurries per treatment were enriched and incubated under "anoxic conditions" inside crimp-sealed 50-ml serum

bottles, after flushing them with nitrogen gas. Aminopeptidase and β-glucosidase activities of each treatment were measured in 15-ml falcon tubes as described in chapter 2, after 48 hours of incubation in the dark at *in situ* temperature.

RESULTS AND DISCUSSION

Short-term incubation

Increasing organic or inorganic nutrients did not change bacterial enzyme activity during the short-term enrichment experiments. Aminopeptidase and glucosidase activities in unamended control samples were not significantly different from the nutrient-amended samples. This result was consistent for most sediment depth intervals (Table 3.1 and Table 3.2). Exceptions were an increase in aminopeptidase activity after adding ammonium in the 1-2-cm interval and an increase in β-glucosidase activity after adding glucose to the top 1 cm and the 4-5 cm deep sediment intervals.

Aminopeptidase and glucosidase activities decreased with depth ($P<0.01$, Table 3.1 and 3.2). The aminopeptidase activity at the top 1-cm interval was about 8 times that in the 9-10 cm deep layer and the glucosidase activity at the surface was about 10 times higher than in the 9-10 cm layer. The addition of nutrients did not change this pattern of decreasing activity with depth. Comparison between the activities of aminopeptidase and glucosidase, in control sediments, showed that aminopeptidase activity was 2 fold faster than that of glucosidase near the surface and up to 5 fold faster in the deep layers.

Long-term incubation

The long-term incubation experiment was designed to examine the response of aminopeptidase and glucosidase activity to organic and inorganic nutrient enrichments

during a 48-hour incubation period. The hypothesis was that resource limitation constrains enzyme activity and the addition of nutrients may allow bacteria to intensify enzyme activity to supply the substrate, which can be assimilated. A relatively long-term incubation period was chosen to allow the microbial community to respond to the enrichments. Benthic microbial metabolism can consume electron acceptors in the nanomolar range hourly (Rowe et al. 2002) and, although variable, bacterial doubling time can range between 1 to 3 days (Crump et al. 2004).

Previous results indicate that microbial enzyme activities can relate to the concentration of limiting nutrients (Hill et al. 2006), but in this study, aminopeptidase and β-glucosidase activities did not change in response to nutrient enrichments (Figure 3.1). There was no significant (ANOVA, $P>0.05$) differences in enzyme activity between unamended control samples and the activity of nutrient-enriched sediments at 0-11, 4-5, or 9-10 cm depth layers. If the microbial community in Aransas Bay sediments were nutrient limited, heterotrophic bacteria would be expected to increase their enzymatic activity in response to enrichments during the incubations. The lack of response to enrichments indicates that extracellular enzyme regulation may be decoupled from nutrient content, perhaps because abundant nutrients were already available in the pore water. Likewise, in an oligotrophic wetland environment, leucine-aminopeptidase and β-glucosidase were not responsive to nutrient additions (Sirova et al. 2006). In other studies, β-glucosidase and aminopeptidase activities in amended sediments were analogous to those of controls, despite negative feedback from the product inhibition of glucose and amino acids (Hoppe 1983; Priest 1992; McQueen et al. 2005).

In control samples, activities of both enzymes were highest at the surface and lower at the 5 and 10 sediment depth intervals. Aminopeptidase activity decreased from 11 μM/h in the top 1-cm interval to 7 μM/h at 9-10 cm, whereas β-glucosidase activity

decreased about 3 fold from the surface to the deeper sediment interval (Figure 3.1). The vertical decrease in activity may be explained in part by sediment TOC and TN content (Table 3.3). Correlations between enzyme activity and sediment geochemical characteristics of control samples indicated that both aminopeptidase and β-glucosidase activities followed the vertical changes in TOC (r=0.79 and r=0.78 respectively). Enzyme-activity changes with depth also correlated significantly with sediment TN content (r=0.84 and r=0.89). In contrast, enzyme activities were not related to sediment total carbohydrate or polyphenol content. The correlations with TOC and TN and the lack of response to the enrichments suggest that nutrient enrichment did not affect aminopeptidase and β-glucosidase activities.

Aminopeptidase activity was higher than β-glucosidase activity at all depths. It was about 2 fold higher in the top 1 cm and about 4-fold higher at the 4-5 and 9-10 cm intervals (Figure 3.1).

CONCLUSIONS

The results of nutrient enrichment experiments support the null hypothesis that nutrient limitation does not reduce extracellular enzyme activities. Mineral nutrient and monomeric organic substrate enrichments did not affect leucine-aminopeptidase and β-glucosidase activity in organic-poor sands of Aransas Bay. The results suggest that the microbial community was not limited by nutrients and imply that mechanisms regulating extracellular enzymes activity may be decoupled from inorganic nutrient availability. The heterotrophic bacteria must have had sufficient inorganic nutrients already in the pore water to satisfy their metabolic requirements (Coveney and Wetzel 1992; Goldman et al. 1987). The absence of apparent organic nutrient limitation may have resulted in part from the conditioning of the microbial community to the “poor quality” of natural organic

matter in the sediments. Labile fractions of TOC and TN may be lacking due to prior removal of the more bioavailable organic substrates. It is also possible that the microbial community in sands is very different from that of organic-rich muds and well adapted to inhabit sandy environments.

The lack of enzymatic response to the nutrient enrichments suggests that confounding environmental factors, such as hydrogen sulfide in anoxic sediment layers, may control enzyme activity (Hoppe et al. 1990). The gradient from oxic to reduced conditions also affects bacterial community composition. Anaerobic bacteria with low energetic efficiency may have less energy available for enzyme synthesis than those with high energetic efficiency (Fenchel et al. 1998). In addition, bacteria may respond to specific cues, which determine whether metabolic benefits obtained from hydrolysis products are higher than the energetic cost for enzyme synthesis (Vetter et al 1998). When successful, the hydrolysis process supplies C, N, and energy to maintain enzyme production and enhance OM breakdown. Therefore, the balance between the energy supplied by OM hydrolysis and the energetic costs needed to maintain metabolism and synthesize enzymes may determine the level of enzyme activity measured *in situ*.

Chapter 4: Labile substrate limitation of extracellular enzymes in Aransas Bay sediments

INTRODUCTION

Most global oxidation of organic matter (OM) occurs in surficial coastal sediments (Wollast 1991; Henrichs 1992; Canuel and Martens 1996; Middelburg et al. 1997). Decomposition processes depend on various factors that affect reaction rates (Mayer 1994; Keil et al. 1994; Fenchel et al. 1998). Benthic microorganisms catalyze reactions involved in OM decomposition and accelerate the diagenetic process. The microbial degradation of OM involves a consortium of metabolic pathways (Henrichs and Reeburg 1987; Canfield 1993; Canfield et al. 1993), which depend on extracellular enzymatic supply of metabolizable substrate (Arnosti and Repeta 1994; Fenchel and Findlay 1995; Holmer 1999; Fenchel 1998). Although enzyme activity *per se* may be independent of electron acceptor conditions (Goel et al. 1997), respective microbial groups can select different electron acceptors to oxidize byproducts of OM hydrolysis to CO_2 and recycle nutrients (Burdige et al. 1999). Thus, enzymatic hydrolysis of complex OM compounds is important to the cycling of carbon and nutrients in sediments.

Extracellular enzyme activities of benthic microorganisms can increase in response to increased loads of OM (Meyer-Reil 1987; Boetius and Lotche 1996, 1999; Goto et al. 2001). For example, the availability of fresh substrate induced the activity of benthic extracellular enzymes up to 3-fold after amendement with labile substrate (Boetius et al. 1996) and after pulses of phytodetritus, from surface water algal blooms, settled to deep-sea sediments (Boetius and Lotche 1996). Aminopeptidase activity correlated weakly with hydrolysable protein content in estuarine sediments (Mayer and Rice 1992). Temporal variations of extracellular activities were related to oscillations of benthic microalgae exudates production (Boer et al. 2008). However, other studies

showed variations in extracellular enzyme activity irrespective of changes in substrate content (Poremba and Hoppe 1995; Arnosti and Homer 2003). The ambiguity between enzyme activities and bulk substrate content suggests that enzymes may respond to specific components of the OM rather than simply to the total amount of total organic carbon (TOC) or total nitrogen (TN) in the sediment.

This study aims to determine whether extracellular enzyme activity in organic-poor sediments of Aransas Bay depends on the availability of specific polymeric compounds. The responses of aminopeptidase and β-glucosidase to respective enrichments of casein and laminarin are examined to increase our understanding of possible factors controlling the potential activities of aminopeptidase and β-glucosidase in organic-poor sediments of Aransas Bay.

MATERIAL AND METHODS

Sampling and sediment preparation

Leucine aminopeptidase and β-glucosidase activities in organic-poor sands incubated were monitored in a closed system over an extended period (13 weeks). Enzyme activities, sediment TOC, TN, total carbohydrate, protein, and polyphenol content, and ammonium concentration were measured throughout the incubation period. After enzyme activities stabilized, casein (protein) or laminarin (β-(1-3)-linked glucose) were added to the sediment samples to determine the effects of substrate addition on aminopeptidase and β-glucosidase activities.

Seventeen Aransas Bay cores were sectioned, within 5 hours after collection, to remove 5-cm thick layers of sediments between 5 and 10 cm deep. The layers were combined in an ethanol-sterilized bucket, homogenized with a sterilized steel spoon, and

sieved through a 1-mm mesh screen to remove large debris (Figure 4.1). Subsequently, 80-g sediment sub-samples were transferred into respective 50-ml polyethylene centrifuge tubes, which were capped and stored inside N_2-flushed gas-tight jars. Although homogenization under air may disturb obligate anaerobes, we assumed that it did not have a significant effect during the long-term incubations (Sun et al. 1991). Three replicate 80-g samples were sacrificed and homogenized for analysis at each time point during the 92-day incubation. Three 1-cm^3 sediment aliquots were "slurried" with core seawater 1:1 (v/v) for immediate enzyme-activity analysis. The remainder of each sample was frozen at – 20^oC and freeze-dried for later analysis of TOC and TN, total carbohydrate, polyphenol, and protein, respectively.

The 80-g samples in the 50-ml polyethylene tubes were centrifuged, and porewater ammonium concentrations in the supernatant water were measured by the phenol hypochlorite method (Parsons et al. 1984). A linear calibration curve was established using standard solutions ranging from 0 to 50 μM. The coefficient of variation of triplicate standard solutions of NH_4Cl was 3% or less and the coefficient of determination of the linear fit (r^2) was 0.99.

Enrichment of pre-incubated sediments

Specific enzyme-substrates were added to the sediment samples after enzyme activities were reached in the 92nd day of incubation. Sediments samples were separated into three treatments: control sediments with no additions, sediments with 50 μg/ml of laminarin, and sediments with 30 μg/ml of casein (Figure 4.1). Three replicate slurries 1:1 sediment to filtered and degassed seawater (v/v) of each treatment per time point were placed into N_2-flushed serum bottles and incubated for 10 more days. Three replicates of each treatment were sacrificed every 48 hours for analyses of

aminopeptidase and β-glucosidase activity, total carbohydrate, TOC, TN and carbohydrate content.

Enrichment of fresh sediment

The hypothesis that aminopeptidase and β-glucosidase activities depend on the availability of compound-specific substrates was tested in fresh sediment samples. Sediment enrichments experiments were conducted on Aransas Bay sediments collected in June 2008 within 4 hours of sampling to minimize digression from natural conditions that can occur in long-term incubations, due to bottle-effects and changes in sediment conditions.

Sediment samples were collected in polycarbonate core liners and brought to the laboratory. Three cores were sectioned with a sterilized metal spatula to remove the top 1-cm and the 9-10-cm deep layers, individually. The three 1-cm layers from each depth interval were placed in respective pre-combusted glass beakers. Pooled sediments sections were homogenized by gentle stirring. The homogenized sediments from each depth interval were slurried by combining 1 cm^3 sediment and 1 cm^3 of 0.2-μm filtered overlying seawater into pre-combusted 50-ml glass serum bottles and mixed by repeated inversion.

Sediment samples of the top 1-cm and 9-10 cm layers were each divided into two treatments: one unamended control and one treatment in which the sediments were amended with either casein or laminarin. The slurries for the aminopeptidase and β-glucosidase assays were enriched with 50 μg/ml casein and 25 μg/ml laminarin, respectively, and the results were compared to those from unamended controls. Oxic samples from the top 1-cm were placed in serum glass bottles and capped loosely to allow gas exchange. Anoxic slurries were crimp-sealed gas-tight and flushed with N_2 by

placing two needles, one for inflow and the other for outflow, of N_2 gas. The slurries were incubated in the dark at the in situ temperature of 29 ^{o}C. Enzyme activity was measured in triplicate slurries of each treatment every 24 hours for 5 days.

RESULTS AND DISCUSSION

Long-term incubations

Sieved and homogenized sediments were incubated under anoxic conditions for 92 days in closed systems, to remove natural variability caused by bioturbation, microenvironment differences, and pore-water advection. Changes in enzyme activity were compared to TOC, TN, carbohydrate, protein, and polyphenol content, which were monitored during the incubation period. Note, that this experimental design provides information about the occurrence, mechanisms, and potential rates of these processes in slurries, but does not relate measured rates of OM decay or enzyme activity to actual process rates in nature.

Sediment TOC content did not change significantly (r^2=0.11, P=0.6) in 92 days. TOC content oscillated within a range from 0.65 to 0.69 μg C/mg dry weight sediment (dws) with a mean TOC value of 0.67 ±0.02 μg C/mg dws (Figure 4.2a). The average value was low compared to other Gulf of Mexico estuaries (Bianchi et al. 1999). The change in TN content with time was also not significant (r^2=072, P=0.7), despite a 7% linear decrease in TN content during the 92 day incubation. The TN values ranged from 0.12 to 0.14 μg N/mg dws with a mean of 0.13 ±0.01 μg N/mg dws during the incubation (Figure 4.2b). Reasons for the stability of the TOC and TN content during the incubation may include low microbial metabolism and the biogeochemical stability of the residual OM in the sediment (Fabiano and Donovaro 1998). The small fraction of specific labile

compounds of the TOC and TN, which bacteria respired to maintain metabolism, could not be elucidated by the assays employed. The 7% loss of N may have resulted from the activities of the microbes that transform organic nitrogen into N_2 gas via denitrification or anammox (Trimmer et al. 2006). The results suggest that bulk measurements of TOC and TN content are insufficient to quantify the amount of OM hydrolyzed in organic-poor benthic environments, where the microbial decomposition rates of labile compounds remaining in the sediments are low, relative to the bulk TOC and TN contents (Arnosti and Holmer 2003

Total carbohydrate content decreased significantly with time (r^2=0.75; P=0.04; Figure 4.3a). The main change happened apparently during the first 20 days. Carbohydrate content decreased by about 30%, from 0.46 ± 0.1 μg/mg dws to 0.32 ± 0.03 μg/mg dws. Despite a high proportion of total carbohydrate to TOC, the decrease in total carbohydrate content did not affect the TOC amount noticeably during the incubation period. One possible explanation for this apparent decoupling is that portions of the extractable carbohydrates were transformed by bacteria (Ogawa et al. 2001; Gruber et al. 2006) or geopolymerization reactions (Hedges 1988; Henrichs 1992) into recalcitrant compounds that occurred prior to the sampling, which would be included in bulk sediment TOC measurements.

Hydrolysable protein content did not change significantly with time (r^2=46, P=0.32, Figure 4.3b), but protein content did vary considerably with average values ranging from 0.1 to 0.8μg protein/g dws. High variability may have been caused by the assay's poor precision in measuring low protein content. Precision of the assay is around 20% of the mean value (Mayer et al. 1986) and the method is reliable down to concentrations of about 0.2 μg/ml (Read and Northcote 1981). During 53 days, protein content averaged 0.4 ± 0.3 μg/g dws. The average protein content was about 325 times

lower than the average value of sediment TN content. This fraction of the TN is small relative to the ranges reported for marine sediments. The proportion of hydrolysable protein usually accounts for about 3 to 10% of the sediment TN (Mayer et al. 1986). Thus, inferences from protein content are unreliable both because the values were near the detection limit and were extremely low in proportion to TN content, relative to other studies.

Total polyphenol content was quite constant during the incubation period (r^2=0.40, P=0.24; Figure 4.4a). Averaged values varied from 0.01 to 0.11 μg polyphenol/mg dws. The mean NH_4^+ concentration was about 40 μM, but had high variability, even though no statistical trend was obvious, i.e. the slope of concentration as function of time was not different from zero (r^2=0.3, P=0.46, Figure 4.4b). Ammonium concentrations in sediments represent a balance between production and re-assimilation (Blackburn 1980 *apud* Jorgensen 1983) in closed systems, but in nature may also be affected by physical-transport processes (e.g. in sandy sediments) and cation exchange. The large differences observed at different sampling times in closed bottles may have resulted from spatial or temporal patchiness in the samples, analytical issues, or may simply reflect the dynamic nature of NH_4^+ in sandy sediments. Estimates of NH_4^+ concentration at each time point represented only one individual measurement, due to the insufficient pore-water volumes obtained from the sandy samples. However, the NH_4^+ assay precision was equal to or less than 3%, based on the coefficient of variation of the mean of triplicate standard NH_4^+ solutions. Low porewater NH_4^+ concentrations in some samples suggest either that the microbial regeneration rates of NH_4^+ in that sample were small, perhaps due to low concentrations of bioavailable organic nitrogen, or that the NH_4^+ uptake rates had kept up with supply rates in the sampled sediment. Benthic

microbes can release NH_4^+ when labile OM substrates have low C:N ratios but may assimilate it when the bioavailable OM has a high C:N ratio (Goldman et al. 1987).

Aminopeptidase activity changed little during the 92-day incubation (r^2=0.005, P=0.91; Figure 4.5a). Enzyme rates oscillated between 1.6 and 2.3 μM/h and averaged 1.9 ± 0.2 μM/h. Aminopeptidase activity during the incubation period fell within the range of values measured in Aransas Bay sediments at the depth interval between 5 and 10 cm (Figures 2.10 and 2.11). Beta-glucosidase also did not show significant changes in activity during the incubation period (r^2 0.41, P=0.17), but did seem to follow a defined pattern over time (Figure 4.5b). Activity rates ranged from 0.1 to 1.3 μM/h with a mean value of 0.8 ± 0.4 μM/h. A drop in activity after day 53 (t=12, P<0.01) cannot be explained by temporal changes of total carbohydrate (r=0.001, P=0.99) or TOC (r=0.77, P=0.13) content.

Results of the long-term (92 days) incubation suggest that aminopeptidase and β-glucosidase activities may operate at low levels in deep sediments, which are isolated from physical disturbances such as bioturbation and porewater advection, which could supply fresh OM or transport nutrients through the sediment. Enzyme activities would remain unchanged unless sediment conditions are modified. For example, the enzyme activity may depend on the diffusion or advection rates of labile substrates. The asymptotic behavior of aminopeptidase and especially β-glucosidase activities in Aransas Bay (Figure 2.10 and 2.11) suggests that such stability of enzyme activities in deep sediments may occur in nature. A conclusion emerging, from the observed pattern of enzyme activity over time in the closed system incubation, is that extracellular enzyme activities in sediments are controlled by the availability of hydrolysable substrates.

Sediment enrichments

Incubated sediment

To test the hypothesis that extracellular enzyme activities in organic-poor sandy sediment of Aransas Bay are controlled by the availability of hydrolysable substrate, the organic substrates, casein and laminarin, were added to sediment samples after the 92-day incubation period. The approach provided a means to quantify the effects of specific substrate additions on leucine aminopeptidase and β-glucosidase activities.

Aminopeptidase activity was quite constant during 92 days averaging 1.9 ± 0.2 μM/h (Figure 4.6a). After casein was added, aminopeptidase activity increased 3.3 fold (2-sample t-test, $P<0.01$) and reached a peak of 7.3 μM/h in 4 days. Activities dropped during the following days to 6.0 μM/h at the end of the experiment (Figure 4.6b). Laminarin additions induced a rapid increase in aminopeptidase activity. Within 48 hours, aminopeptidase activities increased 2.8 fold reaching rates of 7.0 μM/h. The fast response to the addition of laminarin, a source of carbon but not nitrogen, compared to that of casein, which supplied both elements, suggests that the microbial community was carbon-limited and implies that bacteria may have used ammonium as source of nitrogen for enzyme production. The slow response of aminopeptidase to the addition of casein could be attributed to lower carbon content of casein compared to laminarin or to different kinetics of casein breakdown. Casein is cleaved first into peptides, which require further hydrolysis to produce amino acids that microorganisms can assimilate. Interestingly, aminopeptidase activity decreased after peaking. Activity dropped 1.5 fold after the 48-h peak in laminarin amended samples. The decrease in activity likely reflects the reduction in available substrate concentration as the decomposition progressed through time. It also suggests that the enzymes are not long-lived in sediments.

Beta-glucosidase activity also increased with the additions of laminarin and casein during the long-term incubation (Figure 4.7b). Although casein is not considered a specific substrate for β-glucosidase, the activity increased from 0.1 μM/h to 1.3 μM/h in 48 hours. Aminopeptidase hydrolysis of casein may have supplied sufficient nitrogen for the synthesis of new β-glucosidase during the first 48 hours. Glucosidase activity responded to the addition of laminarin; however at a slower rate. Activity increased to from 0.1 to 1.3 μM/h in 4 days (Figure 4.7b). Aminopeptidase and β-glucosidase also increased slightly in control samples, likely due to sample handling effects (Kristensen and Blackburn 1987).

Fresh sediment

For this experiment, Aransas Bay sediment samples were processed within 3 hours after sampling. In contrast to the experiments described previously, sediment samples here were not pre-incubated. Winds were calm during sampling and the bottom-water temperature was 29^{o} C. The presence of a benthic algae bloom was suggested by a green to yellowish "fluff layer," observed at the sediment surface down to a depth of ca. 3 mm. The absence of winds may have allowed phytoplankton sedimentation, and the resulting warm, clear water conditions may have promoted the apparent benthic algal bloom.

Enzyme-substrate was added to determine whether enzyme activity would be affected by the availability of specific compounds in fresh sediment samples. The aminopeptidase activity was higher than glucosidase activity in control samples irrespective of depth. A 25 % increase in aminopeptidase activity occurred within 24 h in the top 1-cm layer (Figure 4.8a) of both the control (t-test, $P<0.001$) and casein amended treatments (t-test, $P<0.001$), but the aminopeptidase activity relative to the control was

not affected by the casein addition. The observed increase may have been caused by the introduction of phytoplankton, from the sediment surface to the entire 0-1 cm sediment layer, during the homogenization step. After the initial pulse of activity, enzyme rates of both the control (23 ± 3 μM/h) and casein-enriched (24 ± 2 μM/h) sediments remained quite constant during the rest of the incubation period.

In the 9-10 cm layer, aminopeptidase activity in both unamended control and casein-enriched samples increased by about 30% in 24 hours (Figure 4.8b). However, the initial pulse in activity was not statistically significant (t-test, P=0.05). Aminopeptidase activity remained constant at about 5.0 ± 0.9 μM/h during the rest of the incubation. After 72 hours, activities in casein-enriched samples were 1.5 times more active than those of control samples (t-test, P<0.01). This isolated difference in activity may have been due to sample variability. Thus, the data is considered inadequate to support the substrate induction hypothesis.

Glucosidase activities increased about tenfold within 24 hours in the top 1-cm layer in both control and amended samples (Figure 4.9a). However, there was no difference in activity between control and laminarin-amended samples. Activities changed from an initial rate of 0.6 μM/h to 5.8 μM/h. As mentioned above, this sharp increase in activity in the control sediment may have been due to the introduction of labile fresh phytoplankton material to the entire sediment layer during sample homogenization. The deposited material, approximately 1 mm thick, was not mixed uniformly in the 0-1-cm layer until experimental manipulation. Underneath this layer, most of the 0-1 cm interval appeared reduced before mixing, as evidenced by its black coloration and sulfidic odor. This potentially anoxic sediment, with substrate concentrated only at the very surface of the sediment, may explain the low initial activity

(0.8 ± 0.2 μM/h) and suggests that the 112-hour long oxygen exposure may have contributed to the substrate-induced increase in activity.

The β-glucosidase response to laminarin addition at 9-10-cm was significant (2-sample t-test, $P<0.01$). Within 24 hours, glucosidase activity in samples amended with laminarin increased from 0.8 μM/h to 5.0 μM/h, while activity in control samples remained at about 0.7 ± 0.1μM/h (linear regression, $P>0.05$, Figure 4.9b). Based on systematic low levels of TOC, TN, and carbohydrate measured in Aransas Bay sediments, low substrate availability is a reasonable assumption at this depth. Substrate competition between natural substrate and laminarin may have allowed β-glucosidase to respond to the addition of substrate.

The overall results of the enzyme-addition experiments with fresh sediments did not verify the hypothesis of substrate-dependence of aminopeptidase activity. Casein additions did not increase aminopeptidase activity either in the top 1-cm or in the 9-10 cm layer. The results suggest that substrate limitation was not the major factor controlling aminopeptidase activity in sediment at those depths at the time of sampling. However, surface phytoplankton detritus or derivative hydrolysable substrates may have competed with the substrate-analog for enzyme binding sites and caused underestimation of enzyme activity in the top 1-cm layer (Hoppe 1983).

Aminopeptidase activity increases in control 0-1 cm samples, suggesting that natural sediment OM, fresh phytoplankton detritus, may have exceeded that of the added labile enzyme-substrates. However, the consistent and pronounced increase in activity of both enzymes during the first 24 hours in both control and treatment samples suggests that the increase in activity was likely due to the introduction of natural fresh material to portions of the sediment where enzyme activities were initially low. The long-term increase of glucosidase activity in the presence of laminarin at the 9-10 cm depth was

statistically significant and suggests that substrate limitation was a factor controlling β-glucosidase activity at that particular depth interval. The results here were analogous to those of other studies reporting ambiguous enzymatic responses to variations in sediment substrate content (Meyer-Reil 1983, 1987; Reichart 1986; Poremba and Hoppe 1995).

CONCLUSIONS

Defining the factors constraining enzyme activities at enzyme-specific background rates in closed systems is relevant to understanding sediment biogeochemical dynamics. The results of the long-term incubations showed that enzyme activity continued at a stable background level until sediment conditions were changed. Aminopeptidase activity remained higher than β-glucosidase activity for the extent of the incubation period. The addition of casein and laminarin induced an increase in aminopeptidase and β-glucosidase activities after 92 days of relatively constant activity without the additions. Such enzyme behavior determined in closed systems under laboratory conditions may be analogous to those of natural sediments, at depths where environmental conditions are quite stable, due to sediment separation from surficial OM sources. Answering questions such as "What is the process or what are the environmental factors that direct extracellular activities to remain constant at a specific level of activity in deep sediments?" may aid our understanding of OM preservation, since sediments in which enzyme are constrained at very low activities for prolonged periods may favor OM preservation.

The recurrent higher aminopeptidase activity compared to β-glucosidase activity, irrespective of sediment conditions, raises questions about the use of enzyme-substrate analogs for environmental studies. Aminopeptidase activity was higher than that of glucosidase in sediment samples incubated for over 3 months as well as in sediment

samples that were processed at the same day of sampling. The activity of enzymes measured with analogs relies on the enzyme specificity to the substrate. The higher estimates of aminopeptidase activity may be related to the closer fit of the leucine-fluorochrome molecule to the mechanism of aminopeptidase hydrolysis compared to that of β-glucosidase and its respective synthetic substrate structure.

Chapter 5: Kinetics and inhibition of extracellular enzymes in estuarine sediments

INTRODUCTION

Organic matter (OM) is biosynthesized in polymeric form, thus found as large moulecular weight compounds in the environment (Boetius and Lochte 1994). Microorganisms use OM as a food and energy source. Extracellular enzymes are released into the environment by microbial organisms to catalyze the breakdown of organic polymers, which provide substrates that are in turn available for uptake by the microbes (Hoppe 1983; Boshker and Cappengerg 1998; Hoppe 1991; Meyer-Reil 1991; Deming and Barros 1993). However, enzymes outside of the bacterial cell are also subject to environmental factors (Chrost et al. 1986; Meyer-Reil 1987; Hoppe et al. 1990; Serrano and Boon 1991; Wetzel 1991; Rath et al. 1993). They may have their hydrolytic capacity altered due to non-specific reactions, such as inhibition by other compounds (Boavida and Wetzel 1998). Enzyme hydrolysis can affect the extent of decomposition and fate of OM in sediment. Enzyme activities in marine sediments present high temporal and spatial variability (Meyer-Reil 1986; Poremba and Hoppe 1995; Mudryk and Podgorska 2005); however, the factors regulating hydrolysis are not understood fully. Non-specific compounds can associate with enzymes and function as activity inhibitors (Boavida and Wetzel 1998). Inhibitors may reduce hydrolytic reaction rates by competing with the substrate for active binding sites or by reducing enzyme affinity (K_m) for substrates (Chrost 1991). Non-specific compounds, which attach to active binding sites, function as competitive inhibitors. This type of interaction does not modify the structural conformation or affect the potential maximum activity (V_{max}) of the enzyme. However, it reduces the number of available active sites and lowers the enzyme affinity for the substrate. Lowering enzyme affinity has the kinetic effect of increasing the K_m values,

which results in a higher substrate concentration requirement to achieve one-half maximum velocity.

Non-competitive inhibitors, on the other hand, do not interact directly with the active binding sites but bind to enzymes, either at the enzyme-substrate complex or at secondary binding sites. These inhibitors change the enzyme configuration and can hinder the formation of enzyme-substrate complexes (Boavida and Wetzel 1998; Lundblad 2007). Therefore, non-competive inhibitors reduce enzyme V_{max} without changing its K_m for the substrate (Marangoni 2003). It can be inferred that inhibitions may occur under conditions of low enzyme-substrate concentrations. When enzyme-substrate concentrations are much higher than those of the inhibitor, the substrate may out-compete the inhibitor for binding sites and hydrolysis rates are unaffected (Marangoni 2003).

Most inhibitions are reversible (Chaiken et al. 1992; Karlsson 1994). Despite the irreversibility of some inhibitions (Turner et al. 1983; Morrison and Walsh 1988), enzyme inhibitions often decrease when the concentration of specific enzyme-substrate increases (Ma et al. 1994; Marangoni 2003). If inhibitions are substrate-reversible, the addition of specific enzyme-substrate should displace the inhibitor from binding sites and reduce the inhibition effect. The relief of inhibition should reestablish the intrinsic hydrolytic capacity of the enzymes.

Potential hydrolytic capacity of enzymes can be assessed by analyzing their V_{max} activity and K_m values. The purpose of this study was to determine whether the hydrolytic capacity of extracellular aminopeptidase and β-glucosidase can be reduced by substrate-reversible inhibition in organic-poor estuarine sediment. First, the kinetic characteristics of aminopeptidase and β-glucosidase were evaluated in organic-poor sediment of Aransas Bay in March 2007 and of Copano Bay in November 2007. Kinetic

curves for each enzyme were determined in sediment samples of both bays at different depth intervals to estimate the enzyme's potential maximum velocity (V_{max}) and affinity (K_m) using an artificial substrates. The potential turnover times of aminopeptidase and β-glucosidase of Aransas and Copano bay sediment were estimated to assess potential hydrolytic OM decomposition in the bays.

In a second experiment, saturation kinetic curves of unamended control and substrate-enriched samples were compared in sediment collected at Aransas Bay in May 2007 to determine whether substrate-reversible inhibition of enzyme activity could occur in the sediment. Consequently, casein and laminarin were introduced to sediment slurries for kinetic studies of aminopeptidase and β-glucosidase, respectively. A third experiment was conducted in sediment collected from Aransas Bay in September 2007 to evaluate polyphenol inhibition of enzyme activity in the sediment.

MATERIAL AND METHODS

Sediment characteristics

Sediment TOC, TN, and total polyphenol and carbohydrate content were measured at intervals during enzyme analyses. The methodology is described in detail in Chapter 2. Briefly, TOC, and TN were determined with an elemental analyzer (Hedges and Stern 1984; Harris et al. 2001), polyphenols were determined colorimetrically with the Folin method (Box 1983; Zang et al. 2006), and total sediment carbohydrates were measured with the phenol-sulfuric acid assay (Liu et al. 1973).

Measurement of enzyme kinetic parameters

Sediment samples were collected at Aransas Bay in March 2007 to determine

kinetic characteristics of aminopeptidase and β-glucosidase. The objective was to evaluate whether enzymes have distinct kinetics at different depths and locations. The depth intervals of interest were 0-1cm, 4-5 cm, and 9-10 cm for Aransas Bay and 0-1 cm and 9-10cm for Copano Bay. Three replicate sediment cores were sectioned in the laboratory to remove a 1-cm sediment section at each depth interval. Wet sediment from each depth was pooled and homogenized in pre-combusted glass beakers. One cubic centimeter of homogenized wet sediment was transferred into a 15-ml falcon tube containing 0.9 ml of filter sterilized core seawater.

Enzyme kinetic parameters of aminopeptidase and β-glucosidase were estimated in Copano Bay sediment collected in November 2007, and compared to those of Aransas Bay sediment collected in May 2007. The objective was to evaluate possible spatial differences in enzyme characteristics and potential substrate turnover times between bays. The sediment depths intervals of interest were 0-1cm and 9-10 cm for both bays. Saturation kinetic curves were determined by plotting enzyme activity as function of substrate analog concentration. Enzyme activity was measured at eight different final concentrations of substrate analog with 5, 15, 25, 50, 100, 150, 250, and 500 μM added to the respective slurry samples. The assay consisted of three replicate slurries for each substrate analog concentration and was performed over a time course of 4 hours. Enzyme activity for each slurry and at each substrate concentration was measured initially soon after the addition of 0.1 ml substrate analog (time zero) and again after 4 hours of incubation. Enzyme rates were expressed as μM/h. Controls, to account for abiotic hydrolysis, were slurries heated at 100 ^{o}C for 30 minutes prior to the addition of substrate analog. Enzyme V_{max} and K_m were calculated, from the non-linear regression of enzyme activity (v) a function of the substrate-analog concentration [S] according to the Michaelis-Menten equation: $v = (V_{max} \times [S]) / (K_m + [S])$, using Sigma Plot software.

This approach assumes a single type of enzyme or different enzymes with identical kinetic parmameters, which may not be the case in natural samples due to the wide variety of microbes.

Substrate-reversible inhibition experiment

A kinetic experiment was conducted to determine whether the activities of the extracellular enzymes, aminopeptidase and β-glucosidase, would have their activity reduced by substrate-reversible inhibition. Sediment samples were collected in Aransas Bay in May 2007. The enzyme parameters V_{max} and K_m were determined as described in the previous section. One-centimeter thick sediment samples were sectioned at 0-1 cm and 9-10 cm intervals, respectively, from three different sediment cores. Pooled triplicate samples from each depth interval were homogenized and slurried as described above. Kinetic curves for both enzymes were determined after three replicate sediment slurries were prepared for each substrate analog concentration. The range of substrate analog concentrations was 5, 15, 25, 50, 100, 150, 250, and 500 μM.

Sediment slurries of the 0-1 cm layer were divided into two treatments: unamended control and substrate-enriched samples. Unamended controls of both aminopeptidase and β-glucosidase experiments consisted of sediment and filtered overlying core seawater (1:1 v/v). Enriched-samples of the β-glucosidase experiment contained 25 μg/ml laminarin and those of aminopeptidase contained 60 μg/ml of casein. Sediment from the 9-10 cm layer was divided in the same manner, except that these treatments received 25 μg/ml of laminarin for glucosidase and 200 μg/ml casein for aminopeptidase assays. Sediment slurries were incubated at room temperature for 1h prior to the enzyme activity assay to allow casein and laminarin to react with the enzyme.

Polyphenol inhibition experiment

Sediment core samples collected in Aransas Bay during September 2007 were enriched with polyphenol to determine its effects on aminopeptidase and β-glucosidase activities. Triplicate sediment cores were sectioned between the 5 and 10 cm intervals to remove a 5 cm section of sediment sample. The three sediment sections were pooled and homogenized to prepare 1:1 (v/v) slurries. Triplicate slurries were prepared for each treatment. Treatments of aminopeptidase assays consisted of (1) unamended control, (2) 60 μg/ml casein, (3) 50 μg/g of tannic acid, (4) 100 μg/g of tannic acid, and (5) 50 μg/g tannic acid plus 60 μg/ml casein. The treatments of β-glucosidase assays were (1) unamended control (2) 25 μg/ml laminarin, (3) 50 μg/g of tannic acid, (4) 100 μg/g of tannic acid, and (5) 50 μg/g tannic acid plus 25 μg/ml laminarin. As mentioned above, slurries were incubated at room temperature for 1 hour before the enzyme assays were conducted.

RESULTS AND DISCUSSION

Sediment characteristics

Geochemical characteristics and sampling conditions are summarized in Table 5.1. Detailed information about Aransas Bay sediment characteristics is provided in Chapter 2. Salinity and temperature were comparable in March 2007 and May 2007 when Aransas Bay sediment was sampled. Aransas Bay salinities varied between 15 and 23 as compared to 9 at Copano Bay. The low salinity at Copano Bay was likely due to the close proximity of the sampling site to the Mission and Aransas rivers. The geochemical components of the sediment decreased in organic substrate content (TOC, TN, and

carbohydrates) with depth. The exception was sediment of Copano Bay where substrate organic content had a distinct mid-depth minimum at 5 cm.

In general, the sediment from 0-1 cm in Aransas and Copano Bays had low TOC and TN contents compared to other coastal areas (Fabiano and Donovaro 1994). In Aransas Bay sediment, TOC content decreased about 30% from 0-1 cm to 9-10 cm. While TOC content in surface sediment was comparable, 1.24 μg/mg dws in March 2007 and 1.87 μg/mg dws in May 2007, the TOC content of 0.80 μg/mg dws was about 2 times lower in September 2007.

The lowest TOC content of 0.60 μg/mg dws occurred at 9-10 cm in September 2007. Samples of March 2007 and May 2007 had slightly higher TOC, with respective contents of 0.87 and 1.28 μg/mg dws. Total N content in Aransas Bay sediment also decreased with depth in all of the samples. Sample TN content ranged from 0.08 μg/mg dws in September 2007 to 0.23 μg/mg dws in May 2007.

Copano Bay sediment had the lowest TOC and TN depth-specific contents at 4-5 cm (Table 5.1). The TOC content decreased from 0.89 μg/mg dws at 0-1 cm to 0.19 μg/mg dws at 4-5 cm and increased to 0.7 μg/mg dws at 9-10 cm. Total N decreased from 0.12μg/mg dws at 0-1 cm to 0.06 μg/mg at 4-5 cm and increased to 0.12 μg/mg from 4-5 to 9-10 cm. The TOC and TN contents of Copano Bay sediment were comparable to the TOC and TN content of Aransas Bay sediment of September 2007.

Total carbohydrate content was highest in the top 1 cm in Aransas Bay sediment (Table 5.1) and decreased by 42%, 34%, and 18% respectively, from the 0-1 cm to the 9-10 cm depth in March, May, and September 2007. In May 2007, the carbohydrate content at 0-1 cm was 60% higher than in samples of March and September 2007. In contrast to Aransas Bay sediment, the Copano Bay carbohydrate content increased from 0.72 μg/mg dws at 0-1 cm to 0.90 μg/mg dws at 9-10 cm. A minimum value occurred at 4-5 cm,

where carbohydrate content was 50% lower than at 0-1 cm. In general, the carbohydrate content of both Aransas and Copano Bays were lower than reported previously for intertidal sediment (Underwood et al. 1995).

Total polyphenol content was quite invariant with depth in both Aransas and Copano Bays (Table 5.1). The polyphenol content of Aransas Bay sediment of March 2007 was 65 and 600% higher than observed in May and September 2007, respectively. The polyphenol content of Copano Bay sediment was about 6 times lower than that of Aransas Bay sampled in March and May 2007. The low polyphenol content was unexpected in Copano Bay sediment, because the Copano Bay sampling site is closer to the local river mouths than the Aransas Bay site and polyphenolic compounds are typically terrigenous in origin (Steinberg and van Altena 1992).

Evaluation of enzyme kinetic parameters

In March 2007, saturation kinetic curves of aminopeptidase and β-glucosidase were determined at three different depths in Aransas Bay sediment to estimate enzyme V_{max} and K_m parameters. The maximum potential velocities of both enzymes were higher at 0-1 cm than at 4-5 or 9-10 cm. Aminopeptidase V_{max} was higher than the V_{max} of β-glucosidase at all three sediment depths (Table 5.2). Aminopeptidase V_{max} decreased progressively with sediment depth from 5.0 ± 0.2 at 0-1 cm to 1.8 ± 0.2 μM/h at 9-10 cm (Figure 5.1), while β-glucosidase V_{max} decreased from 2.4 ± 0.2 μM/h to 0.4 ± 0.08 μM/h μM/h (Figure 5.2). The decrease in enzyme activity may be related to the concentration of available substrates since TOC, TN and carbohydrate content also decreased with depth in Aransas Bay sediment (Table 5.1).

The affinity of both enzymes was lowest at 0-1cm but higher at 9-10 cm. Aminopeptidase K_m values increased from 59 ± 7 at 1 cm to 155 ± 31 μM at 9-10 cm

(Figure 5.1), while those of glucosidase increased from 57 ± 14 at 1 cm to 77 ± 37μM at 9-10 cm (Figure 5.2). Despite the lower value at 4-5 cm, the elevated K_m values at 9-10 cm may indicate that the enzymes affinity for the substrate decreased with increasing sediment depth. This result is interesting because enzymes with high affinity are found, typically, where substrate concentration is low, because enzymes with high affinities are expected to process substrates more efficiently. The paradoxical results found here, therefore, suggests either the synthesis of different enzymes with different kinetic parameters in response to depth specific environmental cues or the presence of non-competitive compounds in the sediment that modified the enzymes kinetic characteristics (Boavida and Wetzel 1998). Non-competitive compounds usually affect enzyme's active binding sites and can cause the substrate to lose affinity (Marangoni 2003; Lundblad 2007).

The potential time required for substrate hydrolysis (turnover time) of each enzyme was estimated using both V_{max} and K_m parameters. Turnover time refers to the estimated time required for the transformation of substrate into product (Unanue et al. 1999). This measurement is a good reference for the potential hydrolytic capacity of an enzyme (Marangoni 2003). Turnover time for both enzymes increased significantly with depth (2-sample t-test, $P<0.01$) in March. Aminopeptidase turnover time increased from 12 hours to 85 hours while the β-glucosidase turnover time increased from 24 hours at surface to 184 hours at the 9-10 cm layer (Table 5.2).

The potential hydrolytic capacity of aminopeptidase of Aransas Bay sediments was analogous to those of the bacterioplankton of eutrophic lakes (Gajewski and Chrost 1995), of Florida Bay waters (Williams and Jochem 2006), and within the range of values estimated for sediments of the Mediterranean Sea (Tholossan et al. 1999). In Aransas Bay, turnover time was significantly shorter than observed for bacterioplankton

aminopeptidase of the oligotrophic Northern Mediterranean Sea, with turnover times ranging from 48 days to more than 1 year (Unanue et al. 1999), and to that of high-latitude sediments with turnover times up to 1.5 years (Donovaro et al. 2002).

Turnover time reflects the combined expression of enzyme potential V_{max} activity and substrate affinity, K_m. Increasing turnover time with depth may result from an increase of sediment OM resistance to degradation (recalcitrance) as OM ages and is buried due to sediment accumulation. These changes in enzyme characteristics may have significant implications to the fate of OM in sediment. The OM may have a higher probability of preservation in sediment where enzyme turnover time is long. In contrast, sediment with a short turnover time likely favors rapid OM remineralization and nutrient regeneration.

Comparison of enzyme kinetics in Copano and Aransas Bay sediments

Kinetic parameters of aminopeptidase and β-glucosidase of Aransas Bay (May 2007) and Copano Bay (September 2007) sediment were compared to evaluate differences in hydrolytic capacity. Aminopeptidase and β-glucosidase kinetic parameters varied with sediment depth and were different between the two bays (Figure 5.3). In Aransas Bay sediment, aminopeptidase V_{max} values were about 4 times higher at 0-1 cm (12 μM/h) than at 9-10 cm (3 μM/h). Higher enzymatic activity at the sediment surface was a common feature in Aransas Bay sediment as discussed in previous chapters. The maximum potential activity of aminopeptidase correlated positively to sediment TOC, TN, and carbohydrate content of May 2007 and negatively with polyphenol content (Table 5.3).

The aminopeptidase V_{max} was almost 1.5 times lower in Copano Bay surface sediment (9 μM/h) than in Aransas Bay surface sediment (Figure 5.3). In contrast,

Copano Bay aminopeptidase V_{max} at 9-10 cm (12 μM/h) was 4 times higher than in Aransas Bay at the same depth. Despite the slightly higher TOC and TN contents of Aransas Bay sediment (Table 5.1), the difference in V_{max} between Copano and AransasBay appeared to be unrelated to TOC and TN content, since TOC and TN content did not vary significantly between the two sites (2-sample t-test, $p>0.05$). There is also a possibility of different microbial communities found at each location.

In Aransas Bay sediment, β-glucosidase V_{max} was higher at 0-1 cm (t-test, P=0.01) (Figure 5.3) than at 9-10 cm. V_{max} values decreased from 8.9 ± 0.5 μM/h at 0-1 cm to 6.9 ± 0.1 μM/h at 9-10 cm. The change in V_{max} for β-glucosidase with depth was not as strong as that of aminopeptidase. β-glucosidase V_{max} values also correlated to sediment TOC, TN, and carbohydrate content (Table 5.3). These positive correlations suggest that enzymes with high V_{max} may predominate in environments with high TOC, TN, and total carbohydrate content.

In Copano Bay sediment, β-glucosidase V_{max} was higher at 9-10 cm than at 0-1 cm (t-test, P=0.01) (Figure 5.3). In contrast to Aransas Bay, β-glucosidase V_{max} at Copano increased 2.5 times from 3.8 ±0.2 μM/h at 0-1 cm to 9.4 ±1.4 μM/h at 9-10 cm (Figure 5.1). The change in V_{max} activity with depth at Copano Bay correlated negatively with TOC content ($r = -0.96$, $P<0.01$) and positively with total carbohydrate content ($r =0.83$, $P=0.04$; Table 5.4), suggesting the V_{max} relates to the potentially labile fraction of OM.

In Aransas Bay, estimates of enzyme affinity for substrate, K_m values, of aminopeptidase and β-glucosidase were lower (high K_m values) at 9-10 cm than at 0-1 cm (Figure 5.4). Aminopeptidase K_m values increased from 47 ± 8 to 62 ±12 μM while those of β-glucosidase increased from 30 ± 3 to 44 ± 5 μM. The increase of K_m values of both aminopeptidase and β-glucosidase with depth correlated with the decrease of TOC,

TN, and total carbohydrate content (Table 5.3). Conversely, K_m changes of both enzymes correlated positively with total polyphenol content (Table 5.3).

In Copano Bay sediment, aminopeptidase and β-glucosidase had contrasting changes in K_m with depth (Figure 5.4). Aminopeptidase K_m values increased from 56 ± 8 μM at 0-1 cm to 231 ± 12 μM at 9-10 cm. Near the surface, aminopeptidase affinity for the substrate at Copano was therefore comparable to those of Aransas bay. However, at 9-10 cm, aminopeptidase affinity at Copano Bay was significantly (t-test, $P<0.01$) lower than that of aminopeptidase at the same depth in Aransas Bay.

In Copano Bay, β-glucosidase K_m values decreased from 56 ± 12 μM at 0-1 cm to 24 ± 6 μM at 9-10 cm (Figure 5.4). Enzymes with high affinity (lower K_m values) are often found in sediment with low hydrolysable fractions of OM since these enzymes can process substrate efficiently at very low concentrations (Gajewski et al. 1993; Unanue et al. 1999; Donovaro et al. 2001). This increase in β-glucosidase affinity at 9-10 cm in Copano Bay could not be explained by the TOC, TN, and carbohydrate content, because their respective contents at 9-10 cm were similar to those at 0-1 cm (Table 5.1 and Figures 2.12 and 2.13). Correlations between glucosidase K_m values with TOC, TN, and carbohydrates content showed ambiguous results, the affinity of β-glucosidase could not be linked to the measured sediment geochemical parameters (Table 5.4).

Turnover time, a ratio of the kinetic constant K_m divided by the V_{max}, is used to estimate the catalytic efficiency with an enzymatic reaction, and thus provides an effective approach to compare 'apparent' catalytic capacity among enzymes (Gajewski and Chrost 1995). Turnover times of enzymes in Aransas Bay were shorter at sediment invervals of 0-1 cm than at sediment depth of 9-10 cm (Figure 5.5). Aminopeptidase turnover of 3.9 ± 0.9 h at 0-1 cm was about 4 fold shorter than the 20.0 ± 0.9 h at 9-10 cm. The increase in turnover time reflected the combined effects of a reduced

aminopeptidase V_{max} and lower affinity (higher K_m) at 9-10 cm. Despite its higher V_{max} at 9-10 cm than at 0-1 cm, β-glucosidase had a shorter turnover time at near surface (3.3 ± 0.2 h) than at 9-10 cm (6.5 ± 0.6 h). The doubling of turnover time at 9-10 cm was likely due to the enzyme's lower affinity (higher K_m). This comparison expresses the importance of enzyme affinity to catalytic efficiency.

The turnover times for enzymes at Copano Bay were enzyme specific. Aminopeptidase had a short turnover time at 0-1 cm (6.0 ± 2.1 h) compared to 18.5 ± 4.9 h at 9-10 cm (Figure 5.5). This difference was related to the enzyme affinity (K_m). Aminopeptidase V_{max} values were comparable between 0-1 cm and 9-10 cm while K_m at the surface was about 4 fold smaller (higher affinity) than at 9-10 cm (Figures 5.3-top and 5.4-top). B-glucosidase turnover time on the other hand was shorter at 9-10 cm (2.5 ± 0.3 h) compared to 15.0 ± 2.6 h at 0-1 cm. In this case, shorter turnover time at depth was a combined effect of high V_{max} and high affinity of β-glucosidase at the surface (Figures 5.3-bottom and 5.4-bottom).

Overall, the results show that aminopeptidase and β-glucosidase have distinct kinetic characteristics. Potential maximum activity and affinity of both enzymes are specific spatially; these parameters differ among sediment depth intervals and between sites. Such features suggest that either enzymes are synthesized as a function of environmental conditions (Gotschalk 1985) and substrate supply (Tholossan et al. 1999; Unanue et al. 1999) or they may be influenced by physical or chemical factors (Nannipieri et al. 1982; Hoppe et al 1990; Kamer and Rassoulzadegan 1995; Boavida and Wetzel 1998; Haslan 1998). The estimates of enzyme kinetic parameters may not represent the natural sediment enzyme characteristics accurately because substrate analogs may not reflect in situ substrates. For example, several families of proteases (Gonzales and Baudouy 1996) and β-glucosidase (Arrieta and Herndl 2001) occur in the

environment. The kinetic characteristics assessed by analogs rely on the spectrum of substrates used in the saturation curves, which, in turn, may result from two or more independent enzymes operating within the range of the substrates assayed. The range of substrate analog concentrations may also fail to resolve enzymes operating at different substrate concentrations. In addition, results obtained from one analog substrate may represent specific kinetic characteristics of that model substrate. Therefore, the spectrum of substrate concentrations and the type of substrate analog used are of concern for studies of enzyme activities in natural samples.

Substrate-reversible inhibition

One objective of this study was to determine whether the activities of extracellular aminopeptidase and β-glucosidase are reduced due to substrate-reversible inhibition. Enzyme-inhibition experiments were conducted on Aransas Bay sediments in May 2007. Enzyme kinetic parameters, V_{max} and K_m, for aminopeptidase and β-glucosidase, of sediments amended with casein and laminarin, respectively, were compared to those of control samples.

At 0-1 cm, addition of casein did not change aminopeptidase kinetic parameters (Figure 5.6). In casein-amended samples, V_{max} of aminopeptidase activity was estimated as 12.0 ± 0.5μM/h as compared to 12.3 ± 0.2μM/h in control samples. Substrate addition did not affect (2-sample-t-test, P=0.43) enzyme affinity, K_m, which were 50.6 ± 2μM/h in substrate enriched samples and 46.3 ±12μM/h in controls. Turnover time of hydrolysis of substrate analogy estimated through the K_m:V_{max} ratio was unaffected with the addition of casein. The aminopeptidase potential turnover of 4.2 hours for casein-enriched samples was comparable to 3.7 hours in controls.

At 9-10 cm, the addition of casein altered aminopeptidase kinetic characteristics (Figure 5.7). Enzyme V_{max} activity increased significantly (2-sample t-test, P=0.013) from 4.6 ± 0.3μM/h in control samples to 7.8 ± 0.7μM/h with the addition of casein. Enzyme affinity however did not change significantly with casein enrichment. Although K_m values of aminopeptidase increased from 90 ± 9 μM to 159 ± 48 μM after casein addition, the change was not statistically significant (2-sample t-test, P=0.10). Despite the increase in V_{max} after enrichments, turnover time in casein-enriched samples of 20.3 ± 4.6h was similar to the 19.5 ± 2.9 h in controls.

The kinetic characteristics of β-glucosidase in samples at 0-1 cm changed with laminarin enrichments (Figure 5.8). Enzyme V_{max} increased significantly with substrate addition (2-sample t-test, P=0.03). Values measured in laminarin-amended samples of 7.5 ± 1.0μM/h were almost 1.5 fold higher than those in controls 10.4 ± 0.1μM/h. However, the K_m of β-glucosidase was comparable in both treatments (2- sample-t-test, P=0.3). In laminarin-enriched samples, K_m values of 27.3 ± 0.4μM were comparable to those of control samples of 33.8 ± 9.2μM. The increase in V_{max} activity was reflected in slightly higher estimates of turnover time for β-glucosidase (2-sample t-test, P=0.04). The turnover time of 2.6 ± 0.1 hours in amended samples was shorter than the 4.5 ± 0.7 h observed in controls.

At 9-10 cm, the addition of laminarin changed β-glucosidase V_{max} activity (Figure 5.9). The enzyme V_{max} increased more than three fold from 2.0 ±0.1μM/h in controls to 6.8 ±0.3μM/h in laminarin-enriched samples. Conversely, the enzyme K_m in amended samples remained comparable to that in controls (2-sample t-test, P=0.08). Beta-glucosidase K_m was 37.9 ± 6.4 μM in controls and 22.8 ± 2.8 μM in laminarin-enriched samples. The significant increase in V_{max} shortened the enzyme turnover time in amended

samples by 5 fold. B-glucosidase turnover was 19.3 ± 2.6 h in control samples but was reduced to 3.4 ± 0.3 h with the addition of laminarin.

The significant changes in enzyme activity with the addition of laminarin suggest that β-glucosidase is more susceptible than aminopeptidase to substrate-reversible inhibition, perhaps due to the broad substrate specificity of β-glucosidase (Parr 1983). The increases of V_{max} after enrichment may suggest either that enzymes are suppressed by the presence of an enzyme inhibitor or that the enzyme active binding sites are not occupied fully. The free binding sites, available to accept substrate, may be related to low relative concentrations of available hydrolysable substrate in the sediment.

Deviations from Michaelis-Menten kinetics

Kinetic curves for β-glucosidase in laminarin-treated samples deviated slightly from Michaelis-Menten kinetics (Figures 5.8 and 5.9). Deviations were most evident at 9-10 cm. At low substrate concentrations, activity showed a hyperbolic pattern as a function of substrate concentration; the data however digressed from the expected Michaelis-Menten pattern as it approached the 500-μM substrate analog concentration. The activity dropped from 7.5 μM/h at the 150 μM to 3.4 μM/h at the 500 μM concentration, which led to a weaker coefficient of determination of the fit of 0.60 (Figure 5.9). Such decrease in activity could be an artifact of fluorescence quenching or product inhibition of activity. Quenching is the decrease in fluorescence due to the excessive fluorescence yield as fluorochrome concentration increases (Robenson and Tilton 1995). Enzyme hydrolysis products may build up at high substrate concentrations and compete with the analog for binding sites or products can bind to the enzyme in an inhibitory way. Product inhibition leads to reduction of activity (Liaw and Penner 1990; Burns and Ryder 2001) and such regulation has been demonstrated for β-glucosidase in

environmental samples (Hoppe 1983; Chrost 1991; Burns and Ryder 2001). Substrate inhibition and quenching effects are detected by lower than expected activity values at the high-end of substrate saturation curves (Seigel 1975; Bell and Bell 1988). This result highlights the importance of conducting detailed kinetic analysis of enzymes to determine the proper experimental conditions for environmental studies.

Although the kinetic curves of enzymes in sediments in both Aransas and Copano Bays approached a hyperbolic function (Figures 5.1 and 5.2), the curves had a common feature. The plots for β-glucosidase, especially at 9-10 cm, could be divided into two phases. One phase was observed at ranges of substrate concentration between 5 μM and 25 or 50 μM and another phase was observed with the full spectrum of substrate concentrations form 5 to 500 μM. Enzyme kinetic curves showing a small plateau at lower substrate concentrations followed by a sharp increase in activity have been interpreted as the coexistence of two enzymes with distinct hydrolytic characteristics: one enzyme operating at low substrate concentrations and the other operating at high substrate concentrations (Vrba et al. 1999; Unanue et al. 1999). A few studies have examined this aspect of enzyme systems in the sediment (Tholosan et al. 1999; Donovaro et al. 2001) and water column (Unanue et al. 1999).

Biphasic kinetics has not been interpreted to propose either the presence of allosterism or cooperativity of enzymes in environmental samples. Enzymes possess binding domains that hold substrate in the correct position and binding sites other than the active binding sites, called allosteric sites, into which regulating compounds bind (Hess and Szabo 1979). A decrease of activity can occur due to non-specific compounds or by-products of hydrolysis binding to allosteric sites. If inhibitors are present or as reaction products accumulate, they bind to allosteric sites and cause a change in the structural conformation of the enzyme that hinders substrate binding at other active sites,

which, consequently, reduces enzyme hydrolysis (Choi et al. 2005). Positive cooperativity can also increase enzyme hydrolysis as substrate activation of one binding site reflects on the activation of other active binding sites or by the removal of an inhibitor from the regulating allosteric site (Seigel 1975).

Slight deviations from the kinetic fit line may suggest a biphasic tendency especially on β-glucosidase, as observed in curves of Aransas Bay in March 2007 (Figure 5.2), Aransas Bay May 2007 in control samples (Figure 5.7-5.9), and on both enzymes at Copano Bay in November 2007 (Figure 5.10 and 5.11). To evaluate this biphasic characteristic of β-glucosidase, kinetic curves with higher resolution were generated in Aransas Bay sediments collected in June 2008. A change of kinetic pattern of the curve and distinct enzyme kinetic parameters determined from the enzyme plots may indicate biphasic kinetics. Enzyme parameters assessed with the full spectrum of substrate analog concentrations showed that β-glucosidase V_{max} activity was 8.4 μM/h at 0-1 cm (Figure 5.12a). In contrast, the V_{max} values at the low concentration range of substrate analog were 2.6 μM/h (Figure 5.12b). The K_m value using the full range of concentrations was 78 μM, indicating a lower enzyme affinity, compared to a K_m of 6 μM estimated at the low range of substrate concentrations (Figure 5.12b).

The difference in kinetic characteristics of glucosidase at 9-10 cm was substantial (Figure 5.13). There was a 5-fold difference between V_{max} values obtained from the curve with substrate analog range from 0 to 50 μM vs. the one using the full range of analog concentration from 0 to 150 μM. The difference between enzyme affinities assessed from the two different curves was large; the K_m changed from 0.2 to 126 μM.

The biphasic kinetics suggests the coexistence of at least two β-glucosidase enzymes with different characteristics. As proposed in enzyme studies in lakes (Vrba et al. 2004), marine waters (Unanue et al. 1999), and deep coastal sediments (Tholosan et

al. 1999), one group with high affinity (low K_m) and low V_{max} operates at low concentrations and another with low affinity (high K_m) and high V_{max} operates at high substrate concentration. Evidence of biphasic kinetics is however new in shallow estuarine sediments, as is the proposition that biphasic behavior relates to the de-repression of allosteric regulation or the effect of positive-cooperativity. In this environment, non-specific compounds such as humic compounds attach to enzymes and inhibit extracellular enzymes (Ladd and Butler 1975; Wetzel 1991; Chrost 1991; Haslam 1998). The increasing analog-substrate concentration for the saturation assay may have detected the substrate concentration at which inhibitors were removed from allosteric sites or concentrations that promoted positive-cooperativity, as analog-substrate binds to one active site initiates the activation of other binding sites.

Positive-cooperativity, allosteric regulation, and the coexistence of distinct enzymes in the same sediment interval underscores the importance of proper methodology for using substrate analogs in estimating enzyme activity in environmental samples. Most studies use analogs at high concentrations and likely exclude enzymes operating at low substrate concentrations. In addition, enzyme kinetics change with increasing substrate content; therefore, improper concentration of analogs used in assays could lead to incorrect estimates of in situ kinetic parameters.

Polyphenol inhibition

Aransas Bay sediments samples collected in September 2007 were enriched with tannic acid to evaluate the effects of polyphenols on enzyme activity in sediments. Polyphenols are potential agents of enzyme inhibition in aquatic environments (Chrost 1991; Boavida and Wetzel 1998; Espeland and Wetzel 2001) and soils (Allison 2006). These compounds originate from vascular plants and macroalgal structures (Steinberg

and van Altena 1992; Stern et al. 1996) and occur in estuarine sediments. Polyphenols, as occur in humic acids, complex with proteins through hydrogen bonding and hydrophobic interactions (Butler and Ladd 1971; Oh et al. 1980; Carlson and Mayer, 1983; Spenser et al. 1988; Haslam 1989; Siebert et al. 1996), which can lead to enzyme precipitation and inhibition of activity (Ladd and Butler 1975; Wetzel 1991; Haslam 1998).

Polyphenols were found at low concentration in Aransas and Copano Bay sediments (Chapter 2). The relationship between enzyme activity and polyphenol content was however ambiguous. In most Aransas Bay sediment samples, both aminopeptidase and β-glucosidase activities correlated positively with total polyphenol content (Table 2.1). However, enzyme activity correlated negatively with polyphenols in Copano Bay sediments (Table 2.2) and in Aransas Bay in May 2007 (Table 5.3).

In September 2007, a laboratory experiment was conducted in Aransas Bay sediments to determine whether polyphenol inhibition is substrate-reversible for aminopeptidase and β-glucosidase. The addition of polyphenol to sediment samples caused a significant (2-sample t-test, $P<0.01$) decrease in activities of both aminopeptidase and β-glucosidase compared to unamended samples (Figure 5.14). Aminopeptidase activity in unamended control samples was 5.1 ± 0.2μM/h and the additions of 50 μg/g and 100 μg/g polyphenols reduced aminopeptidase activity to 3.5 ± 0.2μM /h and 3.5 ± 0.3 μM/h, respectively. The inhibitory effect of 100 μg/g polyphenols on aminopeptidase activity was less significant in the presence of 60 μg/g of casein. Aminopeptidase activity was 27% higher in samples with casein and polyphenol than in samples with polyphenol alone. The difference in activity suggests that casein and polyphenol may compete for enzyme binding sites and implies that polyphenol inhibition can be substrate-reversible.

The effect of polyphenol inhibition on β-glucosidase activity was more pronounced than that on aminopeptidase activity (Figure 5.14). The large effect of polyphenol on β-glucosidase activity may be linked to its broad substrate specificity (Parr 1983), which may allow binding sites to interact with a wide array of compounds and increase the chances of activity inhibition. Beta-glucosidase activity decreased by 80% and 94% in the presence of 50 μg/g and 100 μg/g polyphenol respectively compared to β-glucosidase activity in unamended control samples (Figure 5.14). B-glucosidase activities in samples with a combination of 100 μg/g polyphenol and 25 μg/g laminarin were 15-fold higher (2-sample t-test, $P<0.01$) than those with 100 μg/g polyphenol and about 5-fold higher than those with 50 μg/g polyphenol alone. The difference in activity shows that laminarin reduced the inhibitory effect of polyphenols and suggests that laminarin likely competed with polyphenols for β-glucosidase binding sites.

Enzyme activities in casein- and laminarin-enriched samples were not different from the activities of their respective controls (Figure 5.14). Aminopeptidase activity in unamended control samples of 5.1± 0.2μM/h was slightly higher than that of 5.5 ± 0.3μM/h in casein-enriched samples (2-sample t-test, $P=0.18$). B-glucosidase activity in laminarin-enriched samples with 1.00 ±0.01μM/h was not different from 0.8 ± 0.1μM/h of unamended controls (2-sample t-test, $P=0.20$). These results contrasted with those of May 2007, in which casein and laminarin additions increased enzyme V_{max} activities at 9-10 cm. The difference could be related to 10-fold lower total polyphenol content in September samples compared to those of May. The potential opportunity for substrate-reversible inhibition on enzymes of September was likely low. Thus, substrate additions had no immediate effect on enzyme activities, especially on aminopeptidase activity, which is not always induced by substrate additions (Boetius and Lochte 1996; Mallet and Debroas 2000).

CONCLUSIONS

Overall, the above results show that aminopeptidase and β-glucosidase kinetic characteristics vary spatially. Assessment of V_{max} activity and K_m of both enzymes in March 2007 indicate that in Aransas Bay sediments, enzyme V_{max} activity was highest at the top 1 cm interval and decreased with sediment depth. Affinity for substrate analog also varied spatially in the sediment. Highest K_m values of both enzymes were measured at 9-10 cm indicating that affinity is poor in the relatively deep sediments. Based on enzyme kinetic parameters, the turnover time estimated for hydrolysis of analog substrate varied between enzymes and between the 0-1 cm and 9-10 cm depth intervals studied. Both enzymes had shorter turnover times at 0-1 cm and 4-5 cm compared to those at 9-10 cm. The shortest turnover time was estimated for aminopeptidase at 0-1 cm while β-glucosidase had the longest turnover at 9-10 cm.

Aminopeptidase and β-glucosidase kinetic characteristics differed between Aransas Bay and Copano Bay sediments. The V_{max} activities of both enzymes were highest at 0-1 cm in Aransas Bay, in contrast to highest V_{max} values at 9-10 cm in Copano Bay. Changes of V_{max} activities could be explained by patterns of sediment TOC, TN, and total carbohydrate content in Aransas Bay. However, ambiguous correlations were observed between V_{max} and TOC, TN, and total carbohydrate content in Copano Bay. Aminopeptidase affinity was similar at 0-1 cm and 9-10 cm in Aransas Bay, but significantly lower at 9-10 cm in Copano Bay. In contrast, β-glucosidase had high affinity at 0-1 cm in Aransas Bay as compared to 9-10 cm in Copano Bay. Although glucosidase loss of affinity for the substrate analog with depth could be explained by TOC, TN, and total carbohydrates contents in Aransas Bay, the difference in glucosidase affinity between 0-1 cm and 9-10 cm in Copano Bay was unrelated to TOC, TN, or carbohydrate content. Aminopeptidase turnover was longer at 9-10 cm in both bays.

However, per depth interval aminopeptidase turnover did not differ significantly between bays. Glucosidase turnover was longer at 9-10 cm in Aransas Bay, but shorter at 9-10 cm in Copano Bay.

Substrate-reversible inhibition affected enzyme activity in Aransas Bay sediments. The addition of casein increased aminopeptidase V_{max} activity at 9-10 cm as did additions of laminarin to β-glucosidase V_{max} activity at both 0-1 cm and 9-10 cm. The increase of V_{max} resulted in a short turnover time for β-glucosidase hydrolysis of the substrate analog.

Deviations from Michaelis-Menten kinetics were detected in saturation curves. Aminopeptidase deviations were less significant and likely reflected noise in measurements. However, high-resolution saturation curves of β-glucosidase indicate possible biphasic kinetics. Substrate saturation occurred at both low and high substrate analog concentrations, which may suggest either the coexistence of enzymes with different kinetic characteristics targeting the same substrate analog or a positive-cooperativity effect on enzyme activity.

Polyphenol inhibition of aminopeptidase and β-glucosidase was affected by casein and laminarin, respectively. The degree of inhibition decreased significantly in the presence of enzyme substrate, which may imply that polyphenol inhibition is substrate-reversible. B-glucosidase was more susceptible to polyphenol inhibition than aminopeptidase.

Chapter 6: The role of enzyme hydrolysis on ammonium regeneration rates in estuarine sediments

INTRODUCTION

Estuarine productivity is linked to the input of nutrients from both riverine inputs and biological regeneration processes (D'Elia et al. 1986). Estuarine eutrophication has been increasing in recent decades (Turner and Rabalais 1991). Nutrient supply rates affect estuarine phytoplankton biomass (Howarth et al. 2002; Nielsen et al. 2002) and rivers are a major source of nutrients to most estuaries (Nielsen et al. 2002; Pearl et al. 2002). However, in some cases, the quantities of nutrients supplied by benthic regeneration resemble or exceed amounts originating from rivers (Fisher 1982; Boynton and Kemp 1985; Cowan et al. 1996). For example, south Texas estuaries have restricted fresh-water inflows due to the local sub-tropical semi-arid climate. This region experiences prolonged droughts and significant riverine water inputs depend on short-lived pulses following storm events. As a result, local estuaries have long water residence times and benthic regeneration of nutrients is a significant source of nutrients (Bianchi et al. 1999; Gardner et al. 2006). Corpus Christi Bay waters, for example, have frequent ammonium (NH_4^+) concentration increases in late summer resulting from temperature-induced intensification of benthic OM remineralization (Bianchi et al. 1999).

Primary production is limited by nitrogen in several estuaries (Bianchi 2007). In coastal environments, nitrogen (N) remineralization often occurs in the sediment (Warnken et al. 2000). Ammonium is the dominant reactive N species in the porewater (Berman and Bronk 2003). Nitrogen efflux from the sediment depends on benthic microbial mineralization (Kemp and Boynton 1984), which, in turn, depends in part on protein hydrolysis (Blackburn and Henriksen 1983; Henriksen and Kemp 1988; Galloway et al. 2004). Heterotrophs need to hydrolyze proteins and peptides into amino acids to

allow transport of organic N across their membranes prior to intracellular deamination resulting in NH_4^+ production (Jaffe 2000).

Enzyme hydrolysis is an important process influencing the fluxes of nitrogen between sources and sinks in coastal environments, and thereby relates to global N budget issues. Benthic NH_4^+ production relates to the supply and quality of OM (Blackburn 1991). For example, a large portion of the organic nitrogen deposited in estuarine sediments is recycled to NH_4^+ by bacteria (Wollast 1993; Hansen and Blackburn 2006). The dependence of NH_4^+ production on the availability of organic substrate is suggested by a direct relationship between fluxes of DFAA and sediment efflux of NH_4^+ (Landen and Hall 1998). As mentioned in previous chapters, most organic nitrogen remaining in sediments is polymeric and unavailable to microbial metabolism without initial enzymatic hydrolysis. Assuming that many free amino acids consumed in nature are produced from protein hydrolysis, this link between amino acids availability and NH_4^+ production suggests that aminopeptidase is involved in the NH_4^+ regeneration process. The production of amino acids by aminopeptidase was observed in environmental samples (Cowie and Hedges 1992; Pantoja et al. 1997; Pantoja and Lee 1999). A coupling between aminopeptidase activity and NH_4^+ production is probable but has not been established in marine sediments. A direct relationship between proteolytic enzymatic reactions and regeneration of NH_4^+ in marine sediments would underscore the role of enzymes on both OM decomposition and on nutrient regeneration.

This work aims to examine links between NH_4^+ production and aminopeptidase activity. Much of the NH_4^+ production in marine sediments is hypothesized to depend on proteolytic enzyme activities, assuming that microbial NH_4^+ production is preceded by amino acid formation (Wollast 1993; Landen and Hall 1998) and that proteolytic enzymes supply most of the amino acids (Cowie and Hedges 1992; Pantoja et al. 1997;

Pantoja and Lee 1999).

MATERIAL AND METHODS

Sediment sampling and preparation

Sediment samples were collected in Aransas Bay at a 1.5 m water depth in February 2008 and April 2008. Water temperature was 24^{o}C during both sampling trips. Water salinity was 18 in February and 25 in April. Sediment cores were collected in Plexiglas tubes with an inner diameter of 7 cm. Six sediment cores were brought to the laboratory and sectioned to sub-sample the sediment layer between 5 and 10 cm. Sediment in this interval was assumed to be anoxic based on distinct coloration differences with the top centimeter as well as its sulfide odor. Sediment from this 5-cm section of each core was combined and homogenized in pre-combusted glass beakers. Forty-ml aliquots of homogenized wet sediment were transferred into 100-ml glass serum bottles using a 10-ml truncated syringe. Forty ml of N_2-purged bottom water was added to each bottle to prepare 1:1 slurries by volume. Each slurry bottle was capped with a rubber stopper, crimp sealed gas-tight, and flushed with N_2 gas to maintain the slurry under anoxic conditions during the incubation period at *in situ* temperature.

February 2008

The experimental design to measure NH_4^+ production and aminopeptidase activity included three replicate slurries per treatment. The control treatment consisted of seawater alone, the casein sediment-slurry treatment received 5 μg/ml casein, and the casein-plus-polyphenol sediment-slurry treatment received 5 μg/ml casein and 50 μg/g of tannic acid, a proxy for polyphenols (Alstyne 1995). The concentration of polyphenol

was based on previous experiments that examined the inhibition of polyphenol on aminopeptidase activity (Chapter 5). Slurries were incubated at room temperature in crimp-sealed serum bottles of which 5-ml seawater aliquots were taken for NH_4^+ analysis at time points 0, 4, 24, 48, and 72 hours, using 10-ml syringes. The seawater samples were filtered through 0.2 μm syringe filters, the filtrate was transferred to 10-ml glass vials for storage at -20 ^oC until the last day of the time series.

Ammonium concentration of slurry seawater was measured based on absorbance at 640 nm after chemical chromatophore formation (Parsons et al. 1984). Briefly, 0.5 ml of 0.2-μm filtered slurry seawater was transferred to a glass tube into which was combined with 2 ml of oceanic blue water, 0.1 ml 5% phenol alcohol solution, 0.1 ml of nitroprusside, and 0.25 ml of oxidizing solution. The solution was incubated for 1 h to allow development of a blue color. Sample absorbance was measured at 640 nm. Absorbance values were transformed into NH_4^+ concentrations based on a calibration curve of ammonium chloride with a precision of ± 3% or lower. The regression equation had a coefficient of determination (r^2) of 0.99.

Aminopeptidase activity was measured in slurries consisting of 1 ml wet sediment and 1 ml core seawater. Enzyme analog (L-leucine 7-amido-4-methylcoumarin) was added to slurries to provide a final concentration of 250 μM in the slurry. Three slurries were prepared per treatment for each time point. Slurries were prepared using 10-ml truncated syringes to dispense the 1-ml sediment samples into 50-ml glass serum bottles. Slurry bottles were capped with rubber stoppers, crimp-sealed, and flushed with N_2 gas to maintain anoxic conditions during the incubation period. Slurry samples were sacrificed at time points 0, 4, 24, 48, and 72 hours for measuring aminopeptidase enzyme activity.

A 2-sample t-test was performed to determine whether the NH_4^+ concentrations at each time point were different among treatments. This test compared the means of each

time point among treatments with unequal variance. A Pearson-correlation coefficient was used to determine the degree of linear association between enzyme activity and NH_4^+ concentration in each treatment. All statistical analyses were performed using MINITAB statistical software.

April 2008

The procedures for sample collection and slurry preparation were the same as those for the February experiment, except for the number of treatments and the substrate concentrations for the casein-enriched samples. The experiment was designed to measure NH_4^+ concentration changes with time in four treatments. The control treatment consisted of 1:1 sediment-seawater without any substrate or polyphenol amendments. The other treatments were sediment slurries amended with 100 μg/ml casein, 100 μg/g of tannic acid, and a combination of 100 μg/ml casein and 100 μg/g tannic acid, respectively. Aminopeptidase activity was determined as described in the February 2008 section; however, additions of casein and polyphenols to slurries were 100 μg/ml casein and 100 μg/g polyphenols, the same as those used in the April 2008 NH_4^+ assay.

Sediment slurries were enriched with increasing casein concentrations, of 0, 50, 100, and 150 μg casein/ml, to determine whether NH_4^+ production was directly proportional to protein content. Triplicate respective mixtures of 40-cm^3 wet sediment, and 40 ml filtered core water ratio (v/v), were prepared per casein concentration. Slurries were incubated at room temperature in gas-tight glass bottles under anoxic conditions. A 5-ml seawater aliquot was sampled with 5-ml syringes from each slurry bottle, first when the slurries were sealed and again at the end of the 96-hour incubation period. The 5-ml seawater aliquots were filtered through 0.2 μm syringe filters and the filtrate was stored

at -20 ^{o}C in capped glass vials for five days until colorimetric analysis according to Parsons et al. (1984).

RESULTS AND DISCUSSION

February 2008

Ammonium production and enzyme activity

Samples collected from Aransas Bay February 2008 were treated with casein and polyphenol, and incubated for 72 hours to examine NH_4^+ evolution. Initial NH_4^+ concentration averaged 55 ± 0.6μM in all treatments and almost doubled within about 4 hours of incubation (Figure 6.1). The increases in NH_4^+concentration were slower during the remaining incubation time. In the casein treatment, NH_4^+ accumulated to 130 ± 0.1 μM in 72 hours, and the net NH_4^+ concentration change of 75 μM accounted for 63% of the casein-N added. In control and polyphenol treatments, the final NH_4^+ concentrations were 108 ± 1.0 μM and 107 ± 0.6 μM, respectively. Ammonium concentrations were similar in the control and the casein-plus-polyphenol treatments at each time point. On the other hand, significantly higher concentrations of NH_4^+ were measured in casein enriched samples at 48 and 72 hours (2-sample t-test, $P<0.01$).

Aminopeptidase activity increased with time in all treatments (Figure 6.2). In 72 hours, enzyme activity in controls increased from 1.7 ± 1.0 μM/h to 4.9 ± 0.6 μM/h while in casein-amended samples, the activity increased from 2.4 ± 0.6 μM/h to 6.5 ± 0.7 μM/h. In samples treated with polyphenols enzyme activity increased from 1.8 ± 0.2 μM/h to 3.4 ± 0.1μM/h. After the first 24 hours of incubation, enzyme activity remained lower, at each time point, in samples treated with a combination of casein and polyphenol than in those of the other two treatments ($P<0.01$). The activity of casein-enriched samples was

highest among treatments at time points 24, 48, and 72 hours ($P<0.01$).

Hydrolysis rates can sometimes occur faster than microbial uptake rates (Bruchet and Arnosti 2003; Arnosti et al. 1994). The average rate of hydrolysis for the samples amended with casein was 4 μM/h in 72 hours of incubation, which could produce potentially 288 μM of hydrolyzate during that time interval. However, the net concentration of NH_4^+ produced was 75 μM over the same period. Assuming no amino acid or NH_4^+ assimilation, these results suggest that the potential supply rates of amino acids from enzymatic hydrolysis of protein were 3.8 fold higher than the bacterial NH_4^+ regeneration rates. This faster hydrolytic step agrees with the relationship between hydrolysis of polysaccharides and microbial uptake and remineralization of oligosaccharides (Arnosti et al. 1994). However, aminopeptidase hydrolysis not only produces amino acids, which are readily available for uptake, but also smaller protein molecules and polypeptides which require further enzymatic action prior to microbial uptake. In addition, enzyme activities corresponded to the maximum potential rates, which may be faster than *in situ* rates of aminopeptidase activity.

The increases in NH_4^+ concentration correlated positively with enzyme activity in all three treatments (Figure 6.3). The Pearson-coefficient of linear association (r) between NH_4^+ and aminopeptidase activity was 0.86, 0.89, and 0.71 for the control, casein, and casein plus polyphenol treatments, respectively. The P-values of all correlations between NH_4^+ concentration and enzyme activity were smaller than 0.01.

Ammonium production and casein concentration

Casein was added in 50 μg/ml increments (up to 150 μg/ml) to Aransas Bay sediment slurries to examine the potential relationship between protein substrate added and NH_4^+ released (Figure 6.4). The NH_4^+ produced increased proportionally with casein

concentration at a rate of approximately 200 μM NH_4^+ per 50 μg/ml casein substrate. The linear regression ($r^2 = 0.99$) quantified this relationship and confirmed that the amount of NH_4^+ released was related to the concentration of organic nitrogen (casein) that was available. The direct increase in NH_4^+ concentration with increasing protein content indicated not only that casein is a suitable substrate for microbial metabolism, but also suggests that bacterial proteases hydrolyze it to constituent amino acids, which can be metabolized by the bacteria.

Organic nitrogen constitutes a significant part of the total fixed nitrogen in estuaries (Berman and Bronk 2003). Variations of NH_4^+ concentration in the environment may relate to the types and supply rates of bioavailable organic nitrogen. Proteins derived from phytoplankton can be metabolized and high NH_4^+ regeneration rates have been associated with phytoplankton production (Gardner et al. 1993; 1996). Proteins, however, must be converted to smaller compounds (e.g. amino acids) by enzymatic hydrolysis before microbes can take up and transform it to NH_4^+. Therefore, enzymes must be involved in NH_4^+ regeneration by bacteria (Jacobson et al. 1987).

To evaluate the role of aminopeptidase on NH_4^+ production, NH_4^+ evolution was monitored in sediment slurries containing an enzyme inhibitor in April 2008. In control samples, NH_4^+ concentration reached 59 ± 8μM in 96 hours at a rate 0.5 μM NH_4^+/h. The addition of casein increased NH_4^+ concentration from 14 ± 2μM to 632 ± 28μM at the end of the incubation period (Figure 6.5). The net NH_4^+ concentrations produced indicated that 26% of the N from casein was regenerated as NH_4^+. The overall rate of increase was approximately 6.4 μM NH_4^+/h during the 96-hour incubation ($r^2 = 0.93$), which was 13 fold faster than the rate estimated in unamended control samples. In samples treated with polyphenols, NH_4^+ concentration reached 89 ±7μM in 96 hours, slightly higher than in controls (t-test, P=0.01). Benthic macroinvertebrates can be a

significant source of NH_4^+ in sediments (Gardner et al. 1993). There was however, no visual evidence of benthic macrofauna in the sediment slurries in which NH_4^+ evolution was monitored.

The pattern of of aminopeptidase activity with time was not linear (Figure 6.6). In control samples, enzyme activity peaked from an initial value of 1.1 ± 0.1μM/h to 3.2 ± 0.7μM/h in 48 hours, followed by a decrease to 1.2 ± 1.0μM/h by the end of the incubation period. The significant initial activity increase may have been caused by subtle disturbances of the sediment. Abrasion of sediment grains or damaged microbial cell walls could have enhanced enzymatic activity due to labile organic compounds released from mineral surfaces (Porter et al 2006). In casein-enriched samples, the enzyme activity of 4.7 ± 0.1μM/h, reached at 48 hours, was significantly higher than that of controls (2-sample t-test, P=0.02). In the polyphenol treatment, the pattern of activity was similar to the control. Activity increased from 1.5 ± 0.3μM/h, at the beginning of the incubation, to 3.2 ± 0.2μM/h at 48 hours, and then returned to 1.5 ± 0.3μM/h at the end of the incubation.

The decrease in aminopeptidase activity detected in all treatments may relate to a decrease in labile enzyme substrate. A similar direct relationship between protease activity and substrate concentration was shown in estuarine sediments (Mayer 1989). Substrate dependence may explain the return of enzyme activity to initial levels in both control and polyphenol treatments and the delayed activity decrease in the casein-treated sediments. The slower decrease in activity compared to control samples after 72 hours could relate to casein or hydrolysable peptides remaining in the sediment.

Regression analysis confirmed the theoretical hypothesis that NH_4^+ concentrations are a function of enzyme activity. Assessment of the strength of the linear relationship showed that NH_4^+ concentration correlated positively with aminopeptidase activity in

controls ($r = 0.80$, $P<0.01$), casein-enriched ($r=0.96$, $P <0.01$), and casein plus polyphenol treatments ($r = 0.97$, $P<0.01$) over the first 72 hours. A causal relationship between NH_4^+ concentration and aminopeptidase activity could be inferred from the casein-enriched samples. The induced increases in aminopeptidase activity by casein were reflected in proportionally higher NH_4^+ concentrations. In casein-enriched samples, the regression coefficient of determination (r^2) equaled 0.92, suggesting that 92% of the variation in NH_4^+ concentration could be explained by aminopeptidase activity.

To establish the causal relationship between NH_4^+ concentration and aminopeptidase activity, an additional experiment was conducted to measure NH_4^+ evolution in the presence of polyphenol, a known enzyme inhibitor (Thurman 1983). Ammonium concentrations increased with time in all treatments (Figure 6.7). The net NH_4^+ concentrations in casein-enriched samples indicated that around 30% of casein-N added was regenerated. After 120 hours of incubation, casein additions increased NH_4^+ concentration from 19 ±0.3μM to 737 ±150μM, a final concentration 4.3 fold higher than that of control samples and 2.9 fold higher than that of samples with casein and polyphenol added together (2-sample t-test, $P<0.01$). The addition of polyphenol to casein-enriched samples lowered NH_4^+ production rate by 1.5 fold. Despite the available casein, cumulative NH_4^+ concentration after 120 hours was 1.5 times lower in samples where casein was combined with polyphenol than in samples with casein alone ($P<0.01$).

CONCLUSIONS

Benthic NH_4^+ regeneration in coastal marine sediments has a fundamental role in N-cycling and on the supply of N to primary producers (Nixon 1981; Kristensen 1988). The goal of this work was to determine whether NH_4^+ production is coupled to aminopeptidase activity and protein concentration. The hypothesis proposed was that

NH_4^+ production in marine sediments depends on aminopeptidase activity. The results showed that an increase in enzyme activity, associated with the addition of organic nitrogenous substrate, caused an increase in NH_4^+ production rate. In addition, the presence of aminopeptidase inhibitor reduced the NH_4^+ production rate. Thus, the regeneration of NH_4^+ in the sediment relates to aminopeptidase activity. The dependence of NH_4^+ production on aminopeptidase implies that protease activity can influence estuarine primary productivity and diagenetic processes involved in sediment N cycling.

The influence of bacterial proteases on nitrogen remineralization in estuarine sediments elucidated in this chapter is especially important in estuaries with small tidal amplitude and low freshwater inflow, where benthic regeneration becomes a significant source of nutrients. These types of estuaries are common in south Texas, where low inflow rates have reduced allochthonous loading of nutrients and significant proportions of nutrients are supplied by nutrient recycling. For example, sediment regeneration supplied 90% of dissolved nitrogen to the phytoplankton community in Corpus Christi Bay and maintained relatively stable productivity during long periods of low river discharge (Flint et al. 1986).

A second important result of this work is the observed inhibition of aminopeptidase by polyphenol, which caused reductions in NH_4^+ production rates. This result infers that the remineralization process is affected by the OM source and the presence of enzyme inhibitors. Since phenolic compounds are derived primarily from terrestrial vascular plants, detrital material high in polyphenols may reach the sediments primarily after rain events that increase river discharge rates. The above results demonstrate that the presence of these compounds can reduce enzyme activity, which consequently can reduce OM decomposition and nutrient regeneration rates.

Chapter 7: Summary and suggestions for future research

SUMMARY

The processes involved in organic matter (OM) decomposition, i.e. early stages of diagenesis (e.g. see the schematic of Figure 7.1), affect the chemistry of the atmosphere, soils, and oceans. A significant fraction of particulate OM produced in coastal waters reaches marine sediment where benthic microorganisms mediate most of its decomposition. Consequently, microorganisms dictate the transformations, portioning, and fate of the organic compounds in these systems. Understanding the mechanisms by which heterotrophic prokaryotes process OM in coastal sediment is crucial to addressing such issues as coastal eutrophication and global change.

Extracellular enzymatic hydrolysis is a required step in the microbial processing of OM (Figure 7.1), due to the physical constraints imposed by the bacterial cell wall on the transport of large organic molecules into the cell. Extracellular enzymes therefore contribute to controlling decomposition/transformation rates, and allocation of OM. Factors controlling the capacity of enzymes to process OM must be defined to advance our understanding of early diagenesis in marine sediment. In this investigation, microbiological and geochemical techniques were combined to examine factors affecting enzymatic hydrolysis, a key step in the microbial-mediated processing of OM.

The objective of this research was to examine potential factors controlling extracellular enzyme activity in sandy coastal sediment of Texas estuaries. The motivation for focusing on enzymes was the recurrent identification of "metabolizable OM fractions" in buried relic OM that is considered to be refractory. This evidence raised the question: What factors prevented the microbial extracellular hydrolysis of this apparently "labile" OM? The hypothesis was: The regulation of enzymes either decreases

or prevents hydrolysis of otherwise metabolizable fractions during OM decay in coastal sediment.

Factors limiting enzyme activity in the shallow, organic-poor, estuarine sand of Mission-Aransas estuary, TX, were examined with a combination of geochemical and microbiological approaches. The results show that the availability of labile substrate and substrate-reversible inhibition are mechanisms controlling enzyme activity. The presence of labile substrate induced enzyme activity and its depletion correlated with low enzyme activity. However, in organic-poor sediment, the addition of labile substrate did not stimulate enzyme activity, perhaps due to the presence of enzyme inhibitors or to poor substrate compatibility with the enzyme. Thus, organic compounds normally classified as "labile" via analytical characterization may fall outside the spectrum of enzyme specificity and not be degraded rapidly in sediment.

Results from enzyme-kinetics experiments suggest that inhibitors compete with substrate for enzyme binding sites and change enzyme kinetic parameters. The changes in enzyme parameters, determined experimentally, indicated a substrate-reversible type of inhibition, in which inhibitors decreased the maximum enzyme activity without a significant change in enzyme affinity for the substrate. Enzymes with low V_{max}'s had poor hydrolytic capacities, which were reflected in slow substrate turnover rates. These findings help explain one of the mechanisms responsible for controlling the rate of OM degradation in marine sediment. Biphasic kinetics detected in the sediment suggests extracellular diversity, where the same type of enzymes may have different kinetic characteristics. For example, one enzyme may have a high substrate specificity and low maximum activity at low substrate analog concentrations while another enzyme can operate with a low specificity and high maximum activity at elevated substrate concentrations.

The implication of enzyme inhibition, in limiting enzyme hydrolytic capacity and reducing the degradation rate of OM, was evident from the experimental results showing a significant decrease in ammonium regeneration rates in sediment samples enriched with polyphenol, a natural inhibitor of enzyme activity. These experimental results point to the central role of hydrolytic enzymes in regulating the decomposition rates and fate of OM in shallow, organic-poor sediment (Figure 7.1). They support the dogma that un-hydrolyzed OM components are ultimately preserved in the sediment.

FUTURE WORK

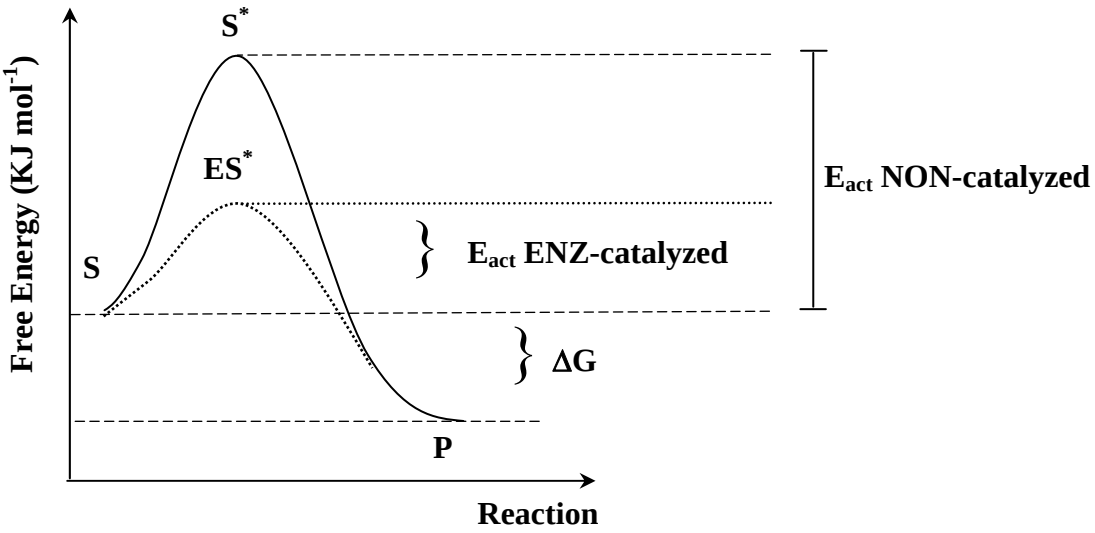
Analytical limitations must be overcome to understand the role of extracellular hydrolysis of natural-sediment OM. The structure and molecular-bonding characteristics of OM are complex and the enzyme analogs may not reflect natural degradation dynamics accurately. Future research efforts should focus on developing new biogeochemical methodologies to identify uncharacterized OM fractions. Further definition of the molecular structure of sedimentary OM would allow development of more precise and representative substrate analogs, which, in turn, would allow improved investigations on the synthesis, regulation, and function of extracellular enzymes. Accurate characterization of OM would facilitate investigations of "preferential OM decomposition" by identifying the mechanisms through which enzymes can selectively target specific substrates of different OM types.

The specific microorganisms responsible for the synthesis of extracellular enzymes, within the total consortium of benthic microbial populations in the sediment, are not yet well described. Research is needed to link enzyme hydrolysis to the composition of specific microbial groups, a goal that may be achievable with molecular techniques (see below). This knowledge would allow improved assessment of hydrolysis

rate-limitation effects on OM decomposition. It would address bottlenecks in understanding how heterotrophic microbial groups obtain metabolizable substrates from natural OM.

Regulation of gene expression is another important aspect for future studies of extracellular enzymes. Prokaryotes use chemical signaling to control metabolic activities (Fuqua et al. 1996). Although several microbial genes have been studied extensively, the environmental or physiological cues that trigger specific genes for the synthesis of microbial extracellular enzymes *in situ* still deserve future investigations. The data of Chapter 6, for example, showed that the addition of hydrolysable substrate initiated a cascade of microbial processes. The presence of a substrate stimulated the benthic microbial community to increase enzyme activity and ammonium production rates. It is likely that various microbial groups are involved in enzyme synthesis and ammonium production. However, the different groups of bacteria must have been interconnected, either physically or chemically, to exhibit the observed responses induced by the introduction of labile substrate to the sediment. How is this microbial function distributed and/or controlled among the different microbial populations? These control mechanisms may not be regulated solely by the presence of substrate. For example, while casein triggered an increase in aminopeptidase in the experiment described in Chapter 6, it did not promote a microbial response in the fresh sediment as presented in Chapter 2. The composite of biogeochemical and molecular approaches can help determining the potential hydrolytic capacity of a marine benthic system by identifying the mechanisms through which the many factors involved in gene expression and metabolic activities control substrate utilization and enzyme synthesis.

Figures



Legend:

S = substrate

P = product

ES^* = enzyme-substrate complex

S^* = substrate in transitional state

E_{act} ENZ-catalyzed = energy of activation of enzyme-catalyzed reaction

E_{act} NON-catalyzed = energy of activation of non-catalyzed reaction

ΔG = change in free energy of the reaction

Figure 1.1: Reaction pathways and free energy of enzyme-catalyzed and non-enzyme-catalyzed reactions (Adapted from Hames and Hooper 2000).

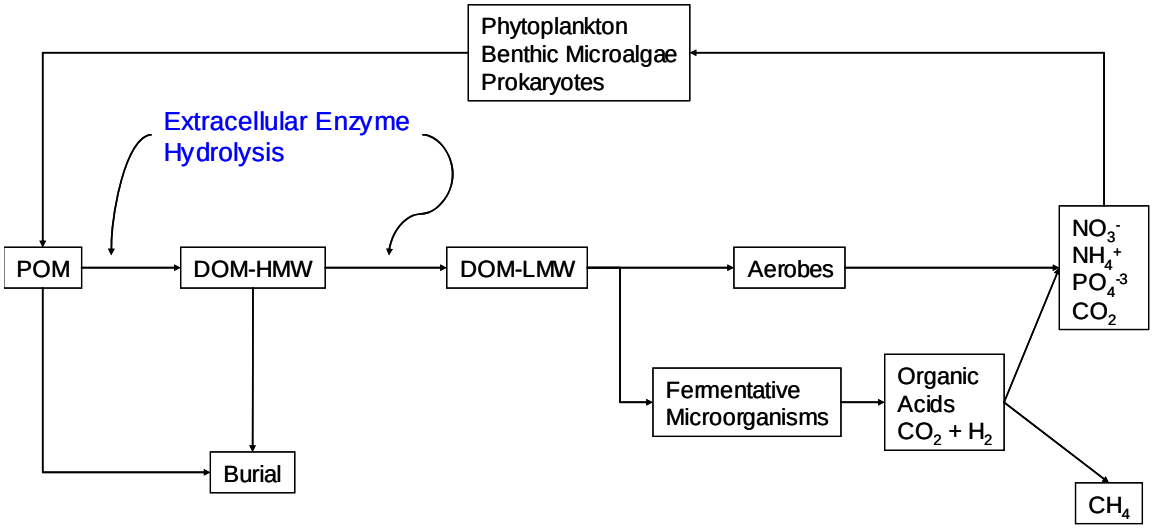


Figure 1.2. Simplified schematic showing the extracellular enzyme-mediated step in the transformations of organic matter in sediments. Not to scale or depth related.

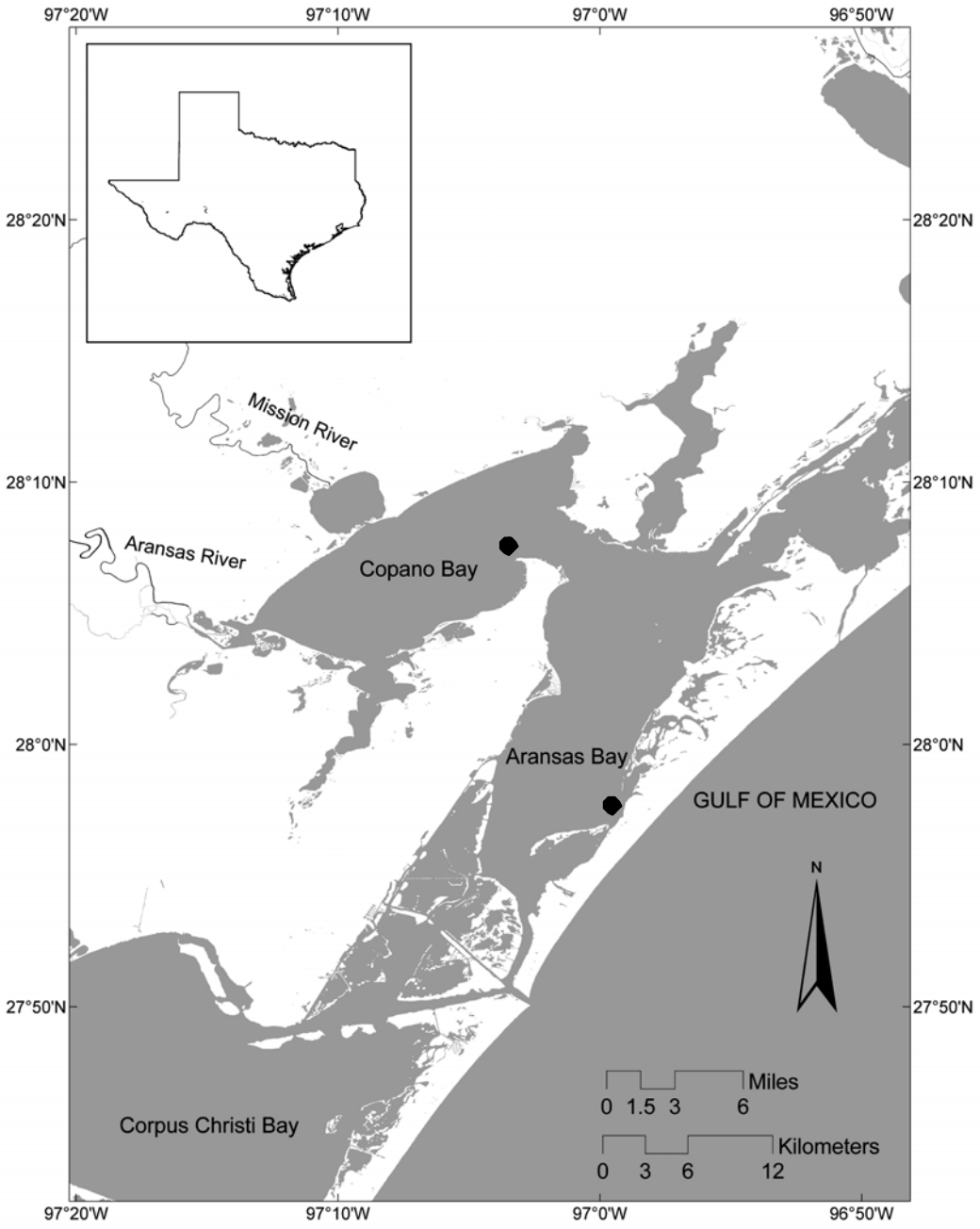


Figure 2.1. Regional map showing the sampling locations in Aransas and Copano Bays, Texas.

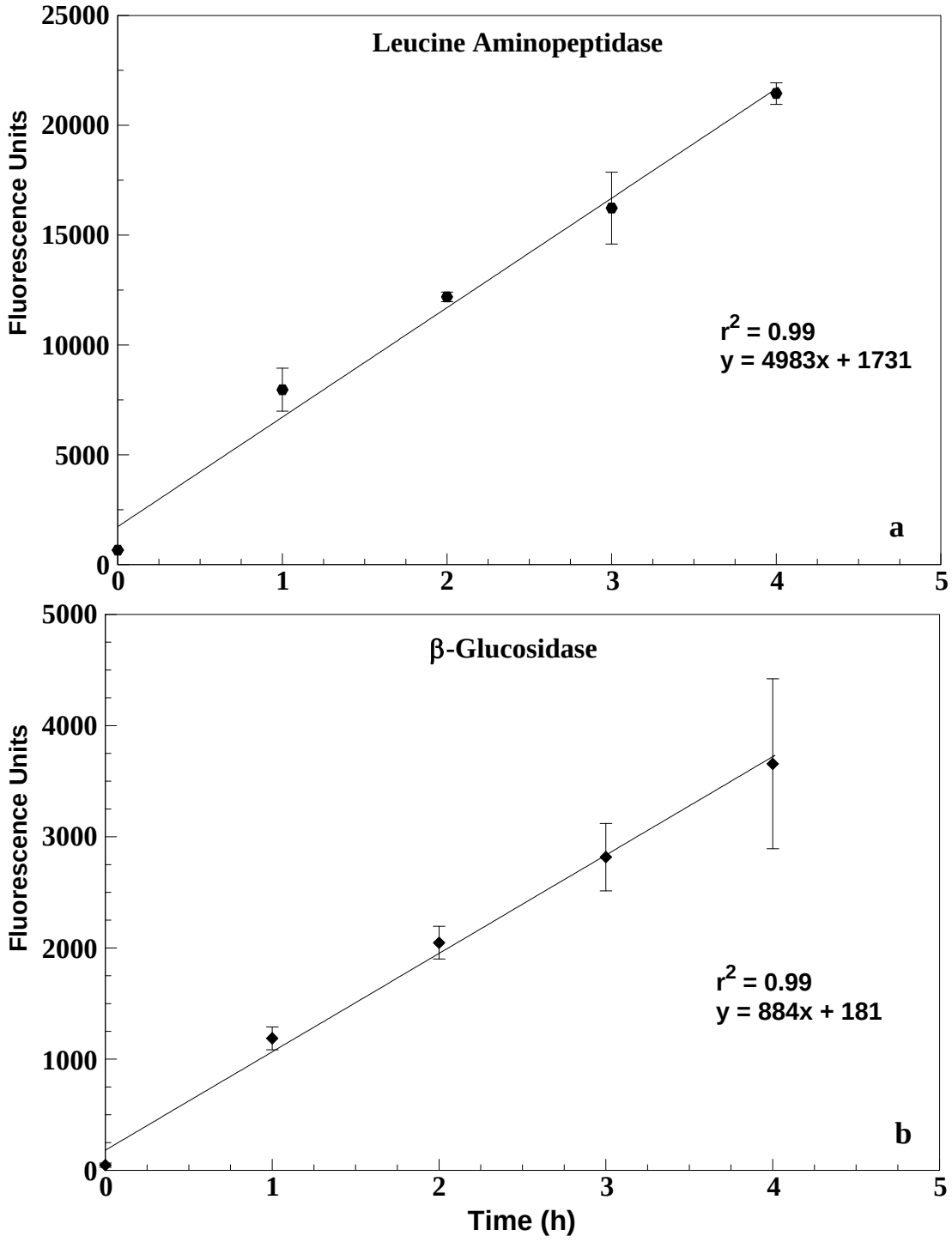


Figure 2.2: Analog fluorescence as function of incubation time for leucine-aminopeptidase (a) and β-glucosidase (b). Error bars are 95% confidence interval (CI) of the sample mean, n=3.

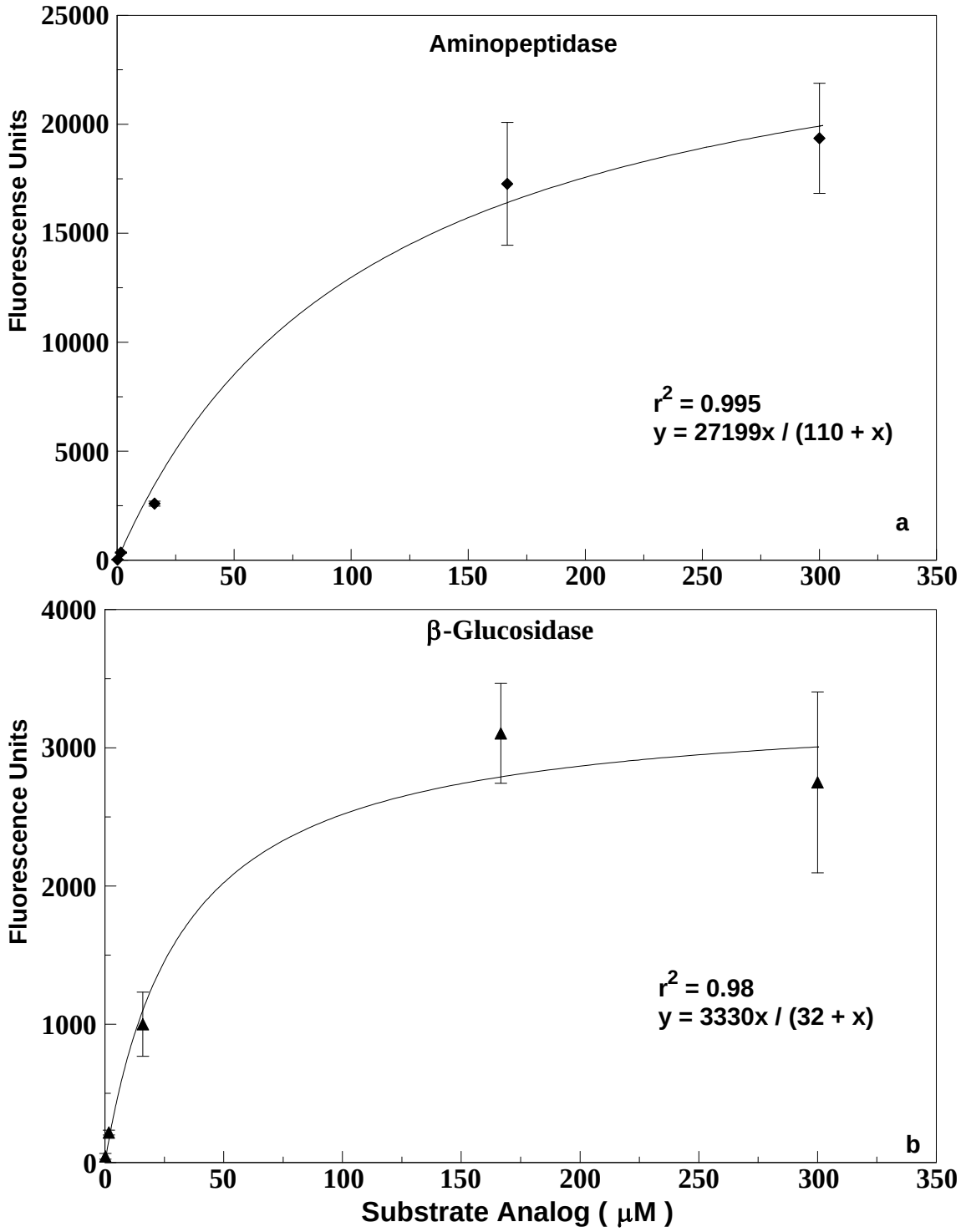


Figure 2.3: Michaelis-Menten curve fit for leucine-aminopeptidase and β-glucosidase. Error bars are 95% CI of the sample mean, n=3.

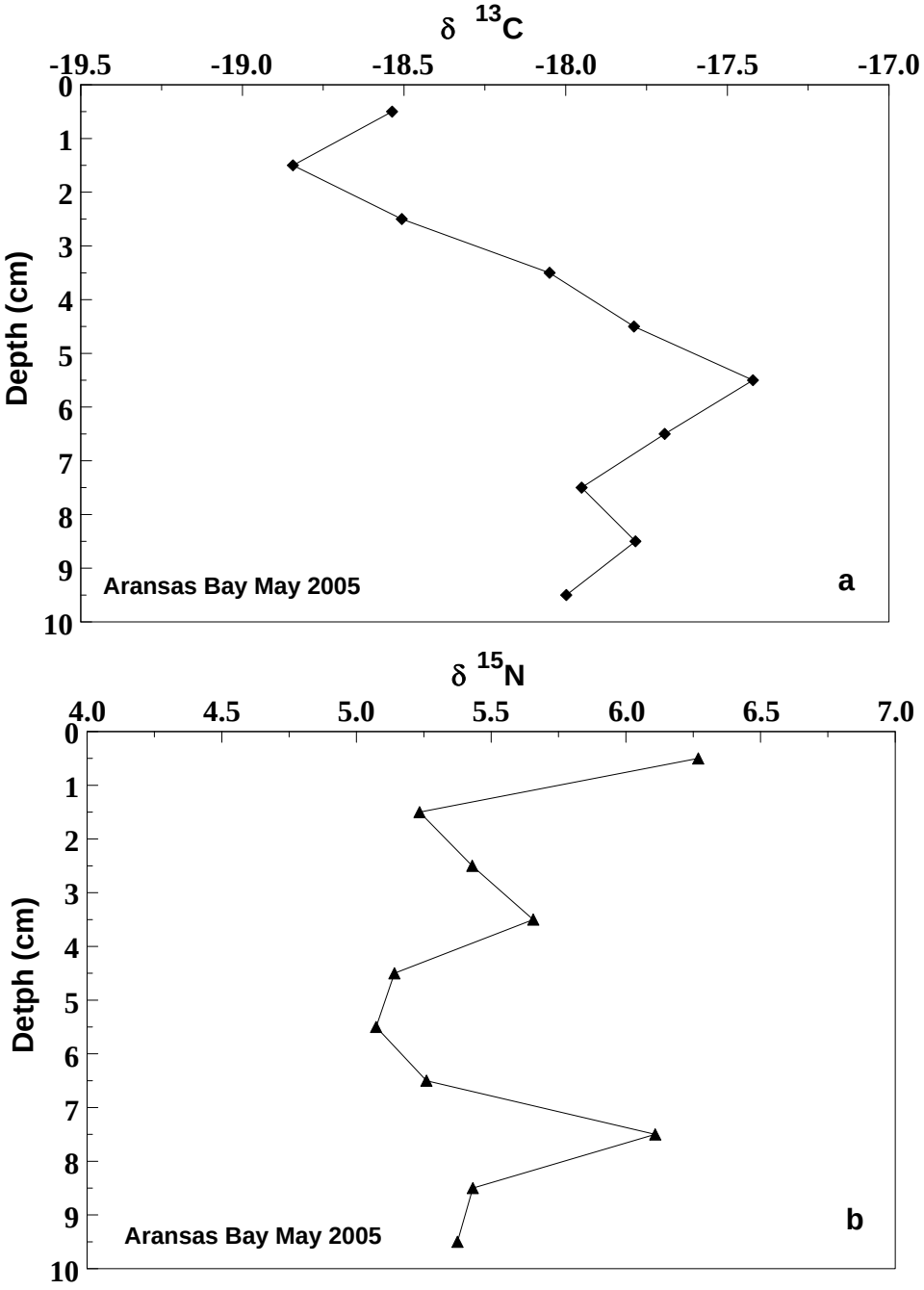


Figure 2.4: Vertical distribution of sediment OM carbon (a) and nitrogen (b) isotopic values in Aransas Bay 2005. (n=1). Each y-axis data point represents the mid-depth of the layer sampled on this and following figures with depth on y-axis.

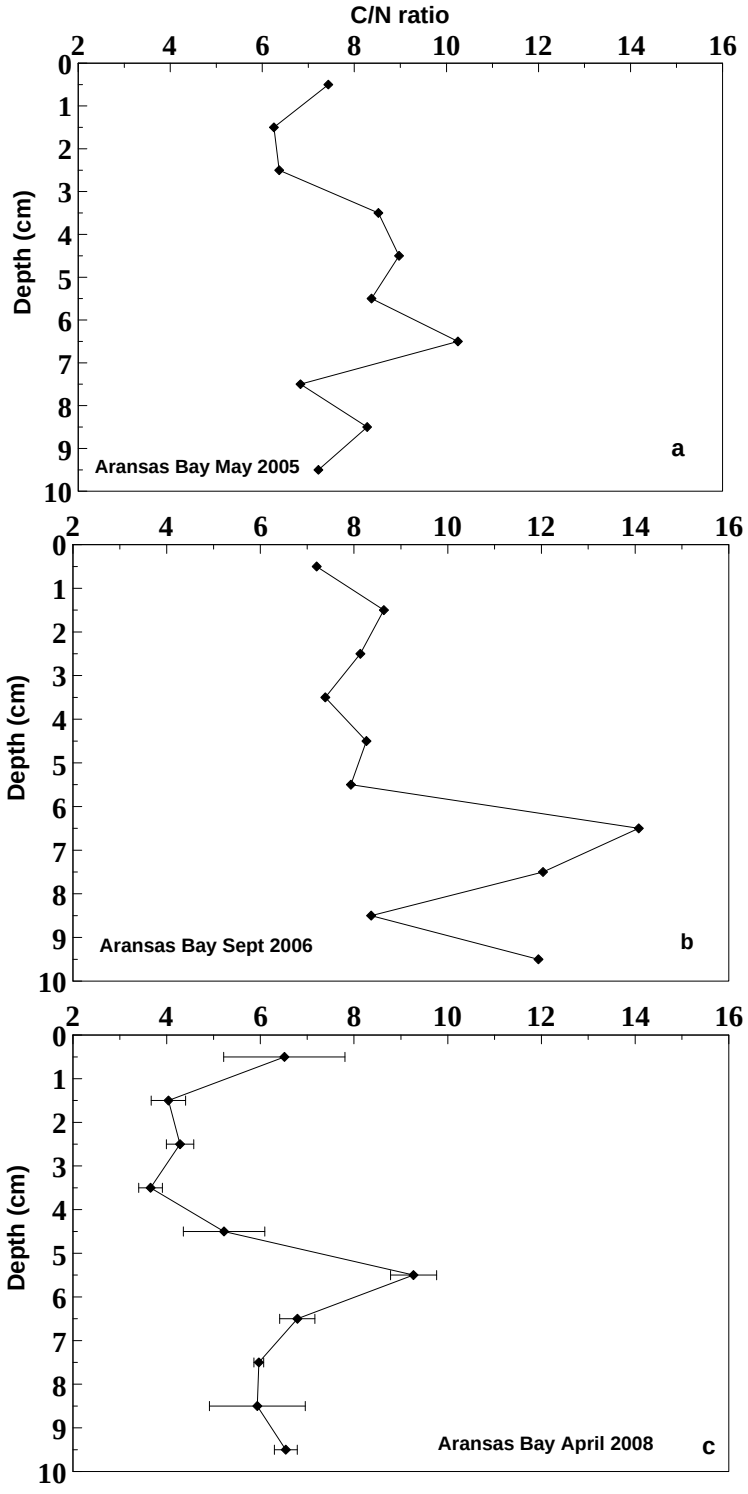


Figure 2.5: Vertical distribution of OM C/N ratio in Aransas Bay sediments. Sample size of a and b is n=1. Error bars = 95% CI of sample size n=2.

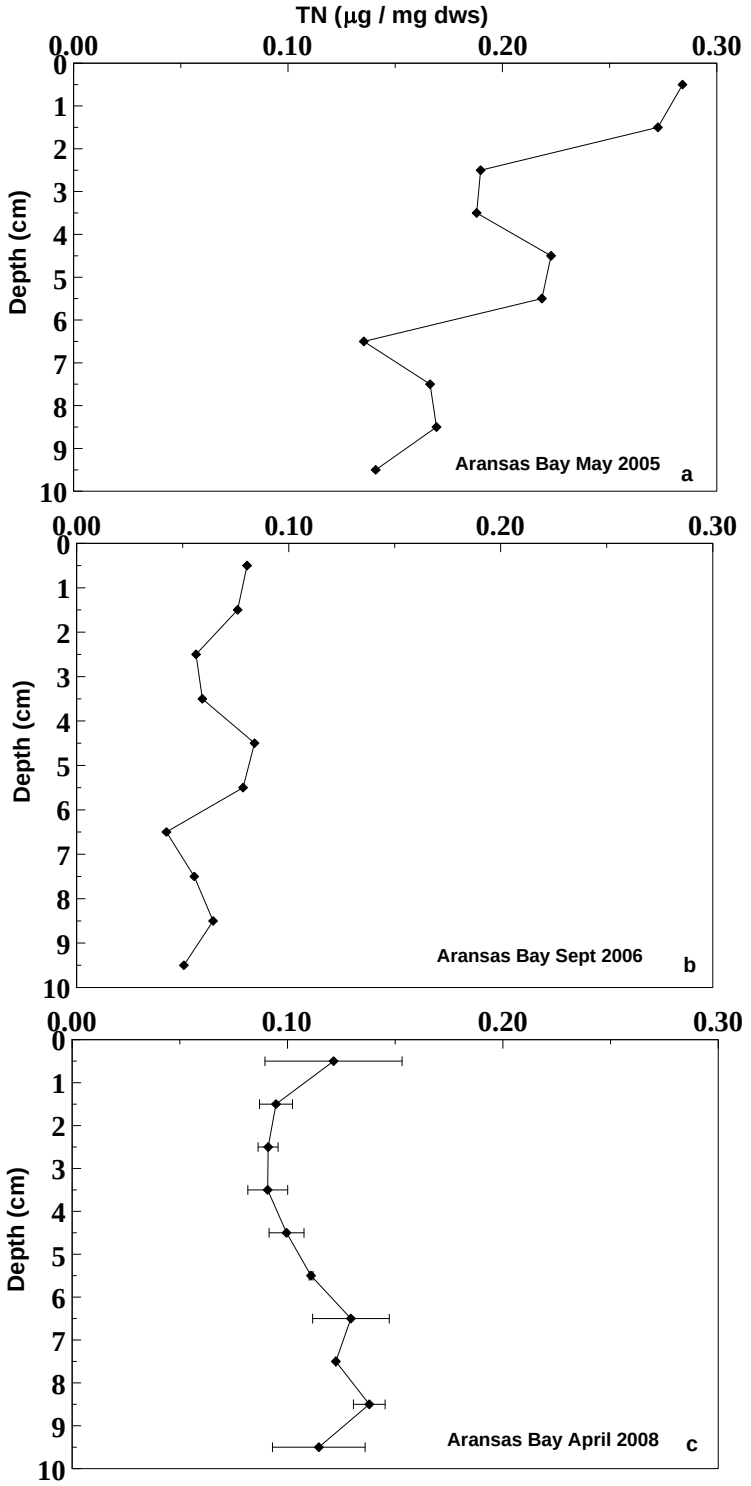


Figure 2.6: Sediment depth profiles of total nitrogen (TN) content in Aransas Bay. Sample size of a and b is n=1.Error bars for April samples are 95% CI, n=2.

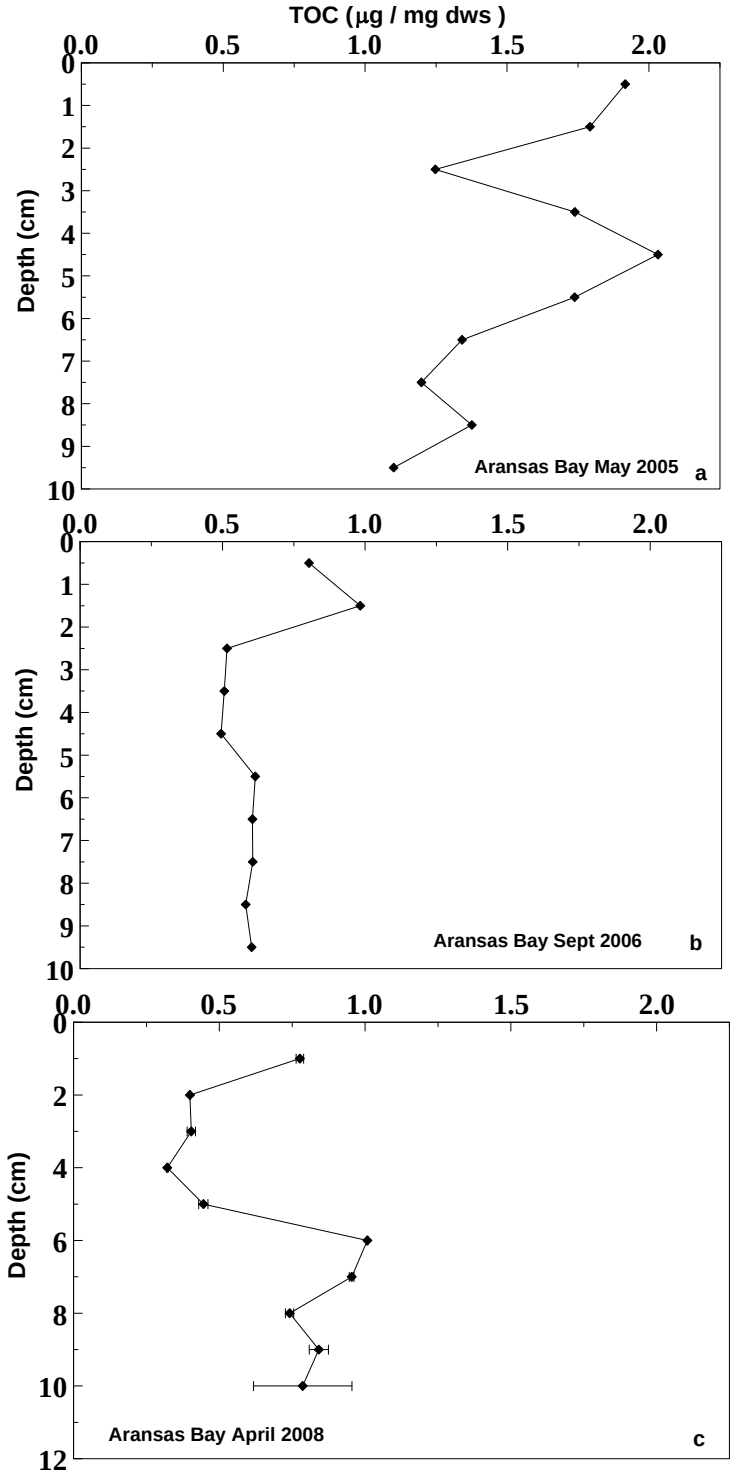


Figure 2.7: Sediment depth profiles of total organic carbon (TOC) content in Aransas Bay. Sample size of a and b is n=1. April 2008 error bars are 95% CI. n= 2.

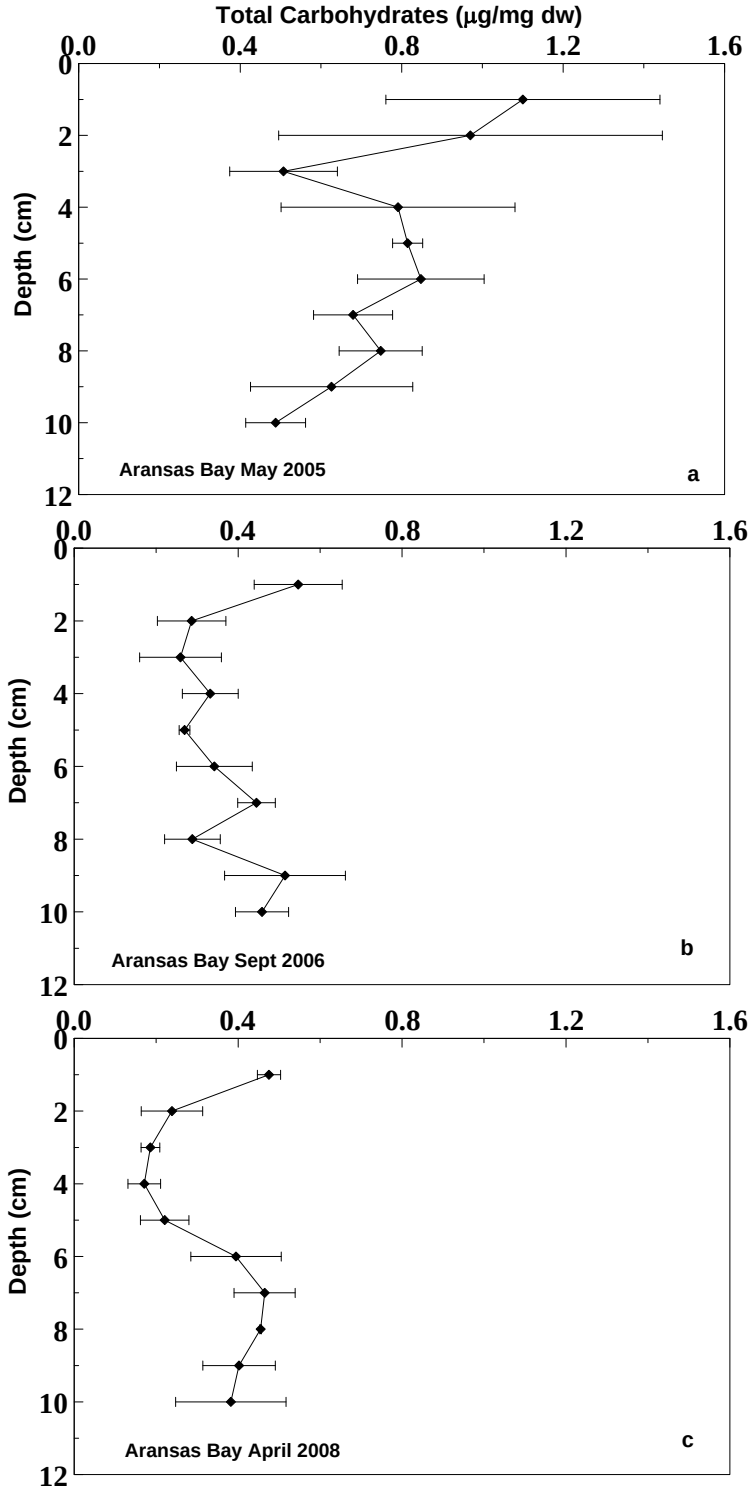


Figure 2.8: Sediment depth profiles of total carbohydrates content in Aransas Bay. Error bars are 95% CI. n=3.

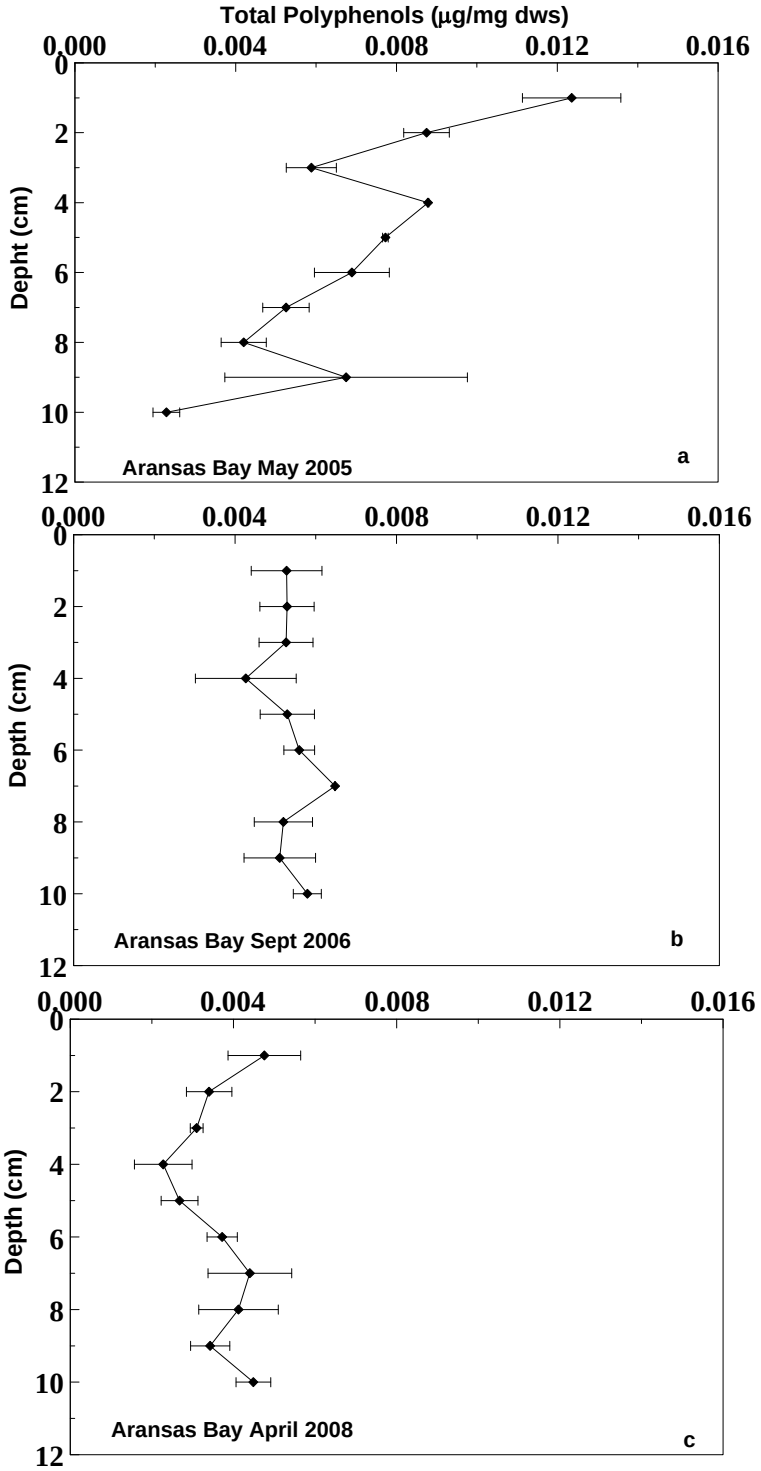


Figure 2.9: Sediment depth profiles of total polyphenol content in Aransas Bay. Error bars are 95% CI. n=3.

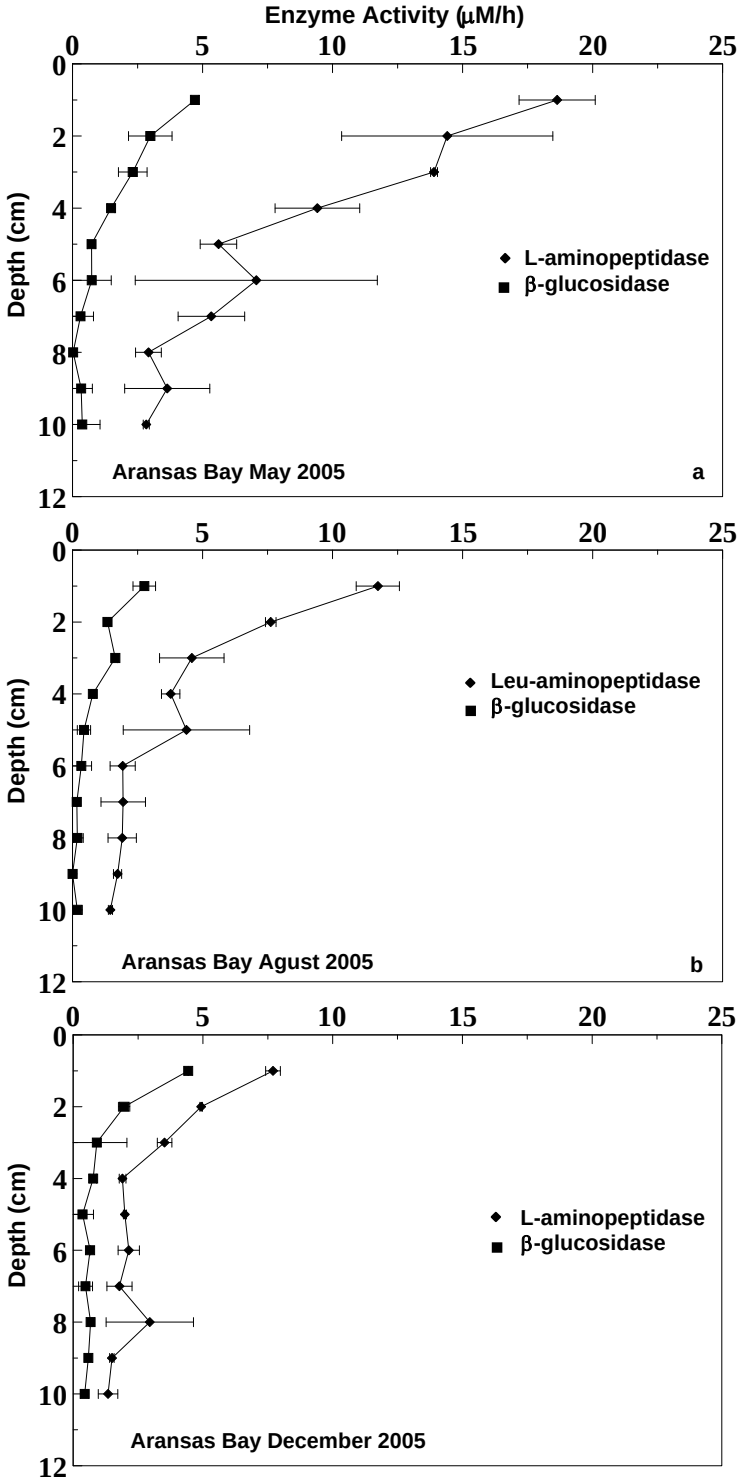


Figure 2.10: Sediment depth profiles of aminopeptidase and glucosidase activity for the year 2005. Error bars are 95% CI. n = 3.

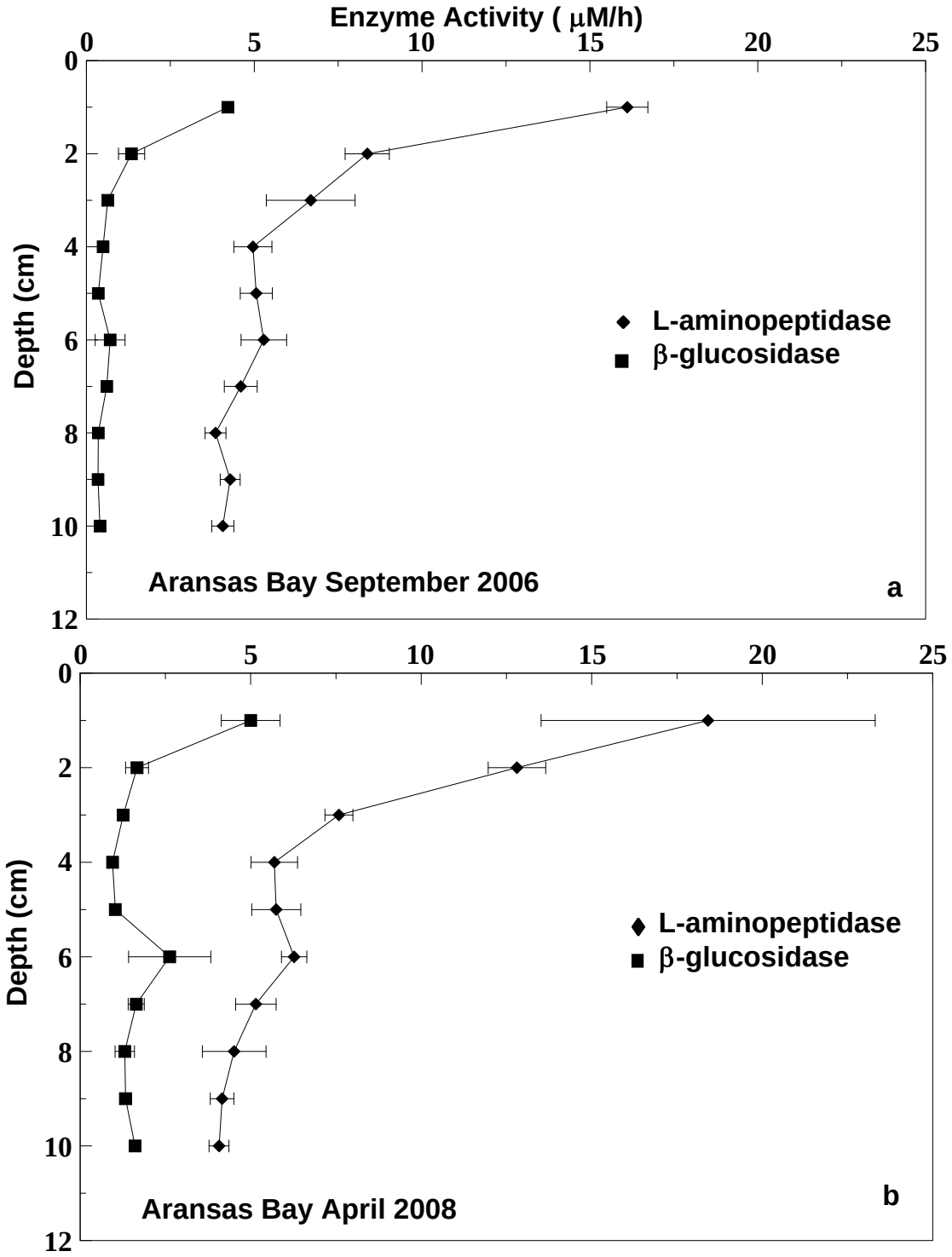


Figure 2.11: Sediment depth profiles of aminopeptidase and β-glucosidase activity in Aransas Bay for the years 2006 and 2008. Error bars are 95% CI. n = 3.

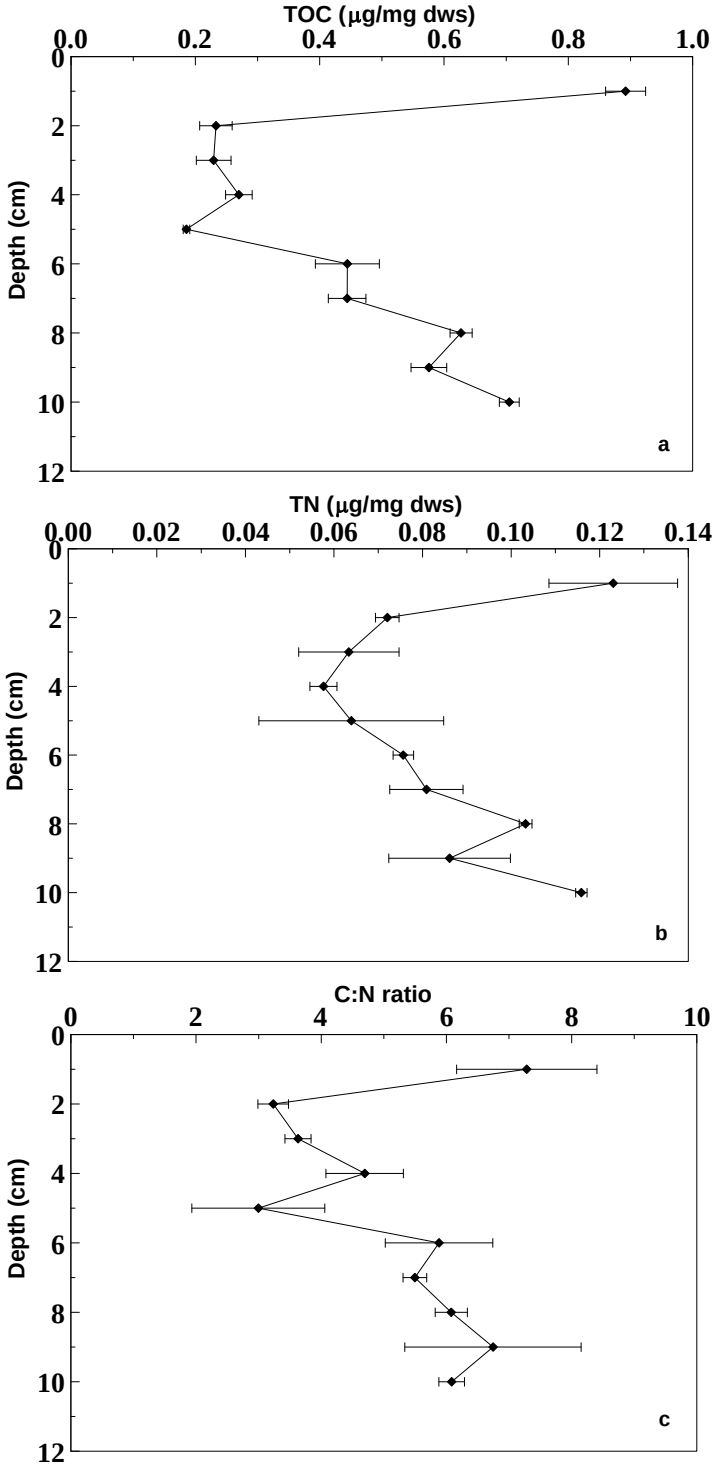


Figure 2.12: Vertical distribution of (a) TOC and (b) TN content and (c) C/N ratio. Copano Bay September 2007. Error bars are 95% CI of n=2.

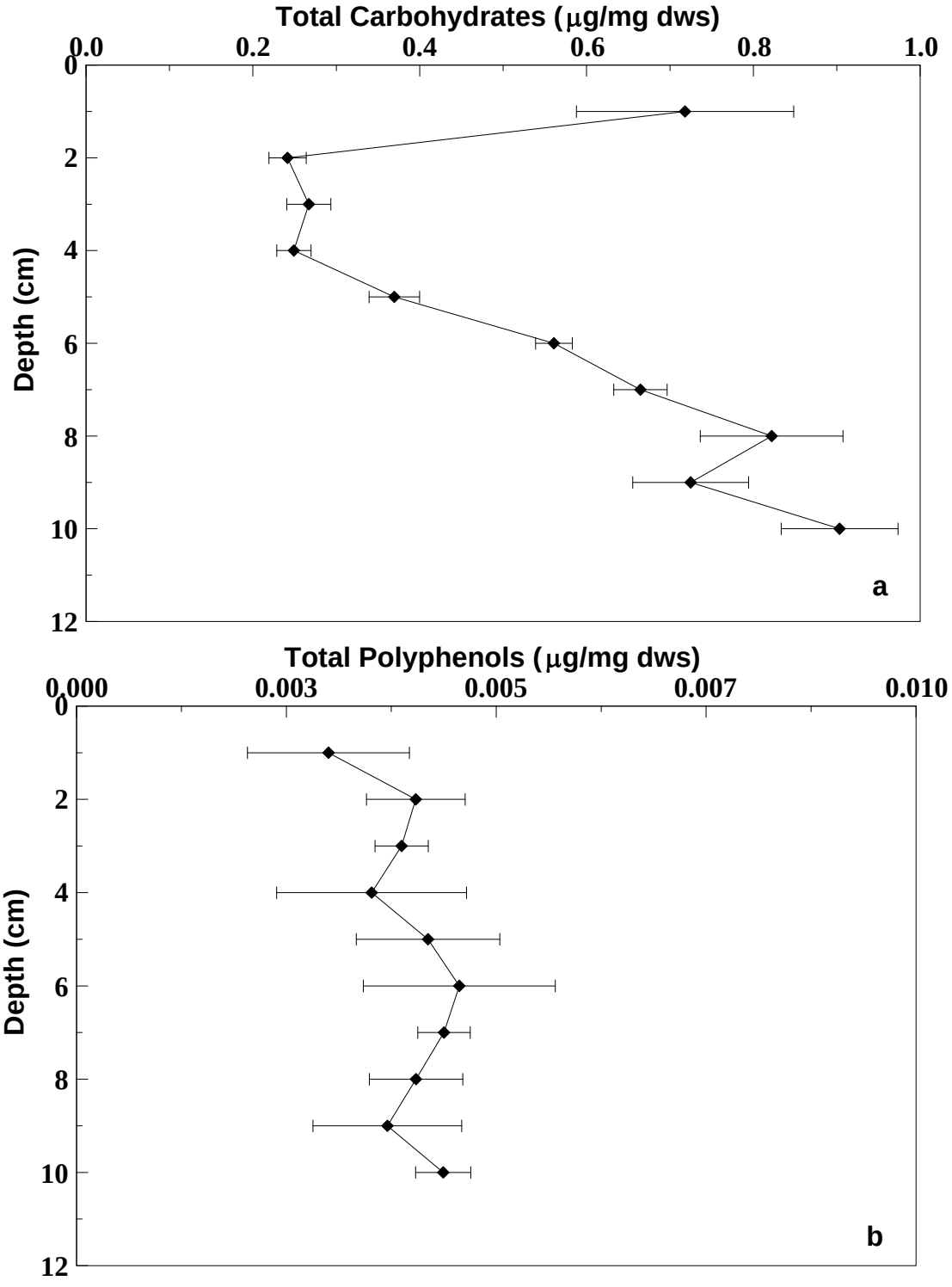


Figure 2.13: Vertical distribution of (a) total carbohydrates and (b) total polyphenol in Copano Bay 2007 sediments. Error bars are 95% CI, n=3.

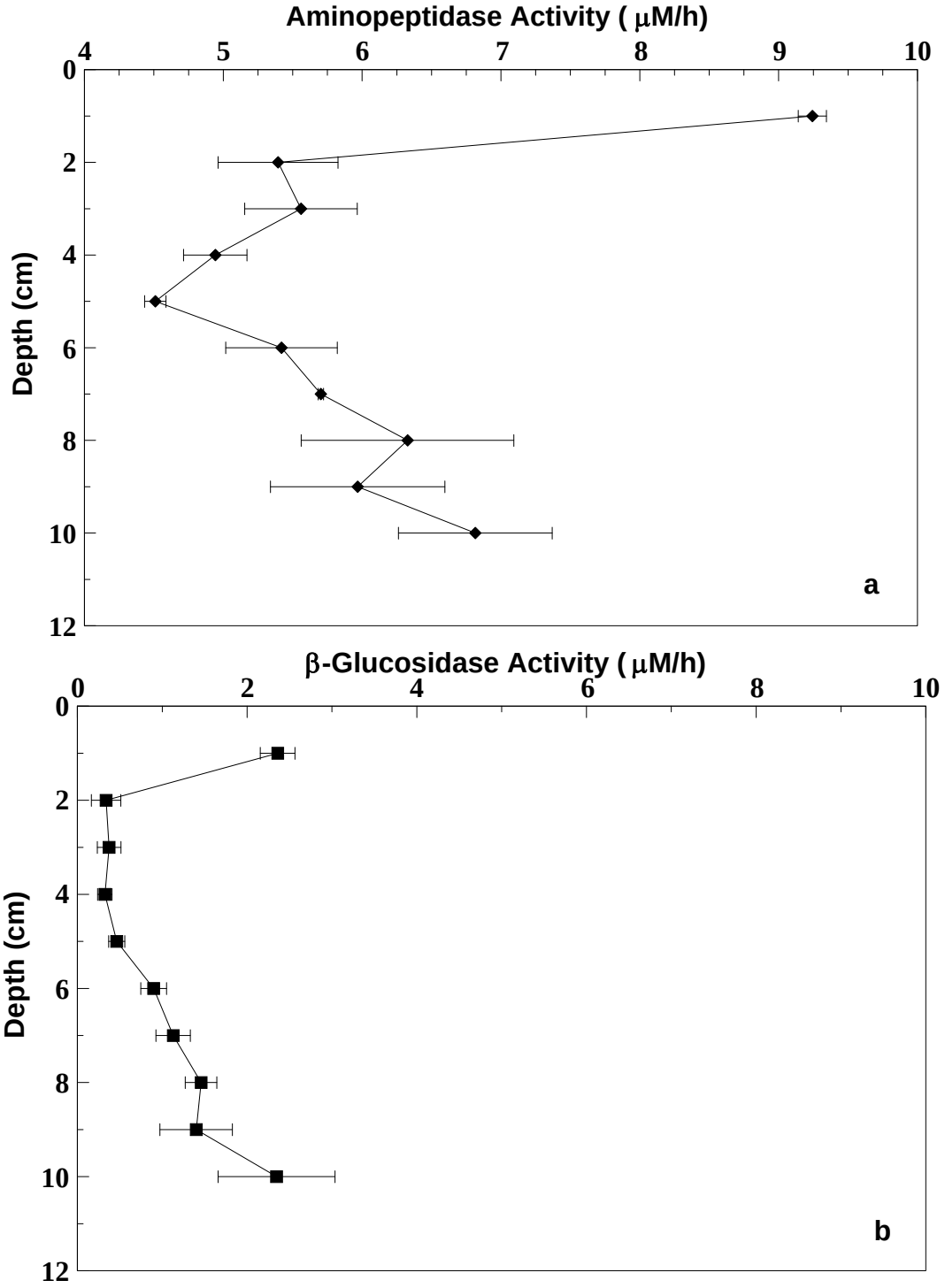


Figure 2.14: Vertical distribution of (a) aminopeptidase and (b) β-glucosidase activities in Copano Bay 2007 sediments. Error bars are 95% CI, n=3.

Table 2.1: Degree of association between aminopeptidase and glucosidase activity and geochemical characteristics of Aransas Bay sediment samples. Values are Pearson correlation coefficients parameters and values in parenthesis are correlation coefficient at $\alpha = 0.05$.

Sediment Parameters	Aminopeptidase	β-Glucosidase
Depth	**- 0.86 (<0.01)**	**-0.61 (0.06)**
TOC	**0.50 (0.15)**	**0.71 (0.02)**
TN	**0.86 (<0.01)**	**0.84 (<0.01)**
Carbohydrates	**0.30 (0.40)**	**0.47 (0.17)**
Polyphenols	**0.72 (0.02)**	**0.72 (0.02)**

Table 2.2: Degree of association between aminopeptidase and glucosidase activity and geochemical characteristics of Copano Bay sediment samples. Values are Pearson correlation coefficients parameters and values in parenthesis are correlation coefficient at $\alpha = 0.05$

Sediment Parameters	Aminopeptidase	β-Glucosidase
Depth	**- 0.15 (0.68)**	**0.37 (0.30)**
TOC	**0.90 (<0.01)**	**0.97 (<0.01)**
TN	**0.88 (<0.01)**	**0.97 (<0.01)**
Carbohydrates	**0.56 (0.07)**	**0.90 (<0.01)**
Polyphenols	**-0.68 (0.03)**	**- 0.44 (0.20)**

Table 2.3: Degree of association between aminopeptidase and β-glucosidase activity and geochemical characteristics of *combined* Aransas and Copano Bay sediment samples. Values are Pearson correlation coefficients parameters and values in parenthesis are correlation coefficient at $\alpha = 0.05$.

Sediment Parameters	Aminopeptidase	β-Glucosidase
Depth	**- 0.67 (<0.01)**	**- 0.43 (<0.01)**
TOC	**0.24 (0.06)**	**0.32 (0.01)**
TN	**0.34 (<0.01)**	**0.39 (<0.01)**
Carbohydrates	**0.25 (0.010)**	**0.40 (<0.01)**
Polyphenols	**0.37 (<0.01)**	**0.28 (<0.01)**

Table 3.1: Aminopeptidase activity (μM/h) in controls and enriched sediment slurries after 5-h incubation. Parenthesis is the 95% CI, n= 3. Letter (**a**) refers to statistically different from control, 2-sample t-test, $\alpha = 0.05$

	Aminopeptidase Activity *(μM/h)*						
Depth (cm)	**KC**	**Control**	**DFAA**	**Glucose**	**NO_3^-**	**PO_4^{-3}**	**NH_4^+**
0-1	1.4 (0.35)	7.7 (0.5)	7.7 (0.8)	7.8 (0.1)	7.4 (0.1)	7.7 (0.4)	8.7 (0.4)
1-2	0.5 (0.02)	4.9 (0.1)	5.3 (0.7)	5.3 (0.6)	5.2 (0.2)	5.2 (0.1)	6.0 (0.1)[a]
2-3	0.3 (0.04)	3.5 (0.4)	3.4 (0.5)	3.9 (0.4)	3.7 (0.3)	4.1 (0.6)	4.4 (0.1)
3-4	0.3 (0.01)	1.9 (0.1)	2.4 (0.2)	2.6 (0.2)	2.7 (0.1)	2.7 (0.4)	3.4 (0.1)
4-5	0.2 (0.02)	2.0 (0.1)	2.4 (0.2)	2.3 (0.3)	1.9 (0.2)	3.2 (2.3)	5.8 (5.8)
5-6	0.2 (0.04)	2.1 (0.5)	2.0 (0.6)	3.3 (1.5)	3.1 (1.6)	2.4 (0.3)	3.5 (0.5)
6-7	0.2 (0.00)	1.8 (0.6)	2.5 (0.8)	2.3 (0.3)	1.7 (0.1)	2.0 (0.4)	2.9 (0.3)
7-8	0.3 (0.07)	3.0 (2.0)	2.8 (2.0)	1.8 (0.1)	1.5 (0.1)	2.0 (0.2)	3.0 (1.1)
8-9	0.3 (0.17)	1.5 (0.1)	3.3 (3.6)	2.9 (2.4)	1.7 (0.1)	1.7 (0.1)	2.4 (0.2)
9-10	0.1 (0.00)	1.3 (0.5)	1.3 (0.2)	1.1 (0.2)	1.2 (0.1)	1.4 (0.4)	1.6 (0.3)

KC: heat killed control

DFAA: Dissolved free amino acids

Table 3.2: β-glucosidase activity (μM/h) in controls and enriched sediment slurries after 48-h incubation. Parenthesis is the 95% CI, n = 3. Letter (**a**) refers to statistically different from control, 2-sample t-test, $\alpha = 0.05$

			b-glucosidase		***Activity***	*(μM/h)*	
Depth (cm)	**KC**	**Control**	**DFAA**	**Glucose**	**NO_3^-**	**PO_4^{-3}**	**NH_4^+**
0-1	0.2 (0.01)	4.4 (0.4)	5.4 (0.5)	8.2 (0.1)[a]	6.4 (1.6)	5.8 (0.8)	3.8 (0.4)
1-2	0.2 (0.02)	2.0 (0.2)	2.3 (1.0)	5.3 (0.6)	2.4 (0.4)	2.3 (0.2)	1.5 (0.1)
2-3	0.1 (0.05)	0.9 (0.3)	0.7 (0.9)	3.4 (0.4)	0.8 (0.2)	0.9 (0.1)	0.3 (0.1)
3-4	0.1 (0.01)	0.8 (0.1)	1.2 (0.3)	2.5 (0.2)[a]	0.8 (0.1)	0.8 (0.3)	0.7 (0.1)
4-5	0.1 (0.01)	0.4 (0.2)	0.5 (0.1)	2.0 (0.3)	0.3 (0.1)	0.4 (0.7)	0.5 (5.8)
5-6	0.1 (0.01)	0.6 (0.1)	0.7 (1.9)	3.3 (1.5)	0.9 (0.5)	1.2 (0.1)	0.4 (0.5)
6-7	0.1 (0.01)	0.5 (0.1)	1.6 (0.1)	2.1 (0.3)	0.6 (0.2)	0.5 (0.1)	0.5 (0.3)
7-8	0.1 (0.01)	0.7 (0.1)	0.7 (0.1)	1.9 (0.1)	1.2 (0.7)	0.9 (0.1)	0.5 (1.1)
8-9	0.1 (0.02)	0.6 (0.1)	0.5 (0.1)	3.0 (2.4)	0.6 (0.1)	0.7 (0.1)	0.6 (0.2)
9-10	0.1 (0.01)	0.4 (0.1)	0.4 (0.1)	1.1 (0.2)	0.4 (0.1)	0.6 (0.2)	0.4 (0.3)

KC: heat killed control

DFAA: Dissolved free amino acids

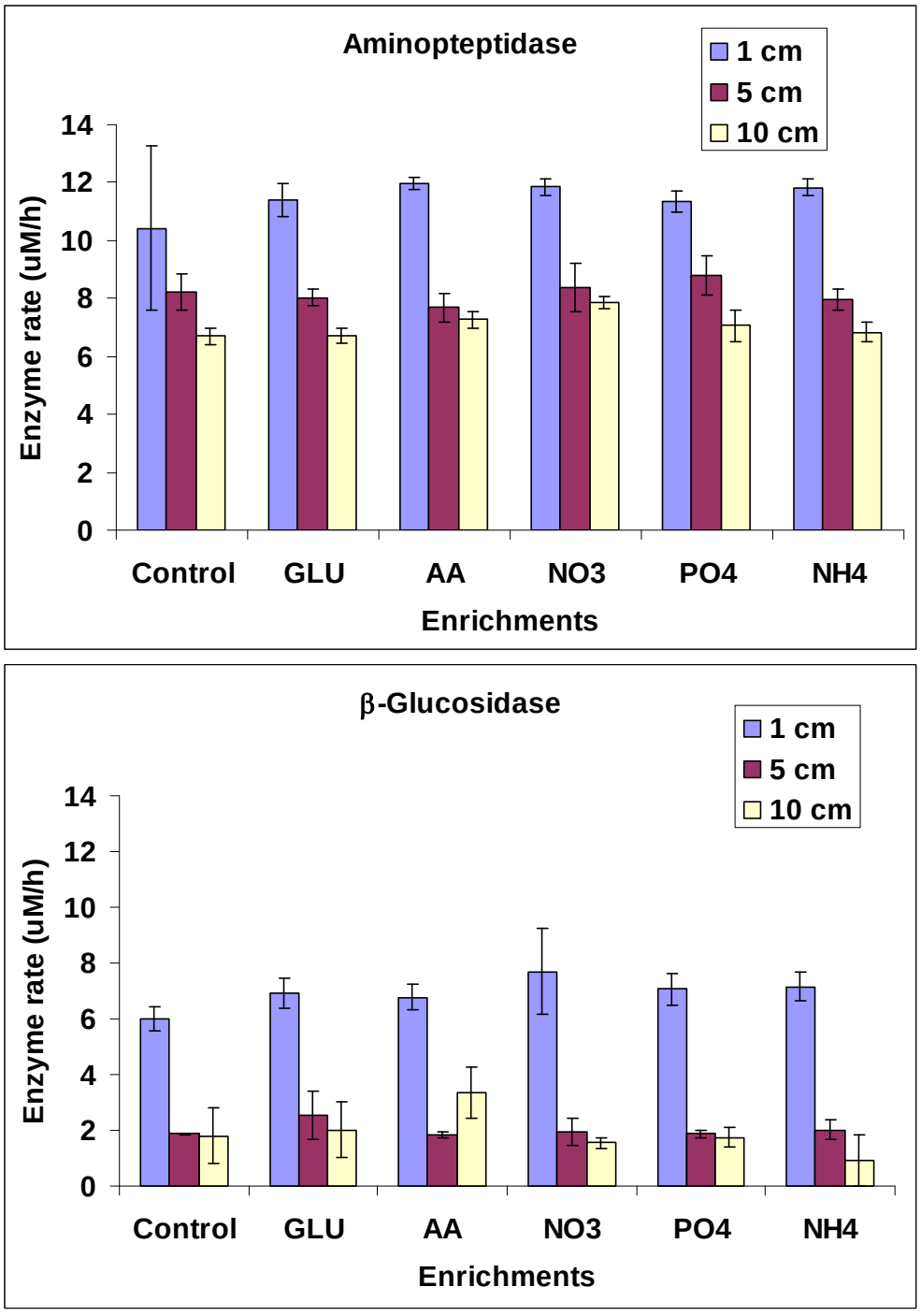


Figure 3.1: Aminopeptidase (top) and β-glucosidase (bottom) activities after long-term nutrient enrichments of March 2007 sediment at depths 1, 5, and 10 cm. Error bars are 95% confidence intervals (CI) around the mean of n = 3

Table 3.3: Correlations between enzyme activity and sediment TOC, TN, carbohydrates, and polyphenol content of controls samples of the 48-h incubation experiment. Values are Pearson correlation coefficients parameters with correlation coefficient at $\alpha = 0.05$ reported in parenthesis.

Sediment Parameters	Aminopeptidase	Glucosidase
Depth	**- 0.77 (0.02)**	**- 0.82 (<0.01)**
TOC	**0.79 (0.01)**	**0.78 (0.01)**
TN	**0.84 (<0.01)**	**0.89 (<0.01)**
Carbohydrates	**0.56 (0.10)**	**0.59 (0.10)**
Polyphenols	**0.19 (0.62)**	**0.07 (0.86)**

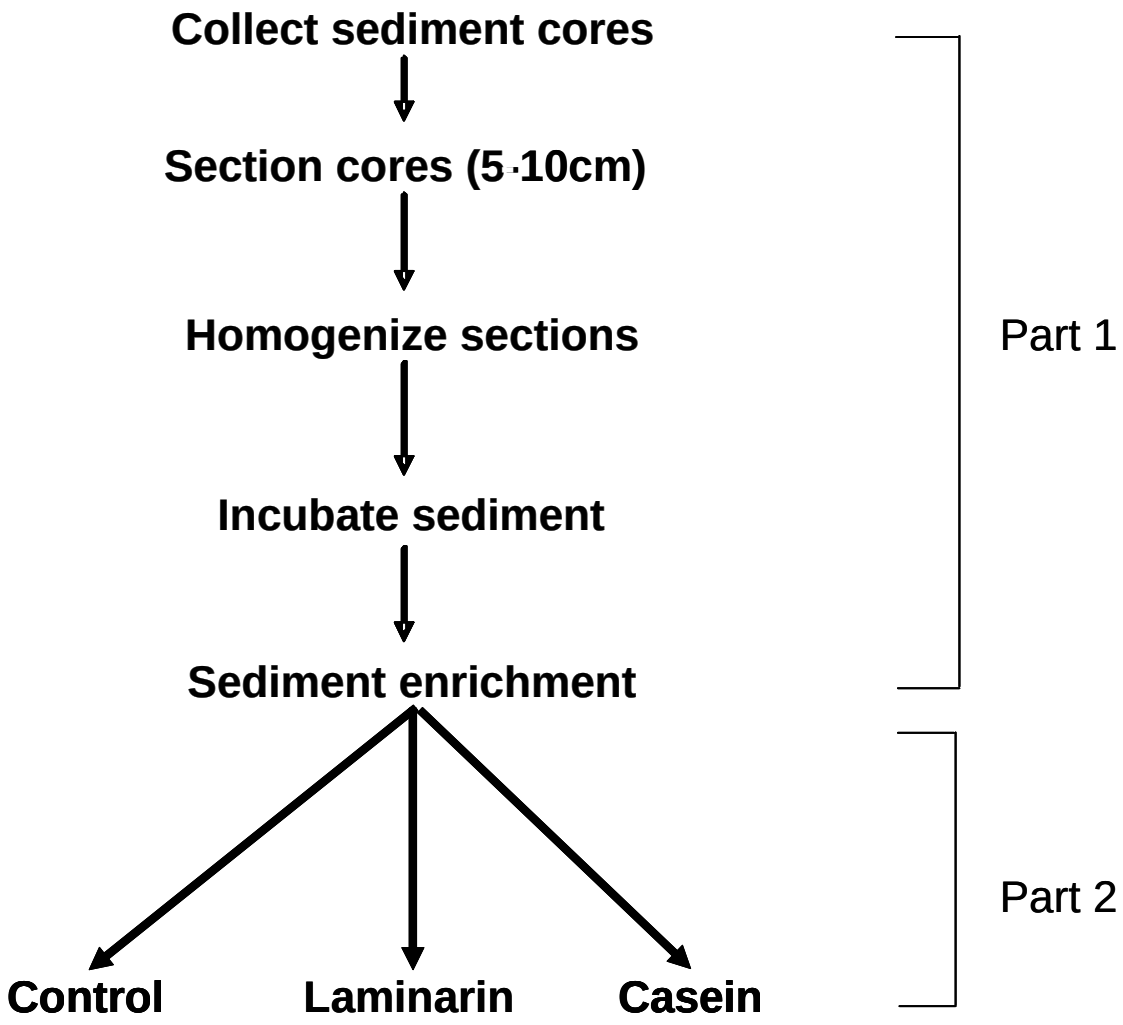


Figure 4.1: Scheme of the sediment manipulations for the long-term incubation and laminarin and casein amendments.

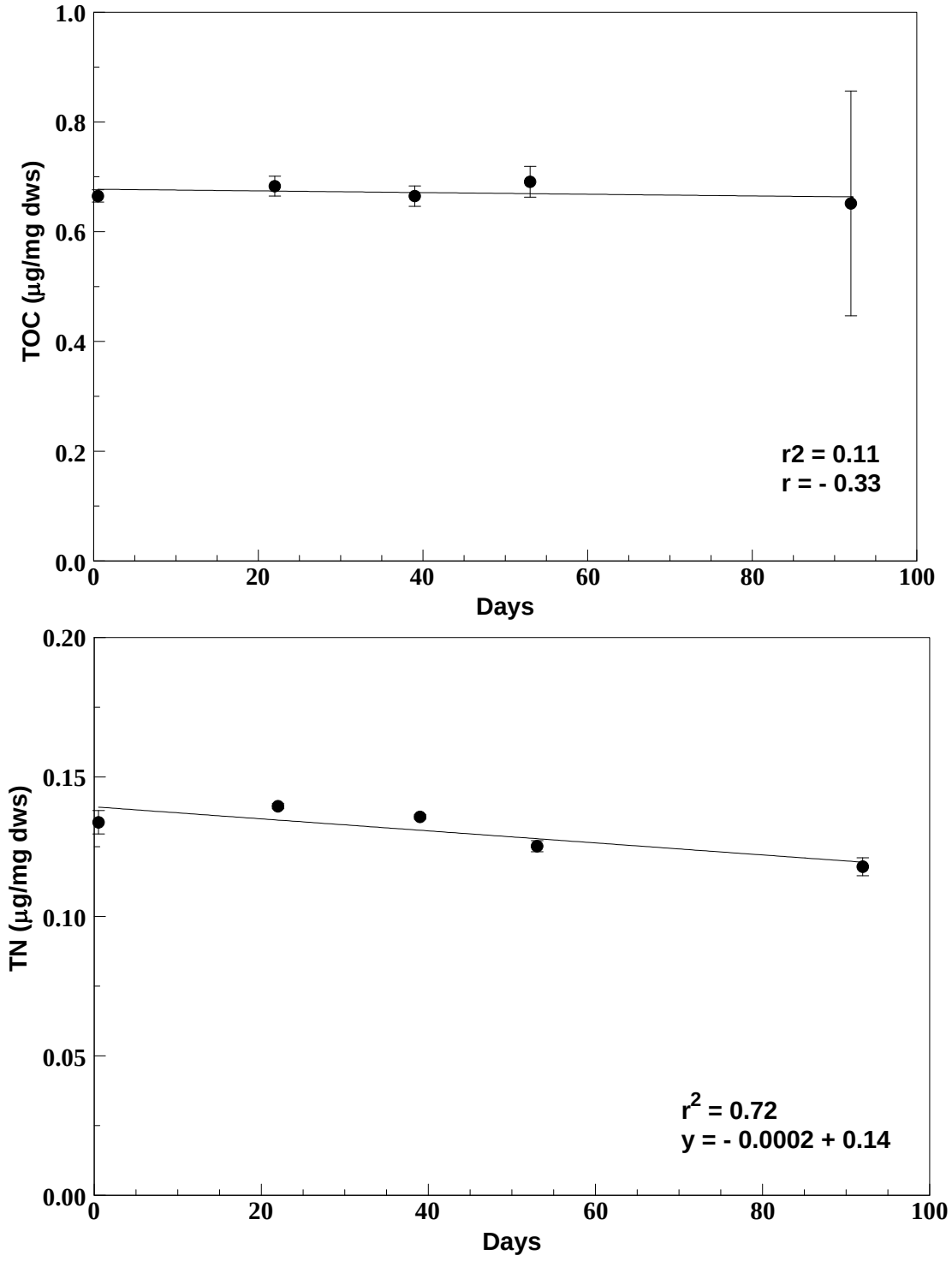


Figure 4.2: Sediment TOC (top) and TN (bottom) content as function of time. Bars are 95% CI, n=2.

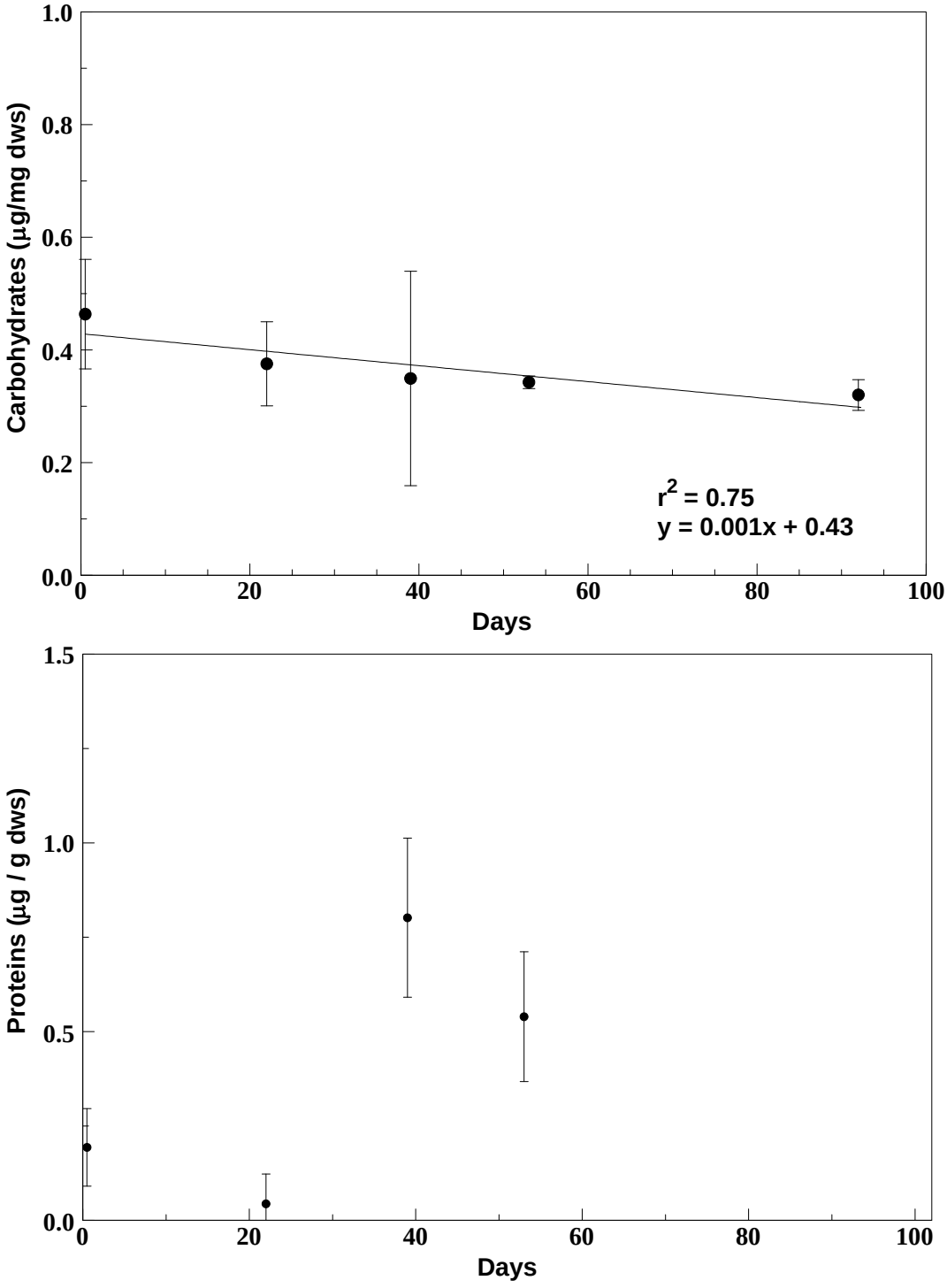


Figure 4.3: Total carbohydrate (top) and hydrolysable protein content (bottom) as function of time. Error bars are 95% CI, n=3.

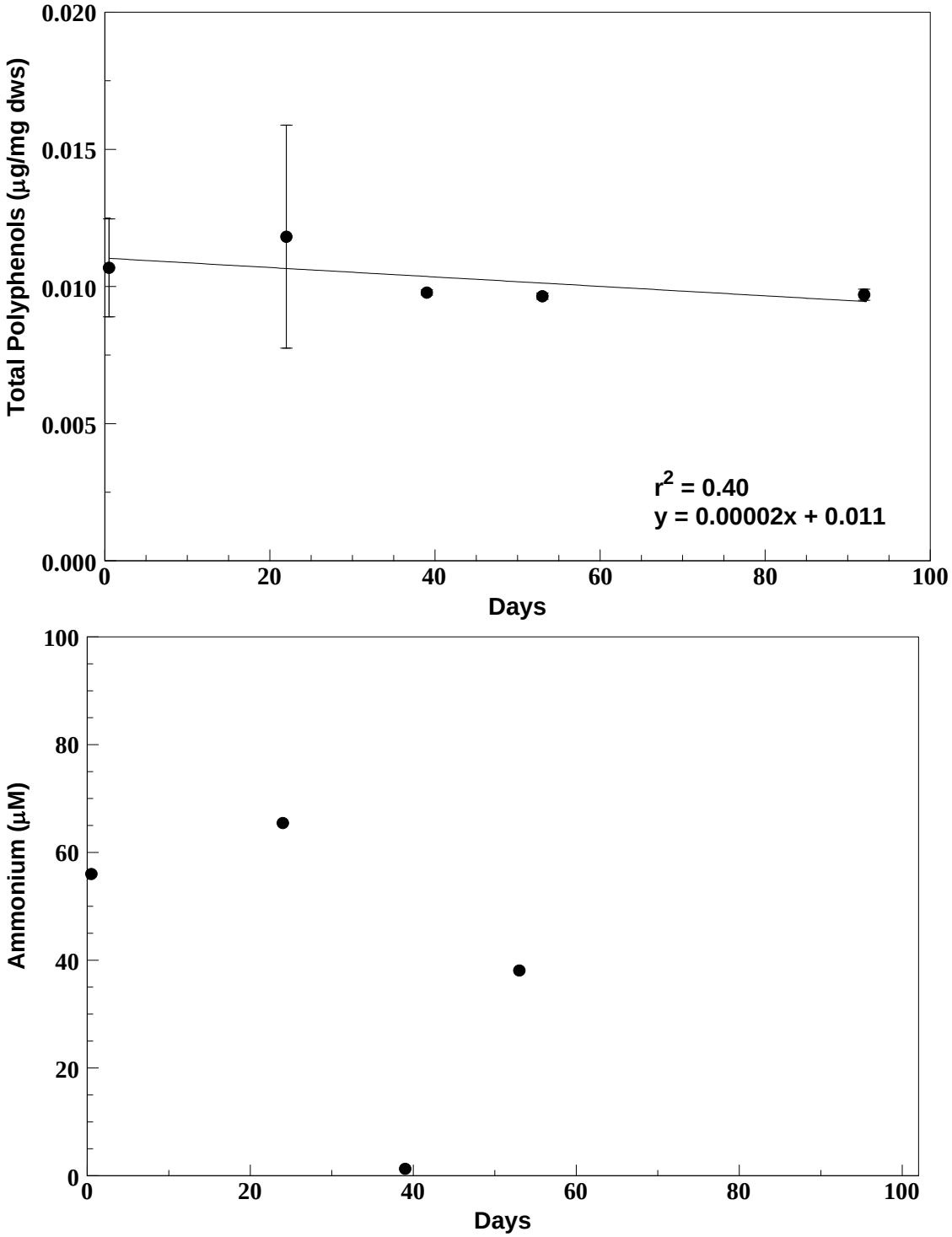


Figure 4.4: Total polyphenols content (top) and ammonium concentration (bottom) as function of time. Polyphenol error bars are 95% CI, n=3, NH_4^+ n=1.

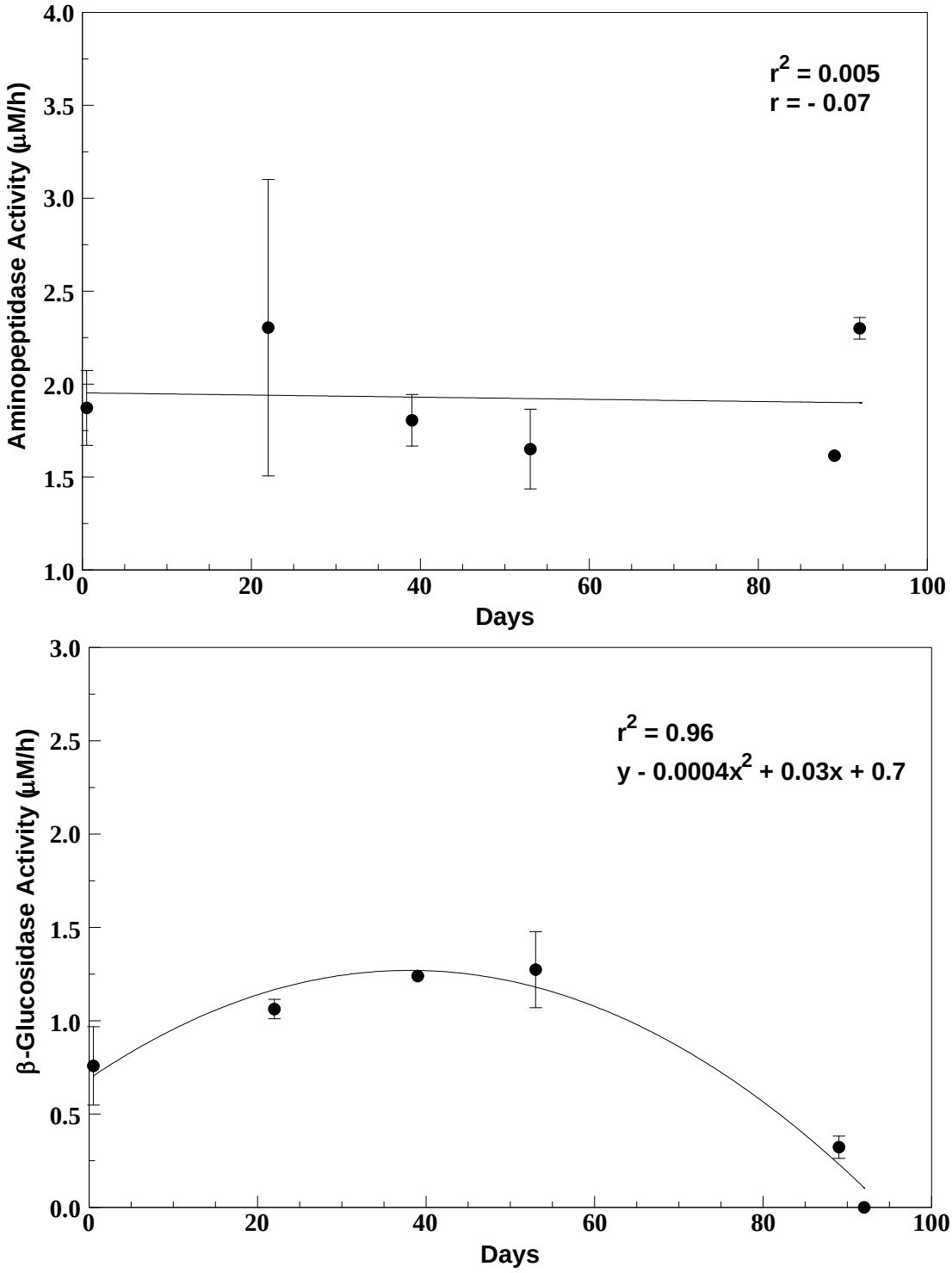


Figure 4.5: Leucine aminopeptidase (top) and β-glucosidase (bottom) activity over the course of incubation. Error bars are 95% CI, n=3.

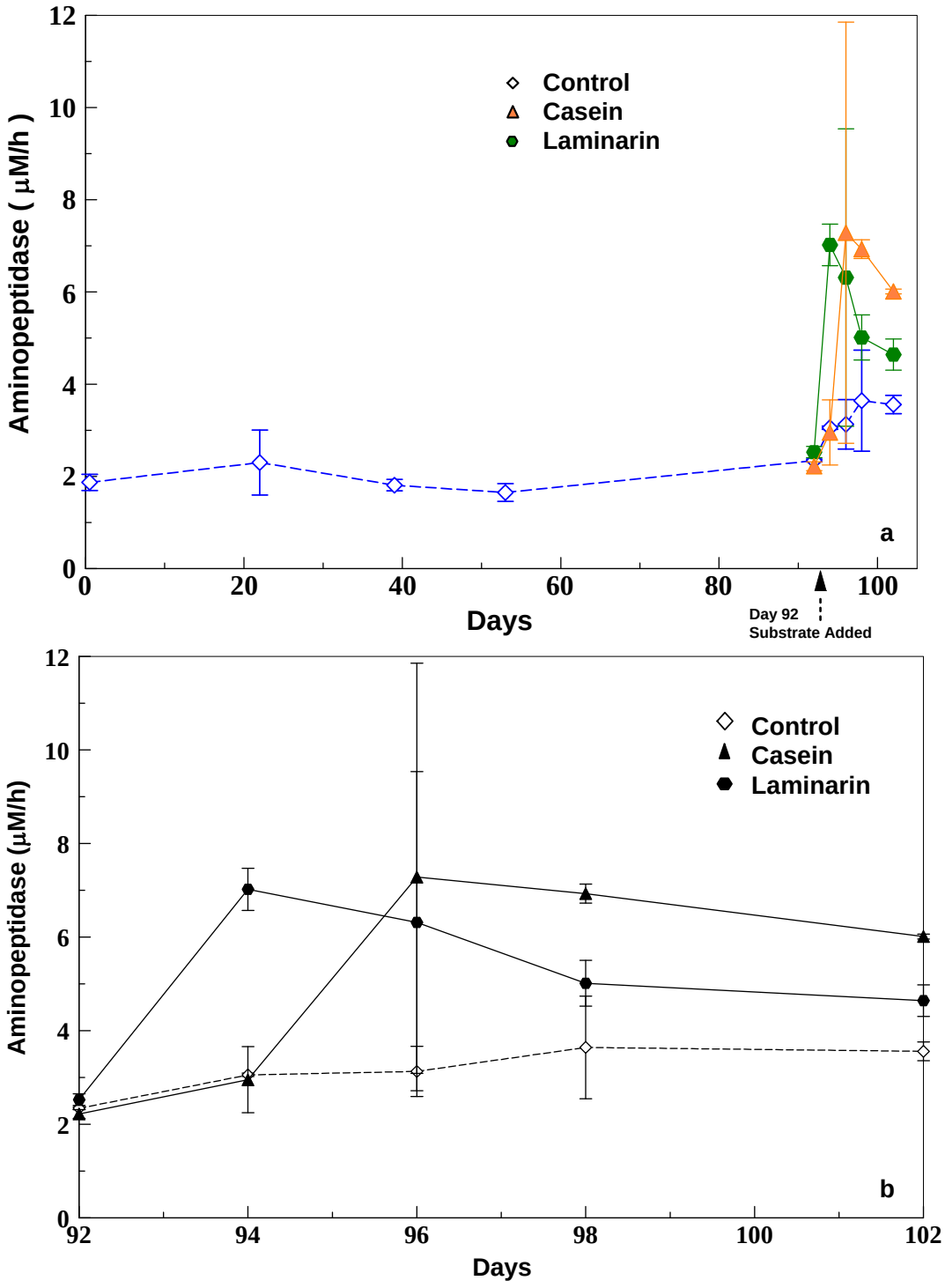


Figure 4.6: Aminopeptidase activity change after the addition of substrate at day 92 (top). Effects of laminarin and casein additions on aminopeptidase activity (bottom).

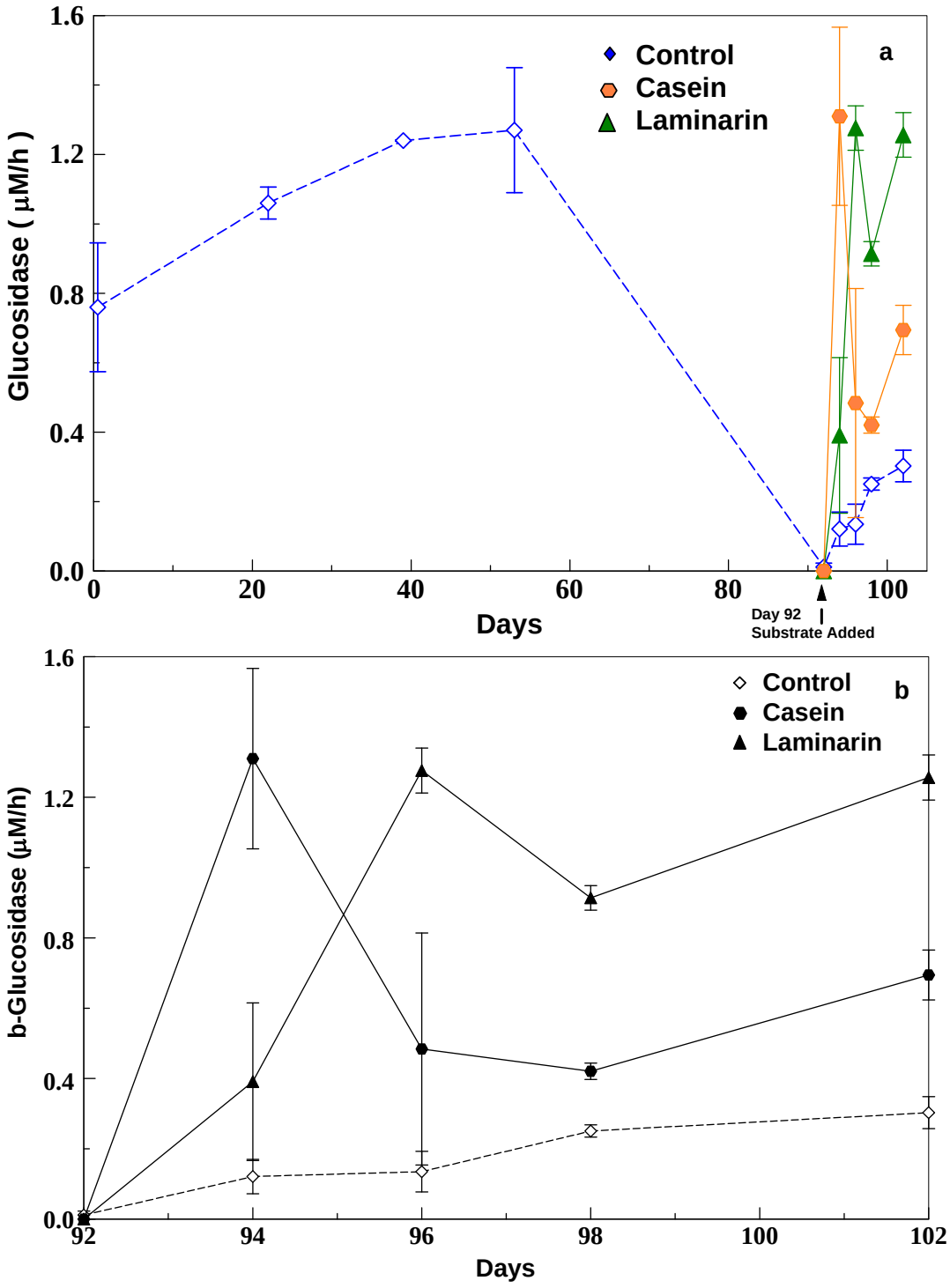


Figure 4.7: B-glucosidase activity change after the addition of substrate at day 92 (top). The effects of laminarin and casein additions on β-glucosidase activity (bottom).

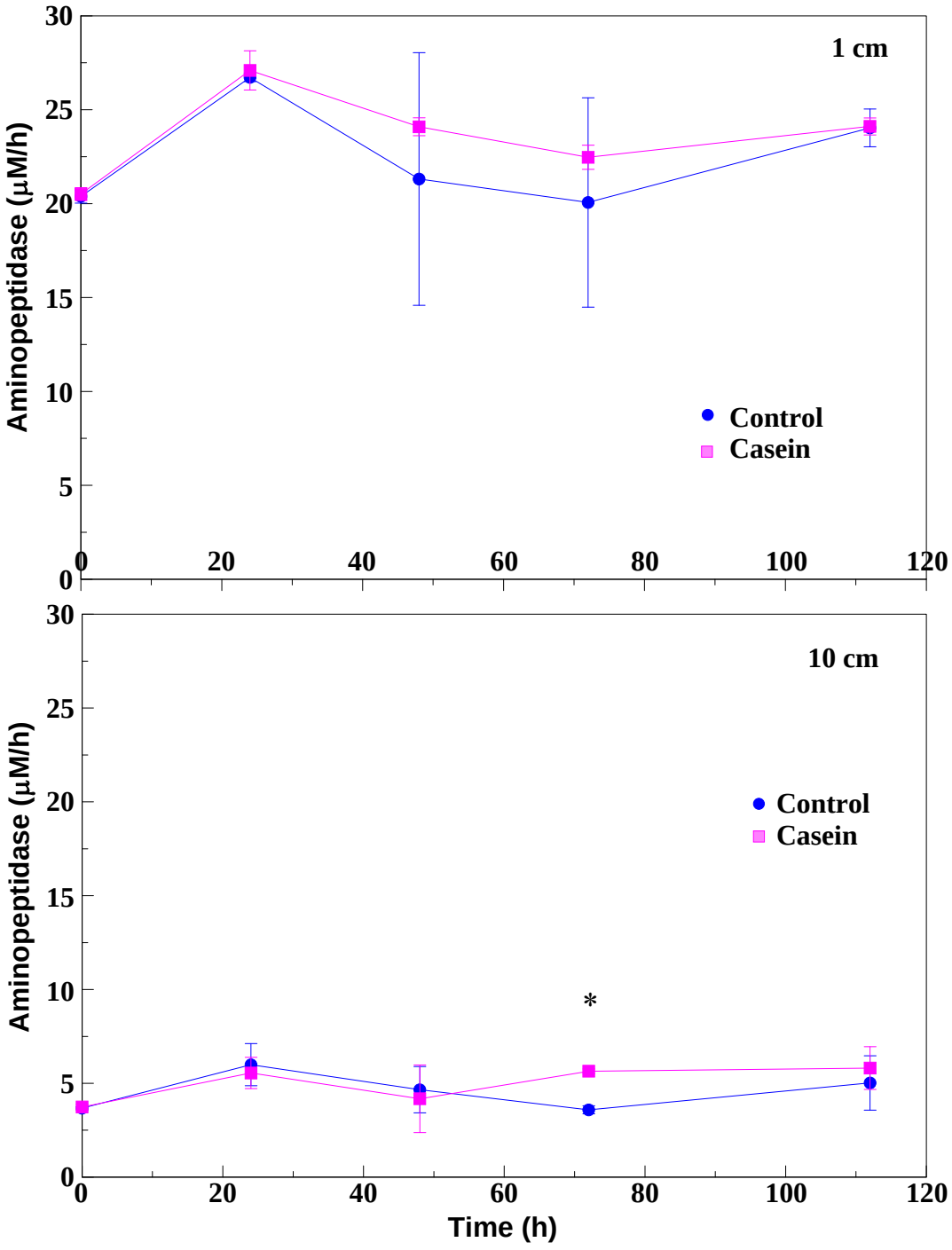


Figure 4.8: Response of aminopeptidase activity to casein addition during 112 hours in control and casein enriched Aransas Bay sediment samples of 0-1cm (top) and 9-10 cm intervals (bottom). Error bars are 95% CI, n=3. (*) = statistically different (t test, P<0.01)

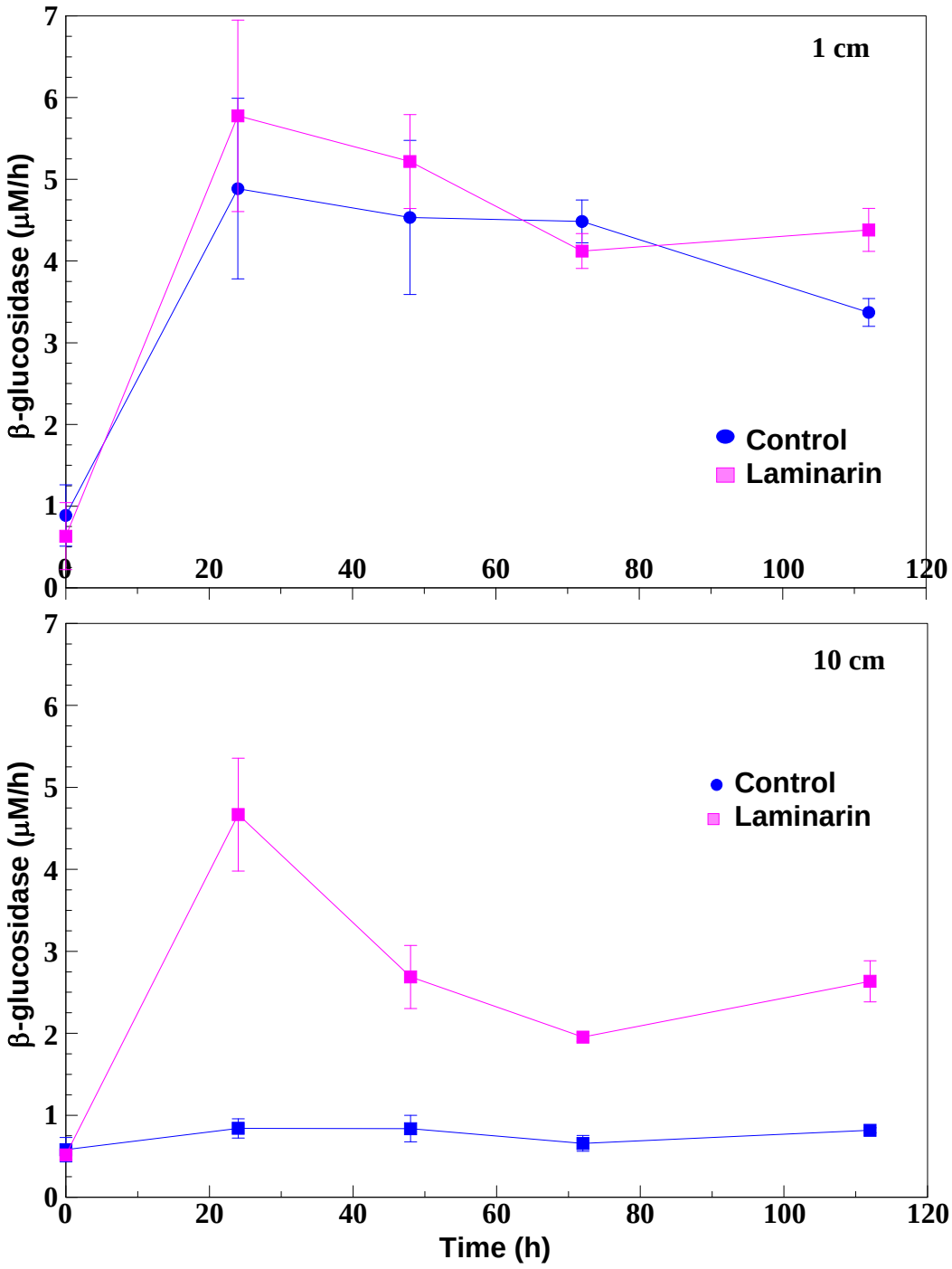


Figure 4.9: Response of β-glucosidase activity to laminarin addition during 112 hours in control and casein enriched Aransas Bay sediment samples of 0-1 cm (top) and 9-10 cm intervals (b). Error bars are 95% CI, n=3.

Table 5.1: Environmental conditions and sediment geochemical characteristics of Aransas and Copano Bays. n.d = not determined.

	Depth (cm)	**Sal** (ppt)	**Temp** (^{o}C)	**TOC** (μg/mg dws)	**TN** (μg/mg dws)	**Carbohydrates** (μg/mg dws)	**Polyphenols** (μg/mg dws)
Aransas Bay							
March 07	**0-1**	**23**	**23**	**1.24±0.01**	**0.17±0.02**	**0.36±0.04**	**0.03±0.002**
	4-5			**1.09±0.04**	**0.11±0.01**	**0.34±0.04**	**0.02±0.010**
	9-10			**0.87±0.02**	**0.08±0.02**	**0.21±0.02**	**0.02±0.002**
May 07	**0-1**	**22**	**25**	**1.87±0.02**	**0.23±0.03**	**0.88±0.04**	**0.04±0.002**
	4-5			**n.d**	**n.d**	**n.d**	**n.d**
	9-10			**1.28±0.01**	**0.17±0.01**	**0.58±0.07**	**0.06±0.011**
September 07	**0-1**	**15**	**26**	**0.80**	**0.08**	**0.56±0.11**	**0.005±0.001**
	4-5			**0.50**	**0.08**	**0.27±0.01**	**0.005±0.001**
	9-10			**0.60**	**0.05**	**0.46±0.06**	**0.006±0.001**
Copano Bay							
November 07	**0-1**	**9**	**24**	**0.89±0.02**	**0.12±0.01**	**0.72±0.13**	**0.003±0.001**
	4-5			**0.19±0.01**	**0.06±0.02**	**0.37±0.03**	**0.004±0.001**
	9-10			**0.70±0.01**	**0.12±0.01**	**0.90±0.07**	**0.004±0.001**

Table 5.2: Enzyme kinetic parameters in Aransas Bay sediment of March 2007. Error bars are 1±standard error of the estimate from the data fit to the M-M model.

Enzyme	**Depth interval (cm)**	**V_{max} (μM/h)**	**K_m (μM)**	**K_m/V_{max}** (hours)
Aminopeptidase	0-1	5.0±0.2	59±7	12
	4-5	3.4±0.5	64±24	18
	9-10	1.8±0.2	155±31	85
β-Glucosidase	0-1	2.4±0.2	57±14	24
	4-5	1.3±0.4	24±25	19
	9-10	0.4±0.1	77±37	184

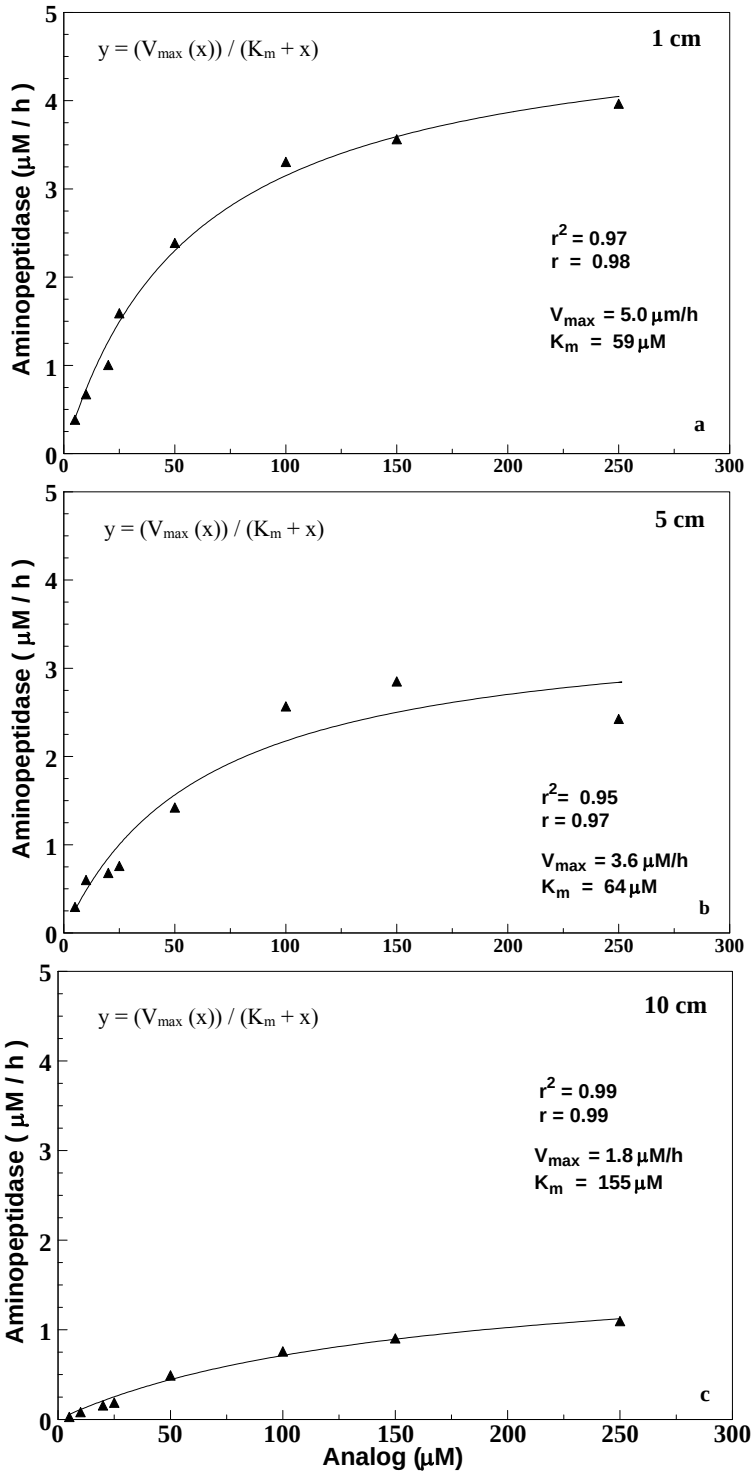


Figure 5.1: Saturation kinetic curves of aminopeptidase at 0-1, 4-5, and 9-10 cm depth intervals of Aransas Bay sediment in March 2007. Sample size, n = 1

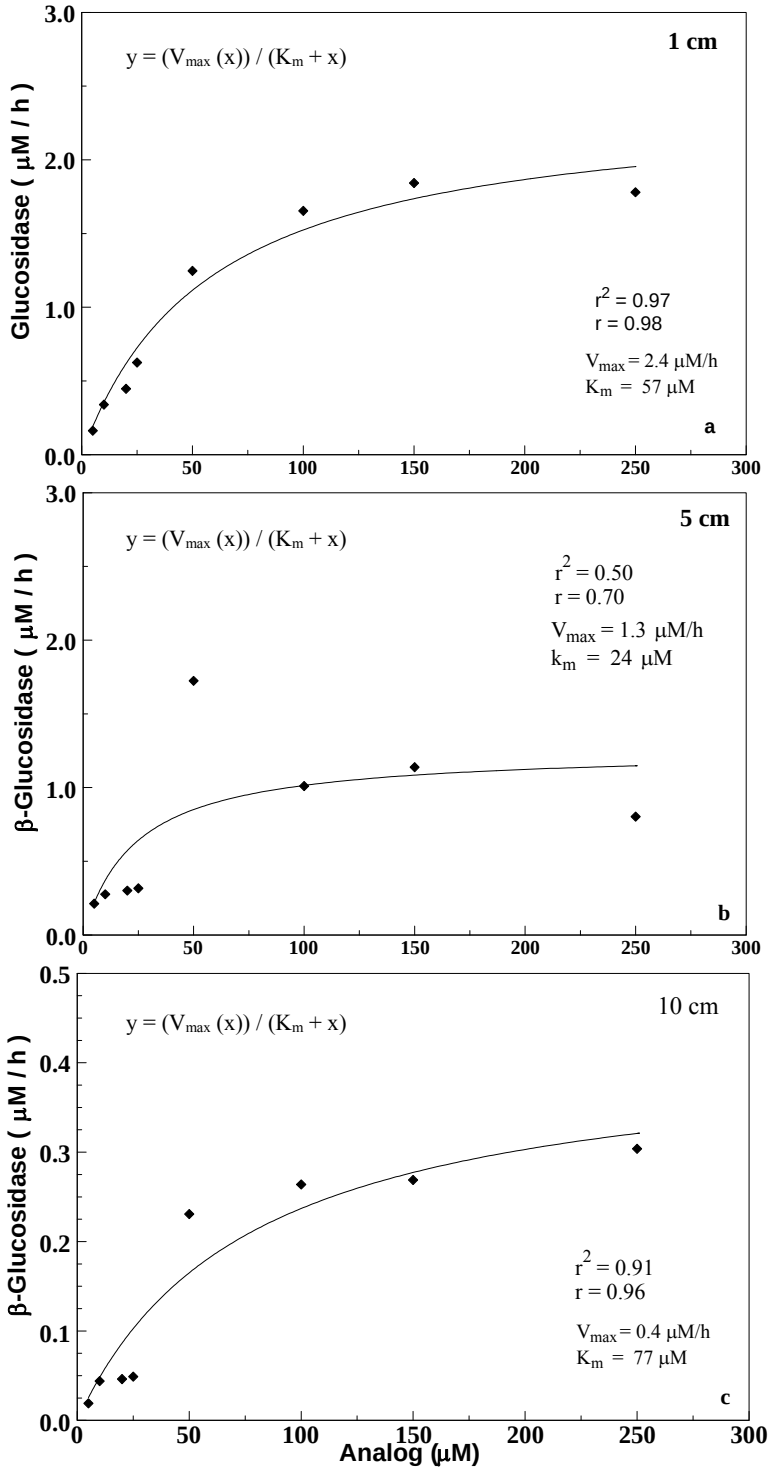


Figure 5.2: Saturation kinetic curves of β-glucosidase at 0-1, 4-5, and 9-10 cm depth intervals of Aransas Bay sediment in March 2007. Sample size, n = 1

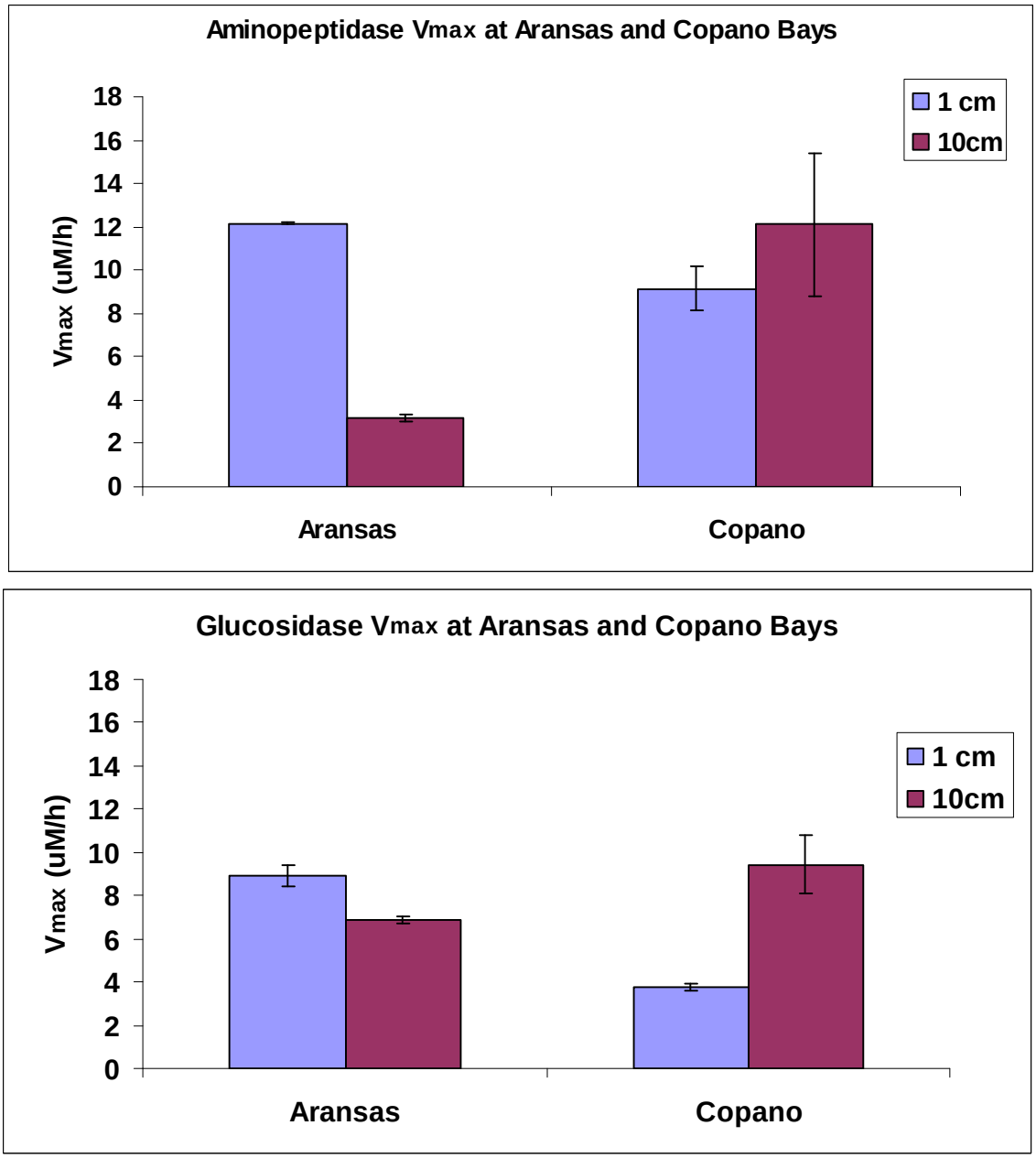


Figure 5.3: Comparison between aminopeptidase and glucosidase V_{max} parameter at 0-1 cm and 9-10 cm in Aransas bay (May 2007) and Copano Bay (November 2007). Error bars are 95% CI, n=3.

Table 5.3: Correlations between enzyme parameters and sediment TOC, TN, and carbohydrate content of Aransas Bay sediment collected in May 2007. Values are Pearson coefficients. (*****) refers to statistically insignificant ($P>0.05$).

	TOC (μg/mg)	TN (μg/mg)	Carbohydrates (μg/mg)	Polyphenols (μg/mg)
Aminopeptidase				
V_{max}	0.99	0.93	0.97	- 0.87
K_m	- 0.71*****	- 0.68*****	- 0.80*****	0.46*****
β-Glucosidase				
V_{max}	0.97	0.85	0.90	- 0.82
K_m	- 0.93	- 0.89	- 0.87	0.87

Table 5.4: Correlations between enzyme parameters and sediment TOC, TN, and carbohydrate content of Copano Bay sediments collected in September 2007. Values are Pearson coefficients. (*****) refers to differences statistically insignificant (P>0.05).

	TOC (μg/mg)	TN (μg/mg)	Carbohydrates (μg/mg)	Polyphenols (μg/mg)
Aminopeptidase				
V_{max}	- 0.64*****	- 0.05*****	0.53*****	0.30*****
K_m	- 0.81	- 0.02*****	- 0.71*****	0.51*****
β-Glucosidase				
V_{max}	- 0.96	0.16*****	0.83	0.77*****
K_m	0.90	0.19*****	- 0.72*****	- 0.87

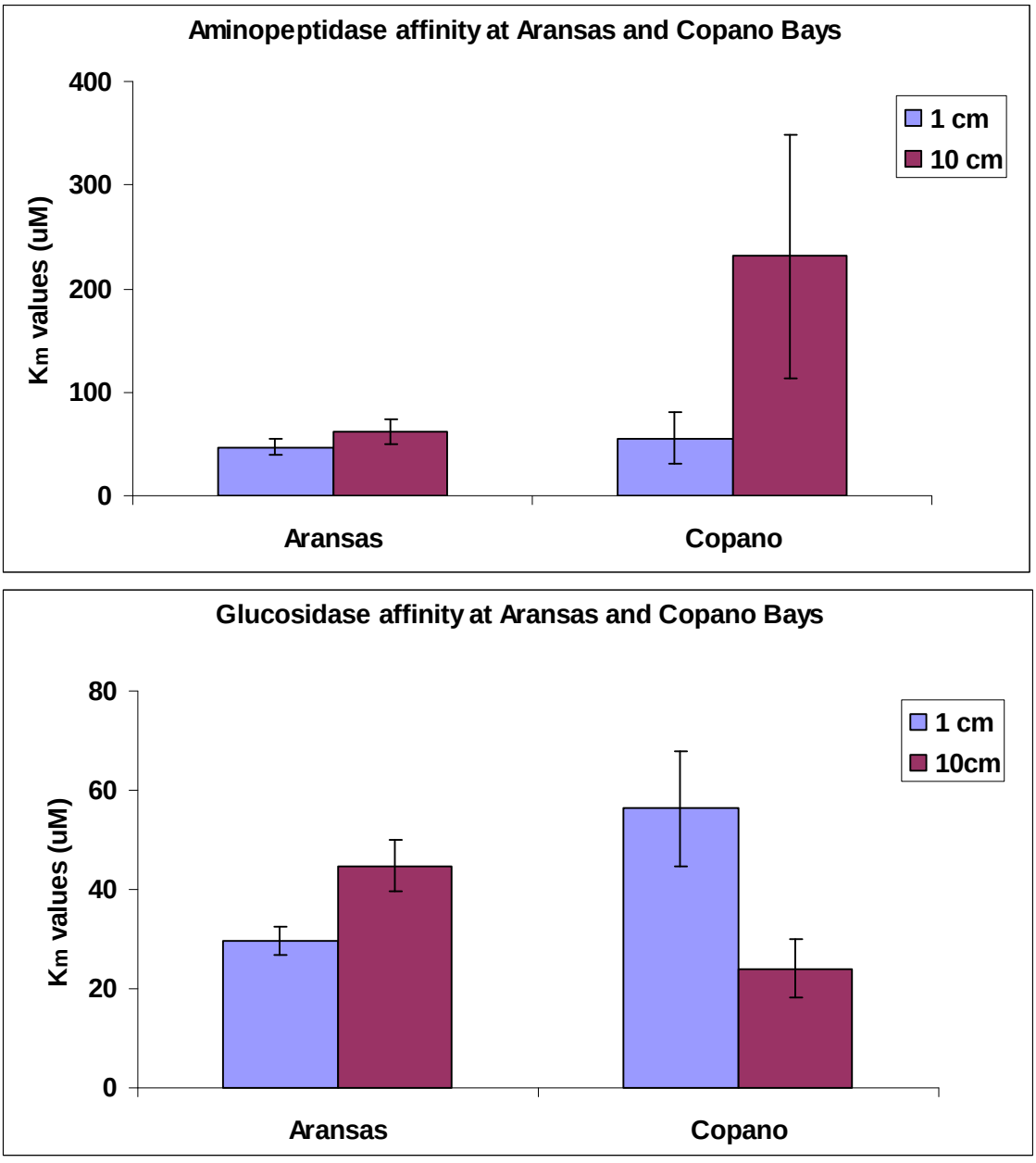


Figure 5.4: Comparison between aminopeptidase and β-glucosidase affinity (K_m) at 0-1 cm and 9-10 cm in Aransas Bay (May 2007) and Copano Bay (November 2007). Error bars are 95% CI, n=3.

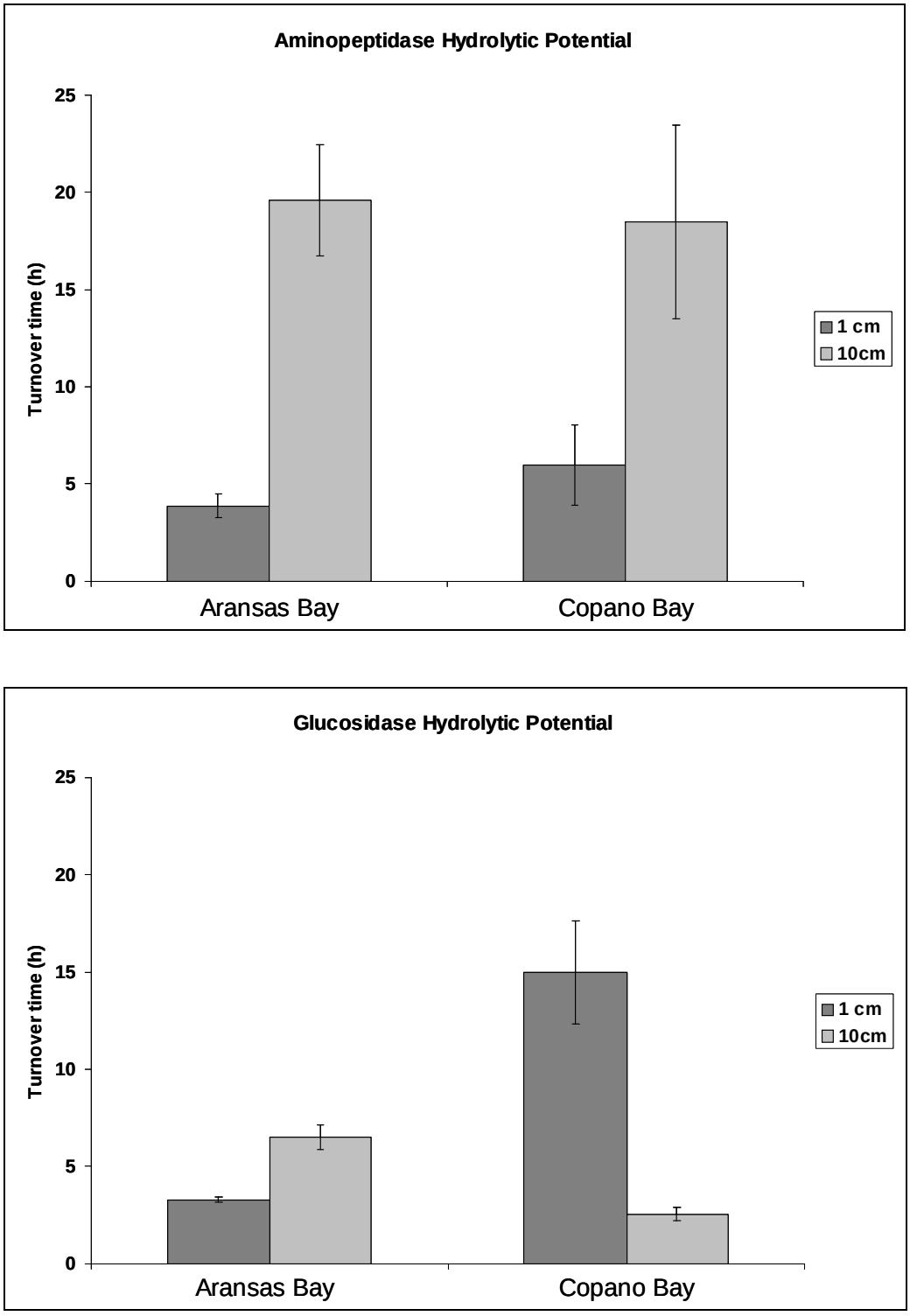


Figure 5.5: Potential turnover time of aminopeptidase and glucosidase in Aransas Bay (May 2007) and Copano Bay (November 2007). Error bars: 95% CI, n=3.

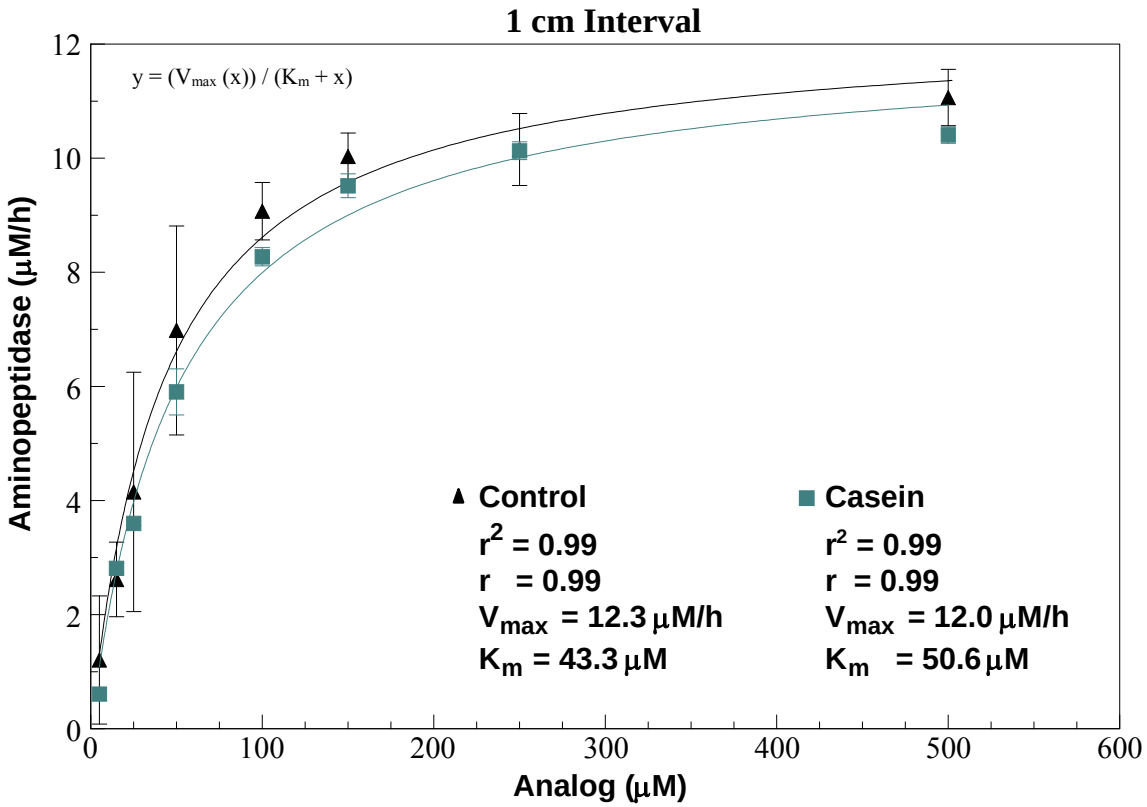


Figure 5.6: Aminopeptidase kinetic curves in control and casein-amended at the top 0-1 cm interval of Aransas Bay sediments in May 2007. Error bars are 95 % CI, n=3.

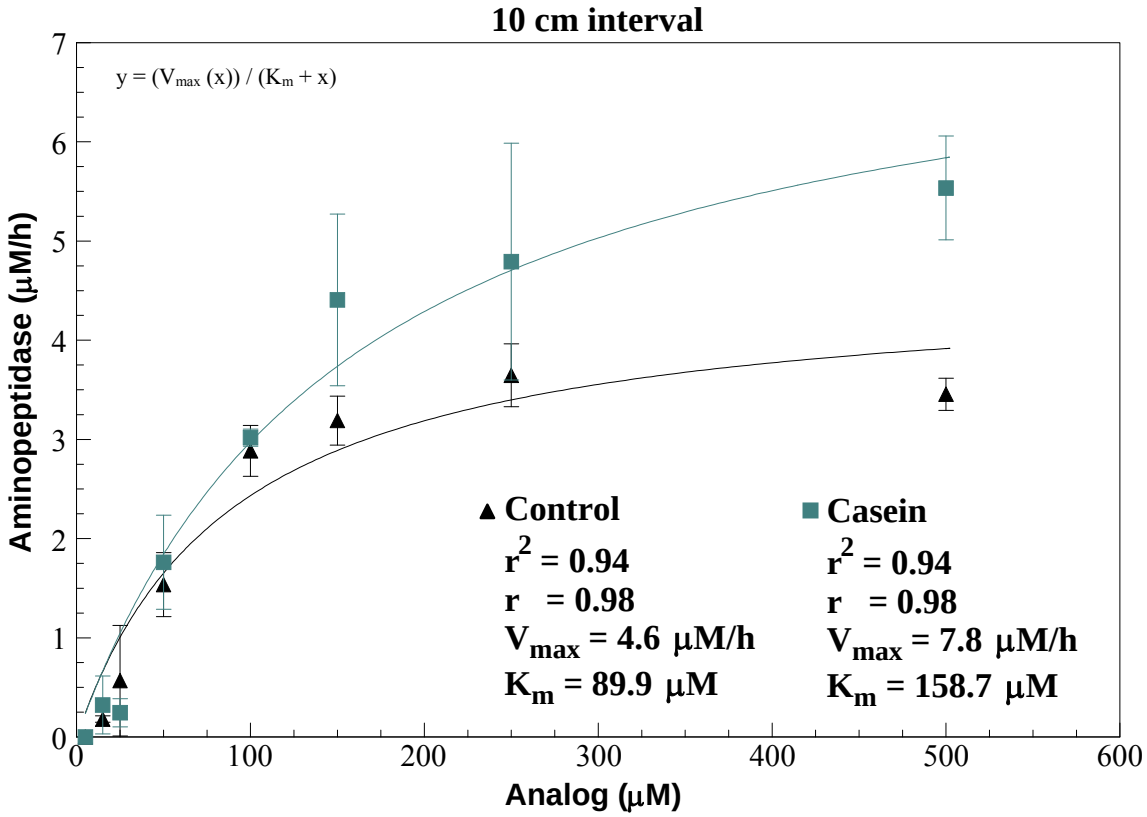


Figure 5.7: Aminopeptidase kinetic curves in control and casein-amended at 9-10 cm interval of Aransas Bay sediments in May 2007. Error bars are 95 % CI, n=3.

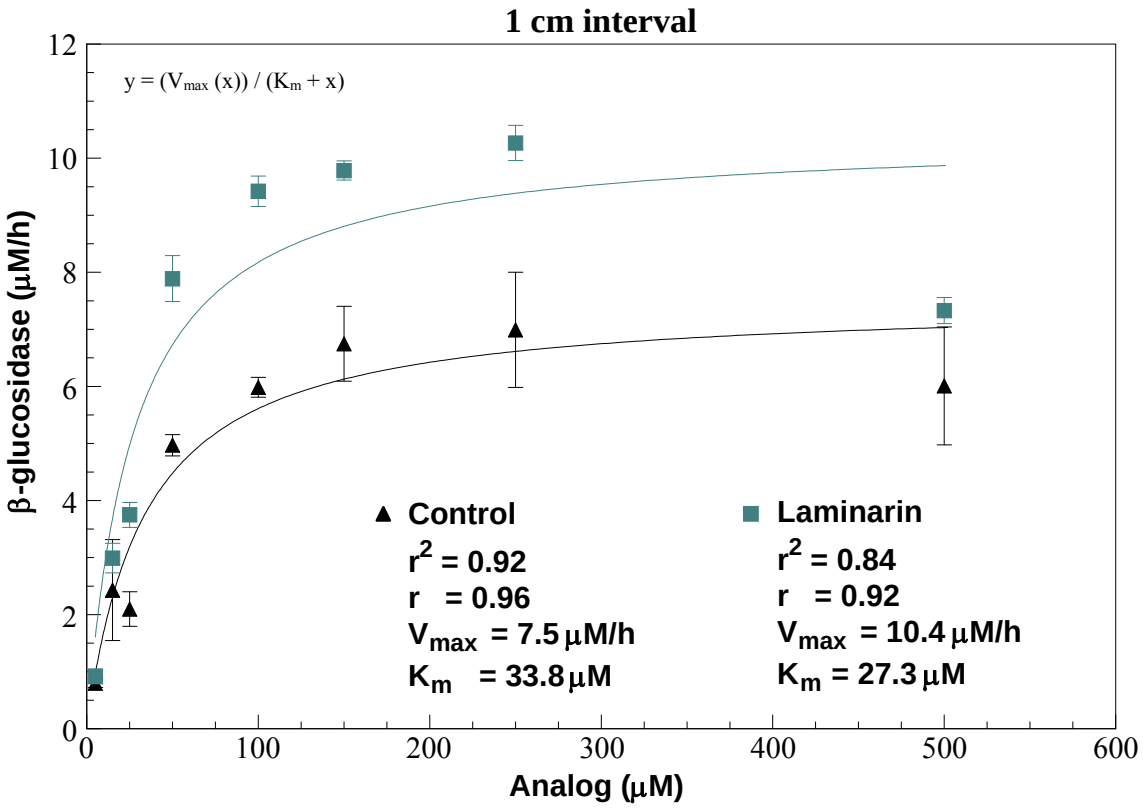


Figure 5.8: β-glucosidase kinetic curves in control and laminarin-amended at 0-1 cm in Aransas Bay sediments in May 2007. Error bars are 95 % CI, n=3.

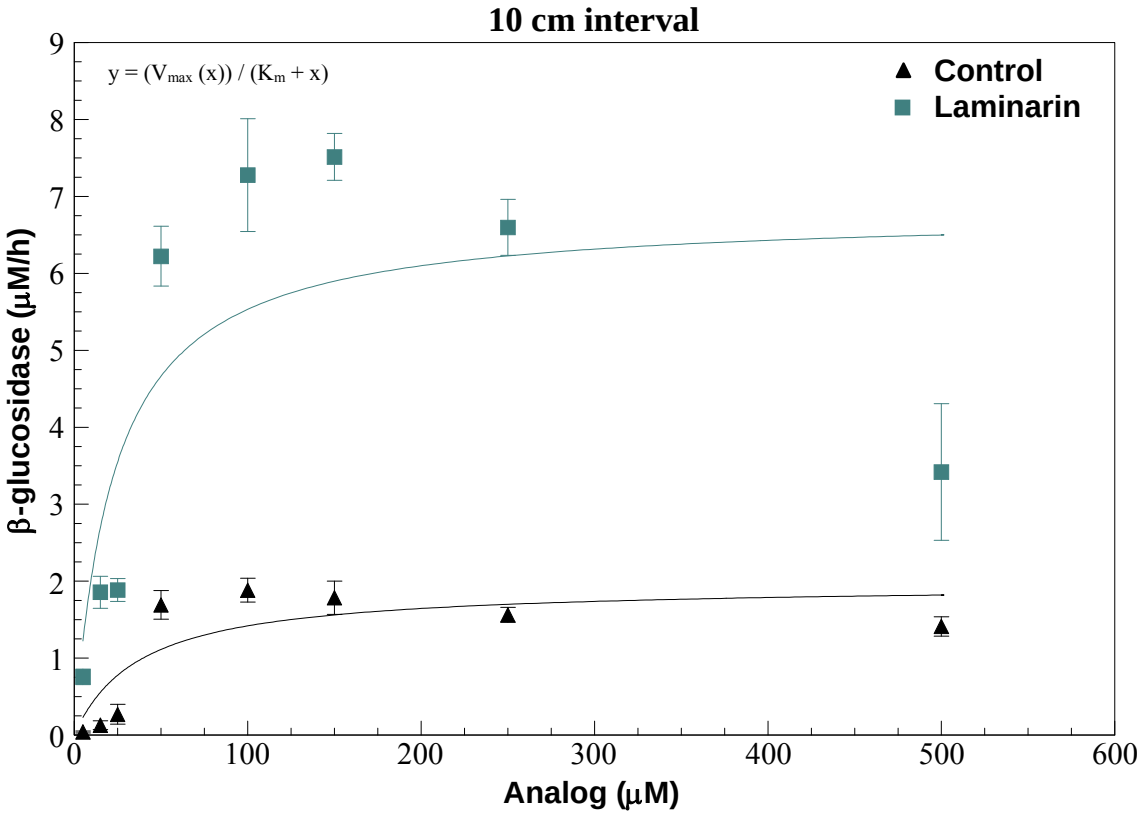


Figure 5.9: β-glucosidase kinetic curves in control and laminarin-amended 9-10 cm Aransas Bay sediments in May 2007. Error bars are 95 % confidence CI, n=3.

Control	Laminarin
$r^2 = 0.72$	$r^2 = 0.60$
$r = 0.86$	$r = 0.78$
$V_{max} = 2.0$ μM/h	$V_{max} = 6.8$ μM/h
$K_m = 37.9$ μM	$K_m = 22.8$ μM

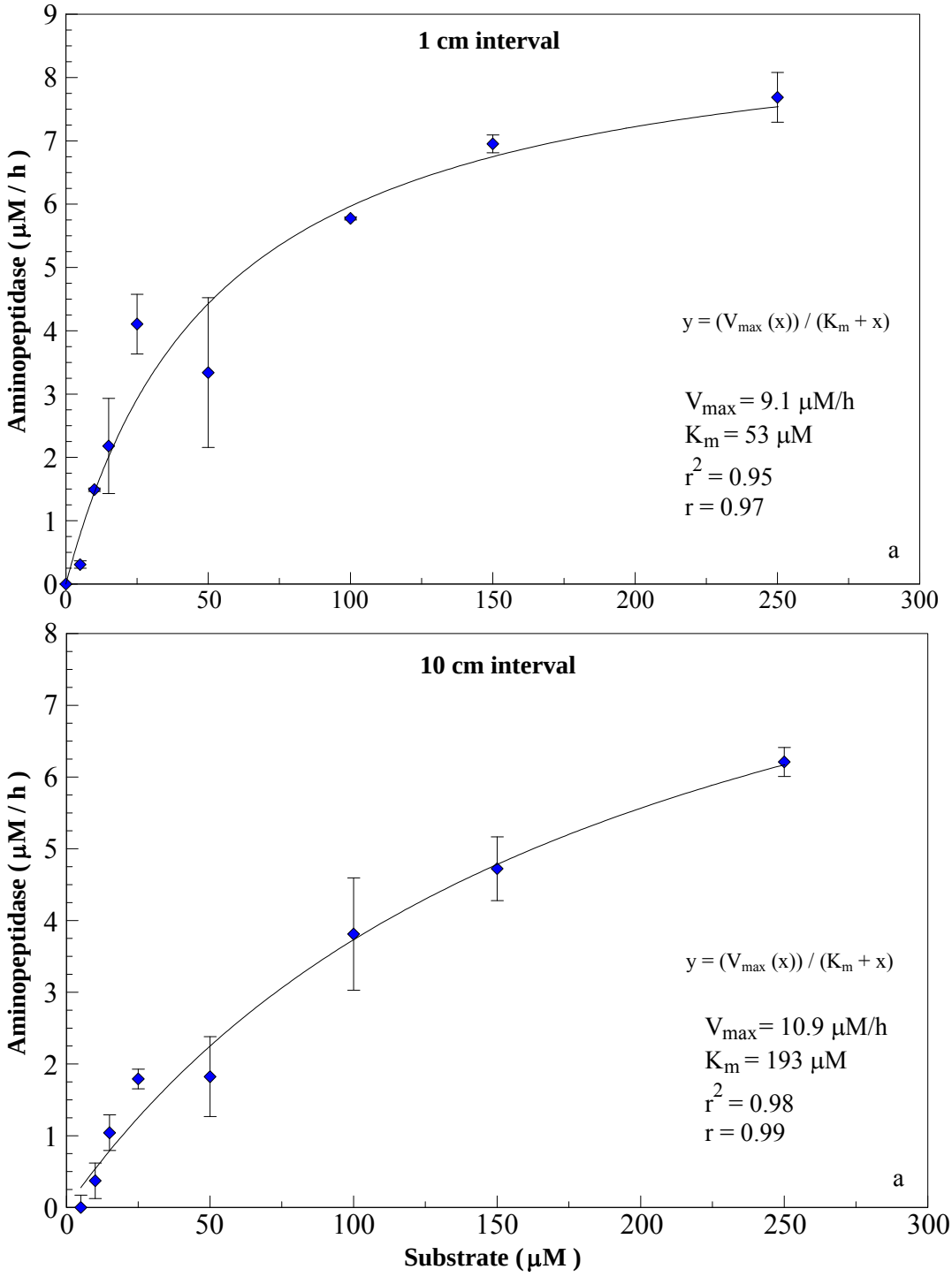


Figure 5.10: Aminopeptidase kinetic curves at 0-1 cm and 9-10 cm intervals in Copano Bay sediments, November 2007. Error bars are 95% CI, n=3.

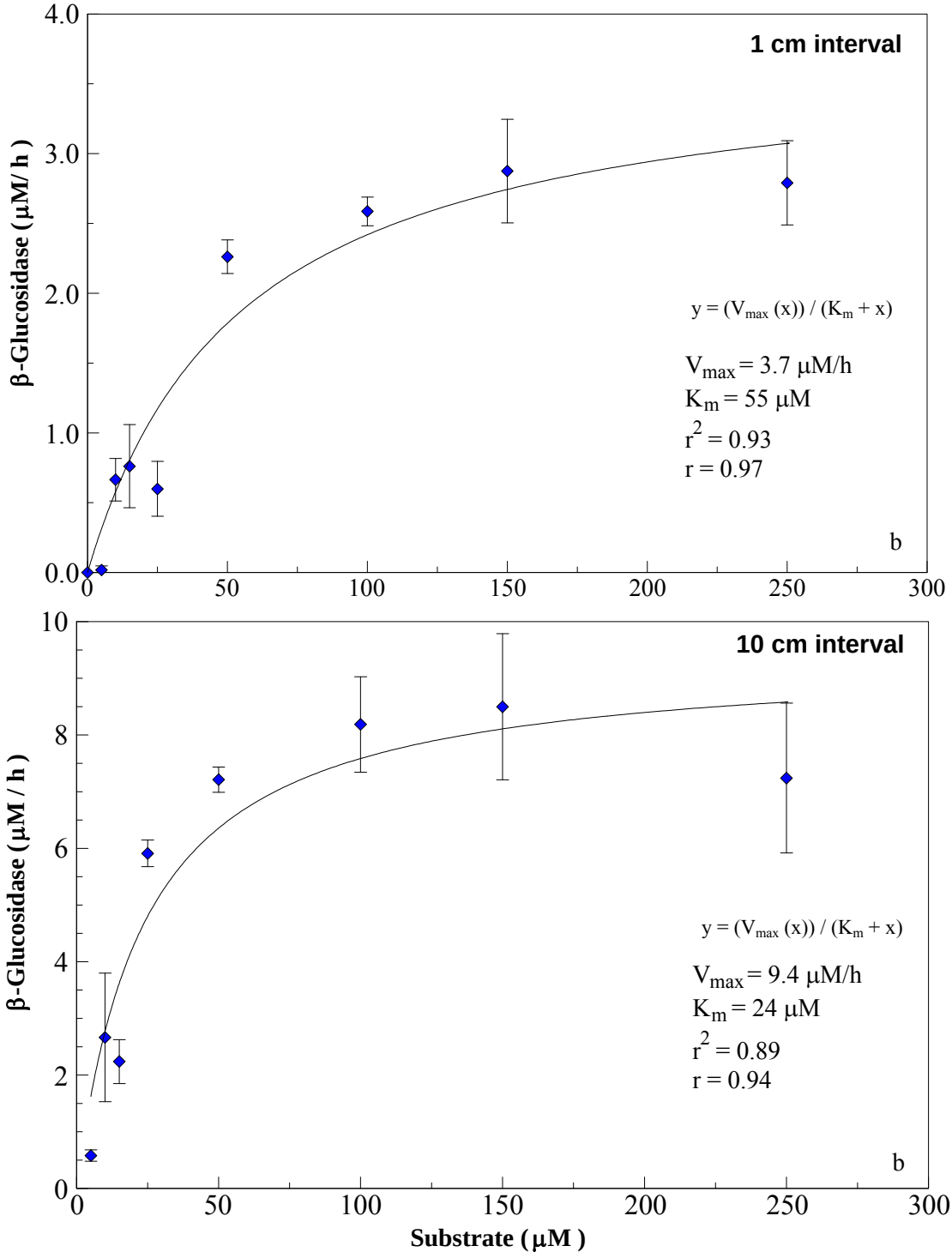


Figure 5.11: β-glucosidase kinetic curves at 0-1 cm and 9-10 cm intervals in Copano Bay sediments, November 2007. Error bars are 95% CI, n=3.

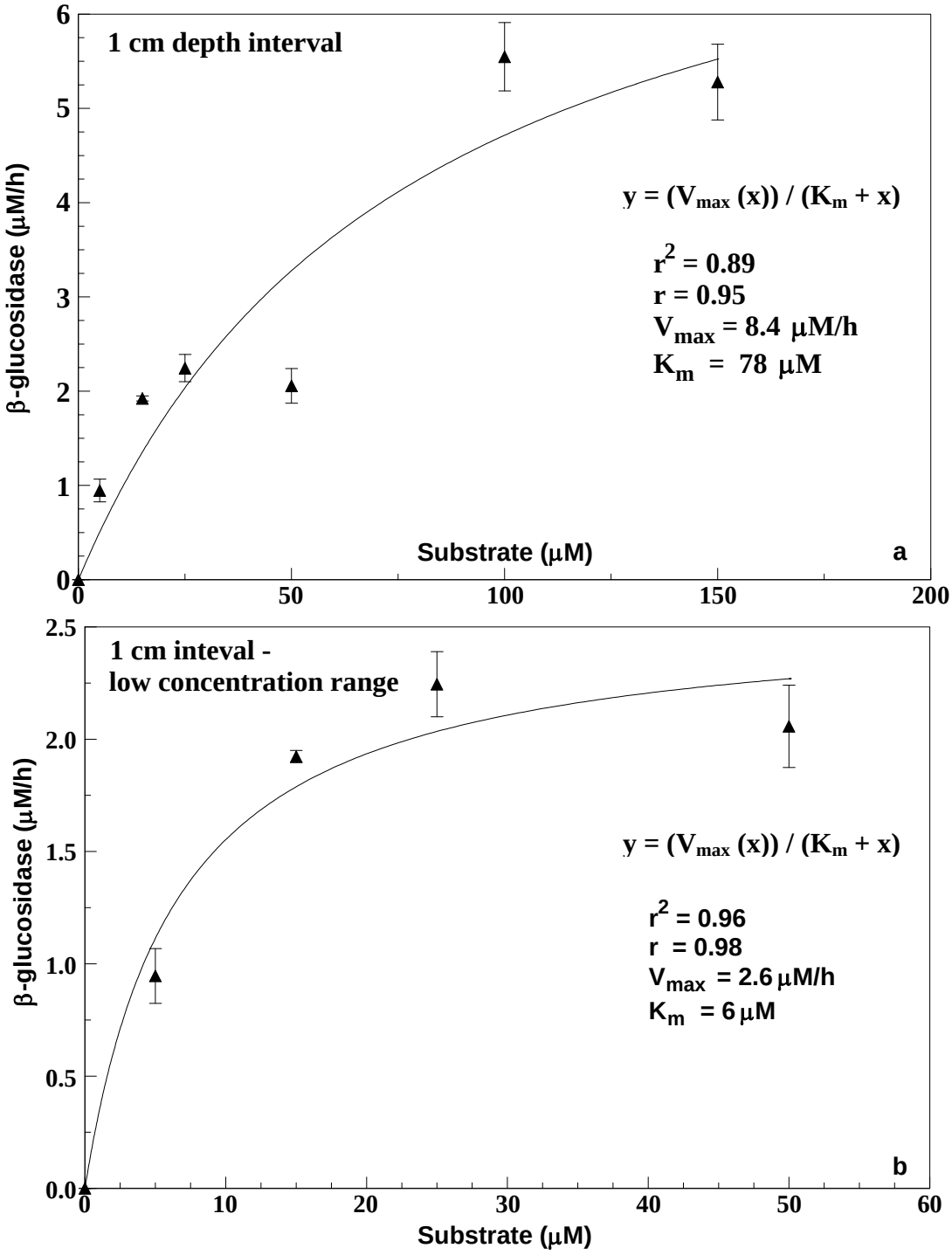


Figure 5.12: B-glucosidase biphasic saturation kinetics in Aransas Bay (June 2008) at sediment 0-1 cm interval (a) and kinetics at low range of substrate analog concentration (b). Error bars are 95% CI, n=3.

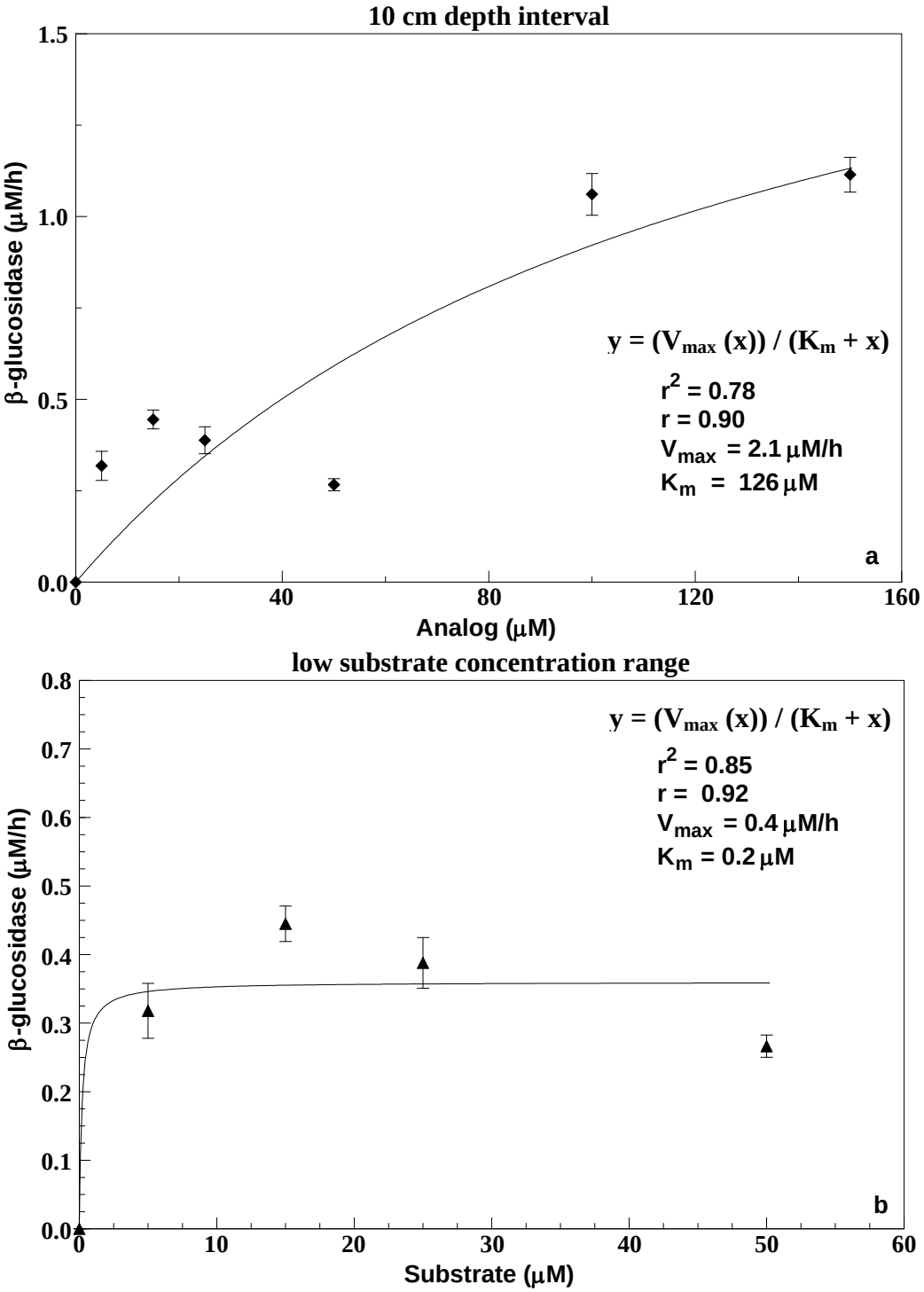


Figure 5.13: B-glucosidase biphasic saturation kinetics in Aransas Bay (June 2008) at sediment 9-10 cm interval (a) and saturation kinetics at low range of substrate analog concentration (b). Error bars are 95% CI, n=3.

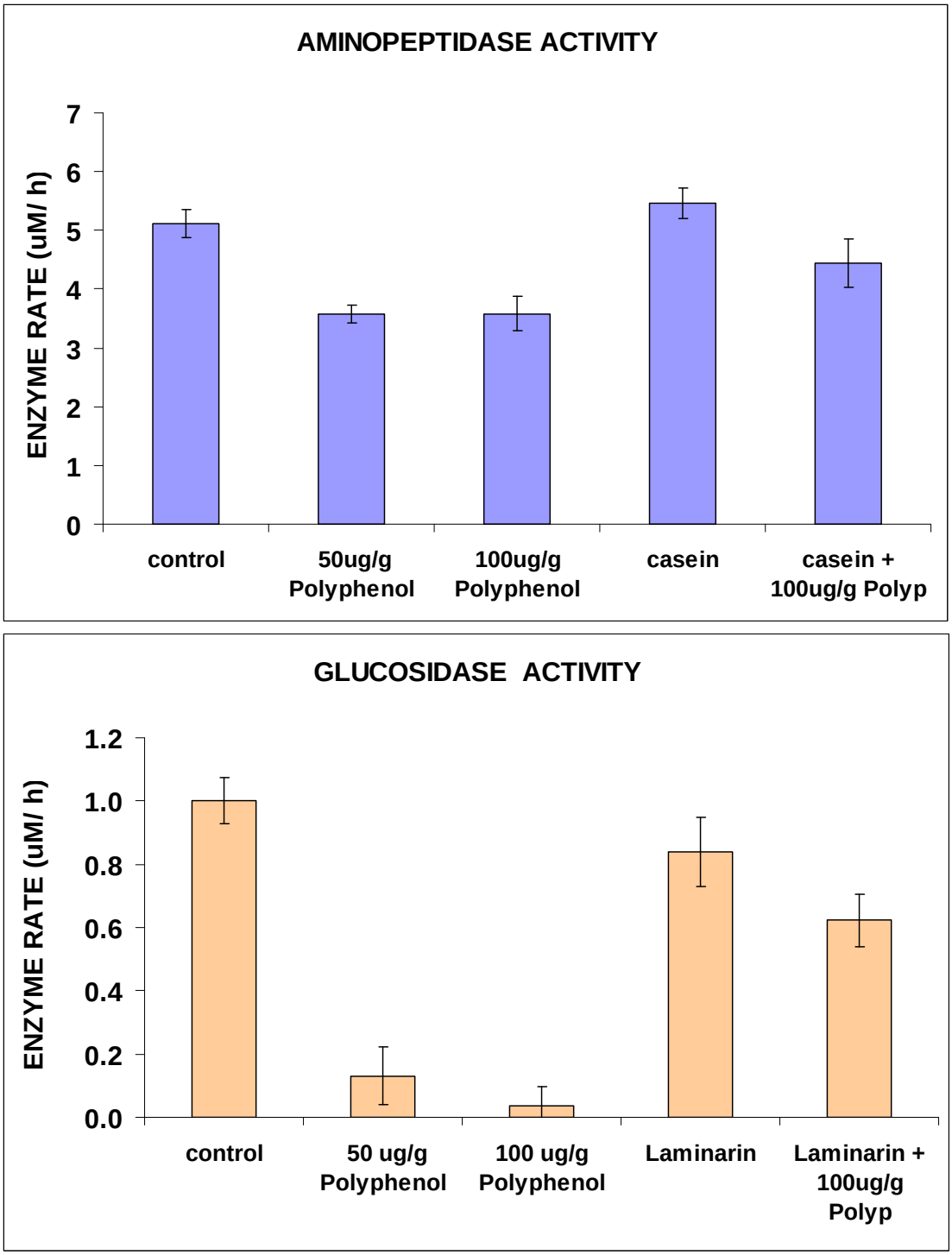


Figure 5.14: Effect of polyphenols, casein, and casein + polyphenols additions on aminopeptidase (top) and polyphenols, laminarin, and laminarin + polyphenols on glucosidase activities. Error bars are 95% CI, n=3.

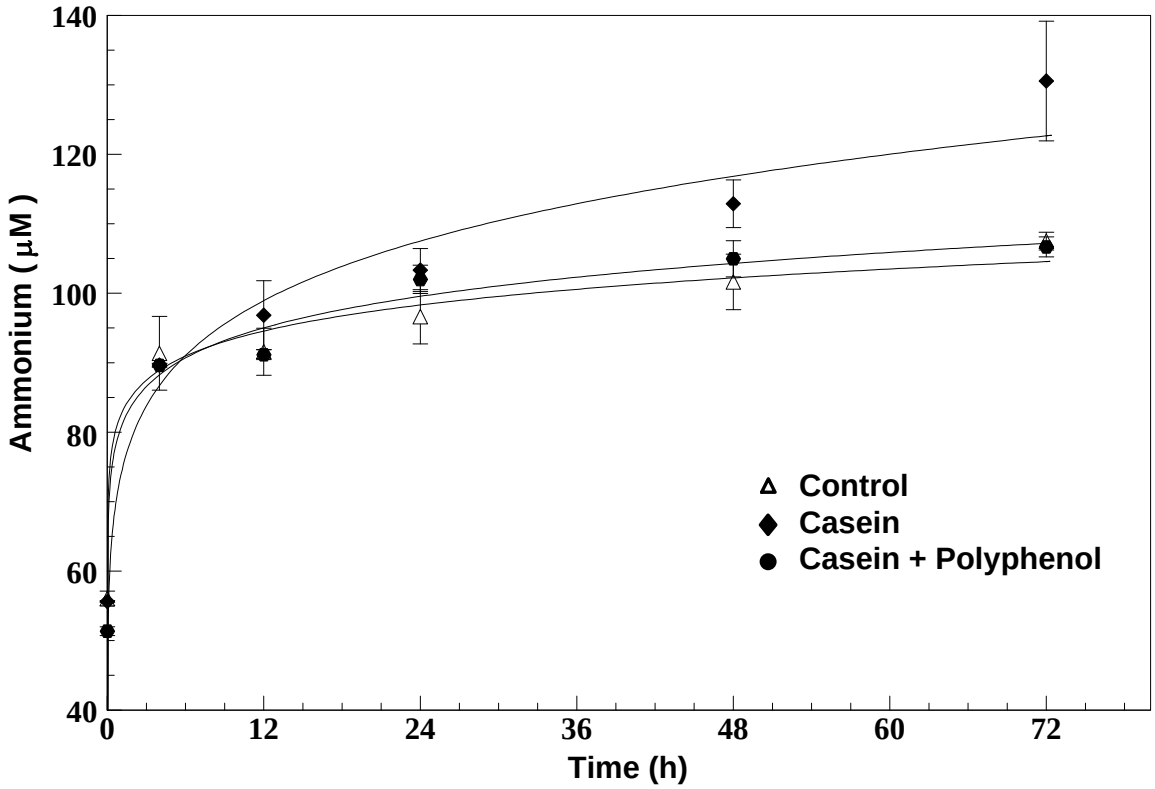


Figure 6.1: Ammonium evolution through time in Aransas Bay sediment slurries, February 2008. Error bars are 95% confidence intervals of n = 3.

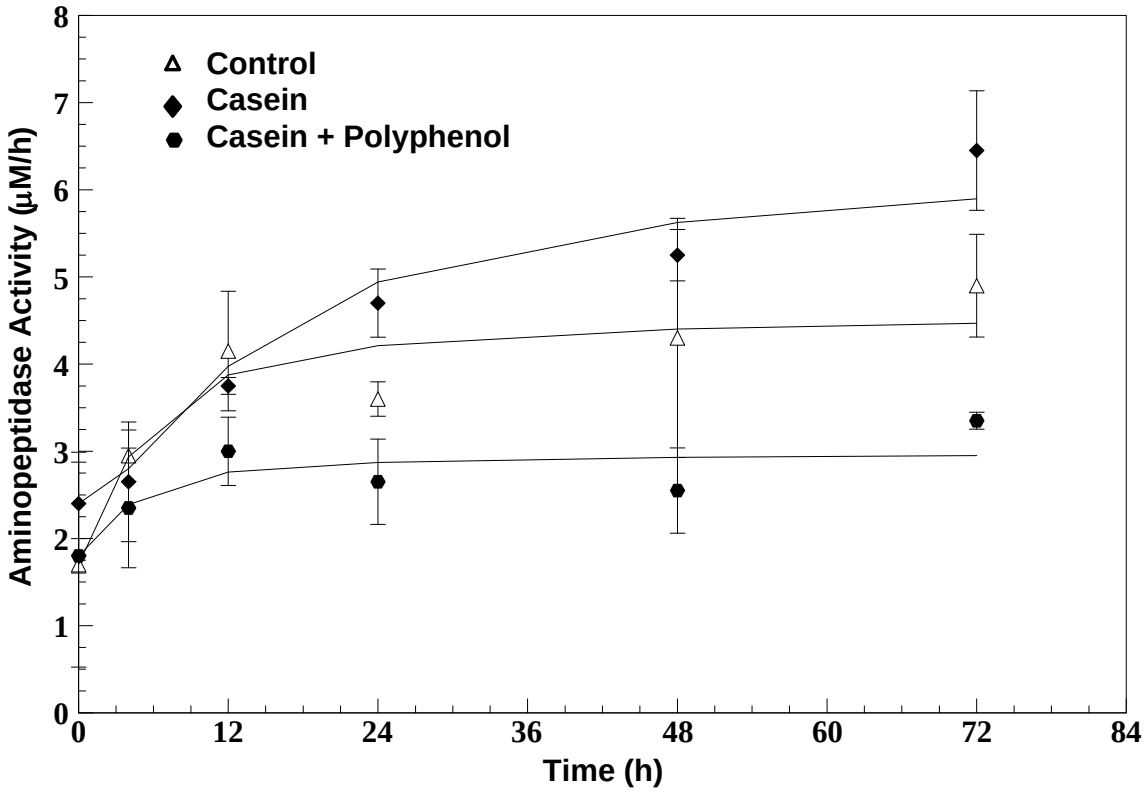


Figure 6.2: Aminopeptidase activity as function of time in Aransas Bay sediment slurries, February 2008. Error bars are 95% CI of n= 3

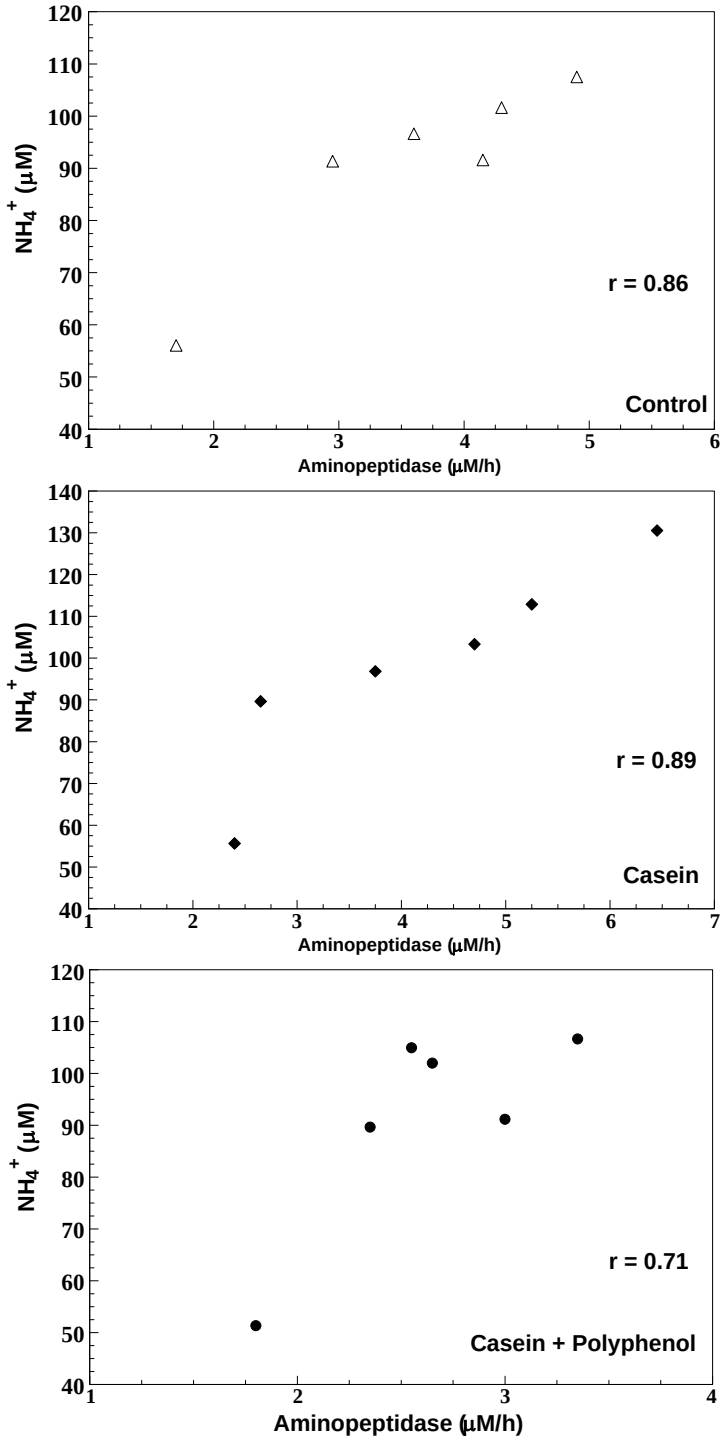


Figure 6.3: Linear correlations between ammonium concentration and aminopeptidase activity in control, casein, and casein + polyphenols treatments, February 2008. r = Pearson's correlation coefficient.

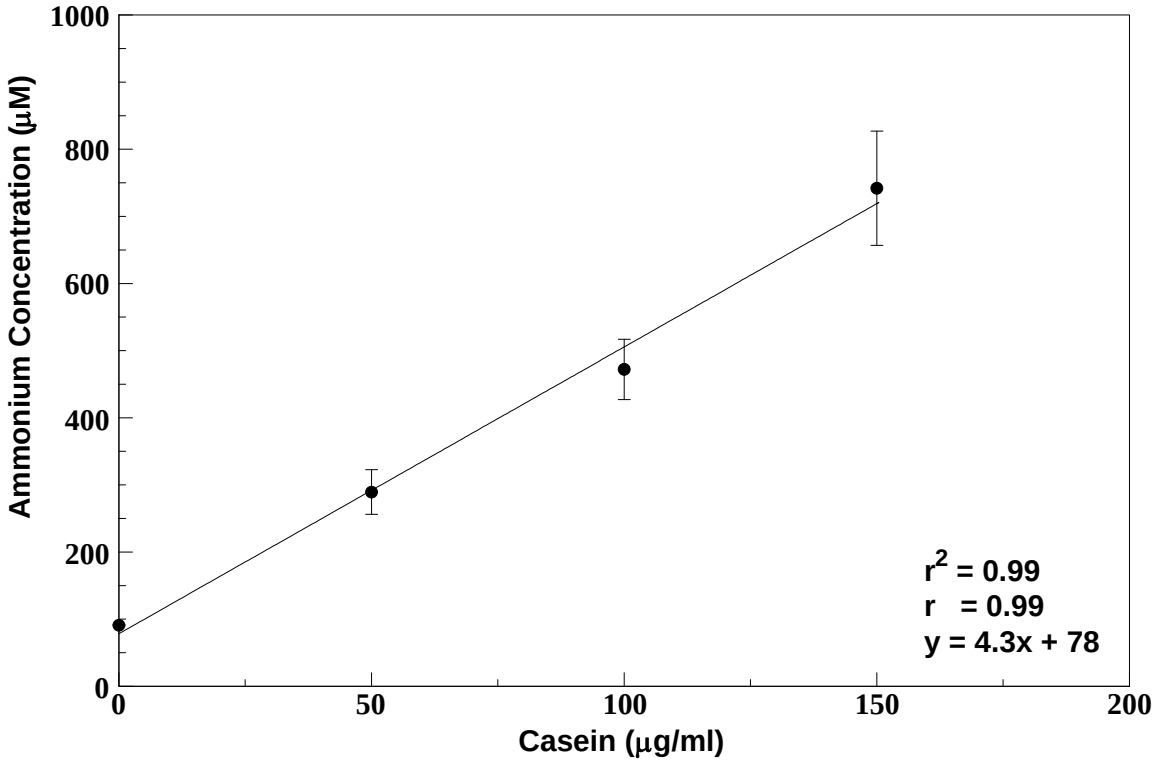


Figure 6.4: Linear relationship between organic nitrogen substrate (casein) and ammonium concentration, February 2008. Error bars are 95% CI of n= 3.

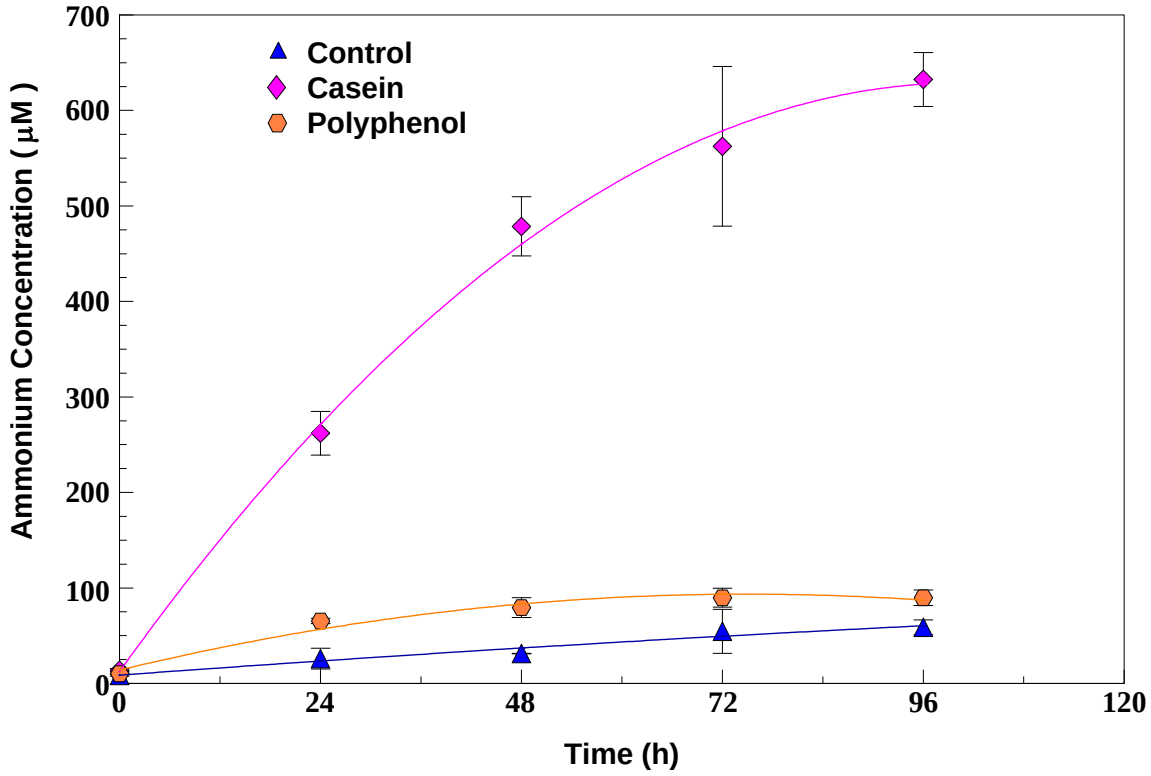


Figure 6.5: Ammonium concentration as function of time in Aransas Bay (April 2008). Error bars are 95% CI of n =3.

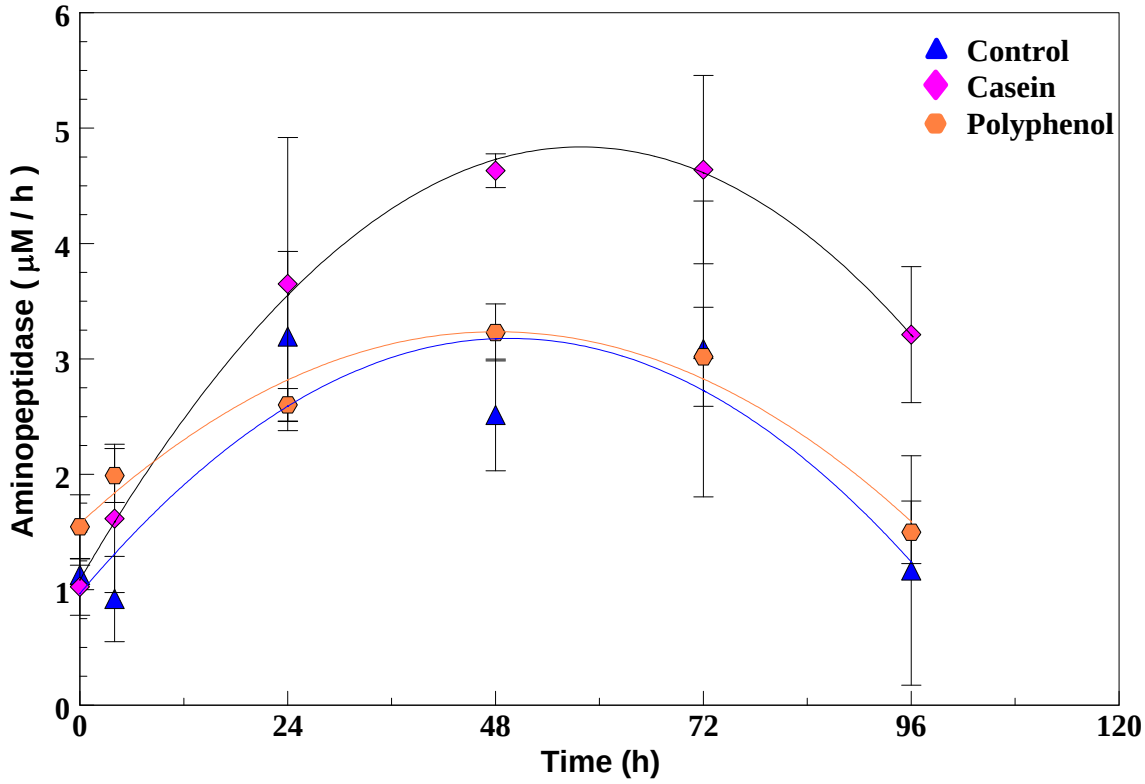


Figure 6.6: Aminopeptidase activity as function of time in Aransas Bay sediment (April 2008). Error bars are 95% CI of n =3.

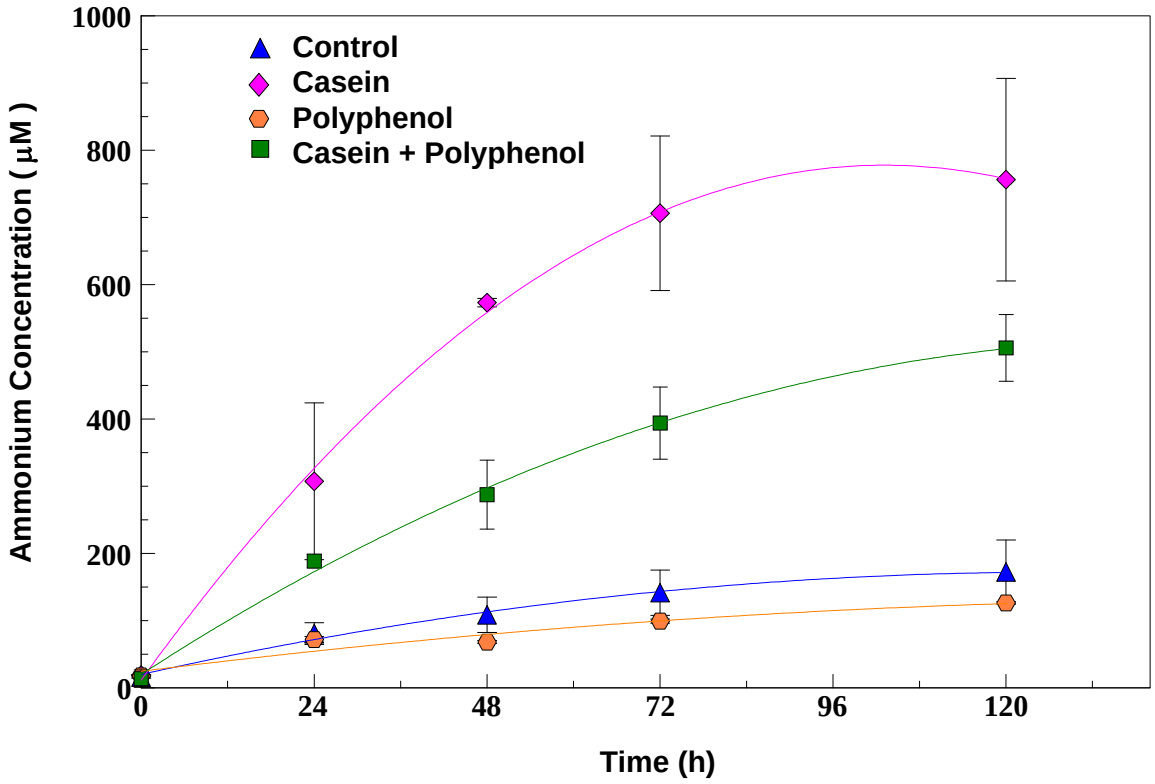


Figure 6.7: Ammonium concentration as function of time in Aransas Bay (April 2008). Error bars are 95% CI of n =3.

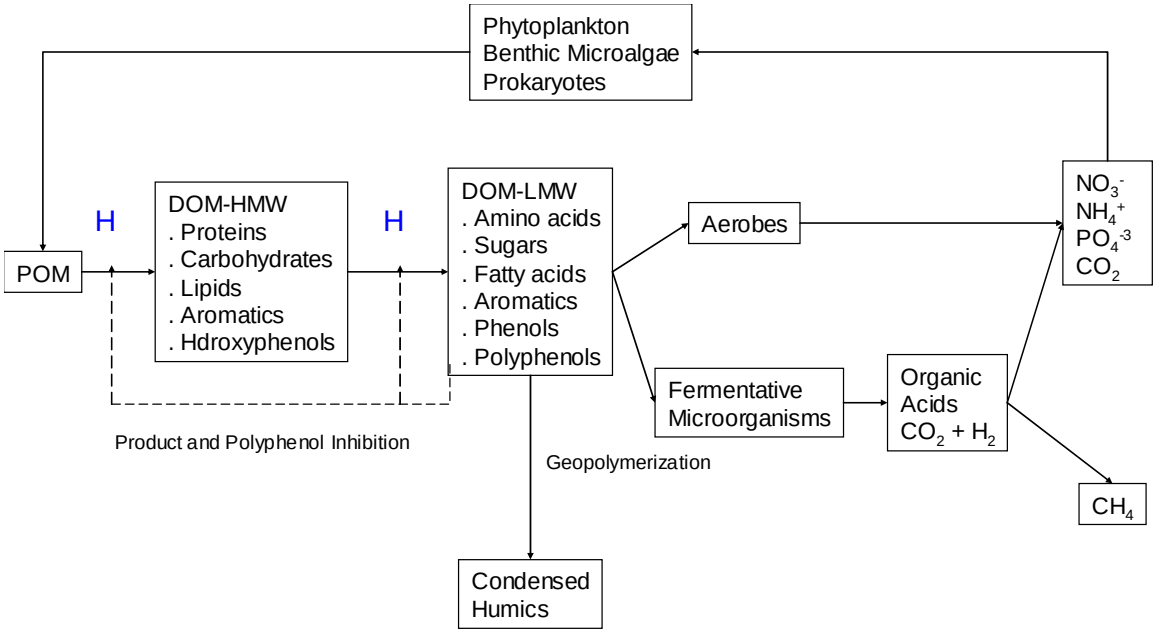


Figure 7.1: Schematic of bacterial biogeochemistry and the role of enzyme hydrolysis in the process of OM remineralization. Modified from Rashid (1995) and Fenchel and Jorgensen (1977). H: enzyme hydrolysis; DOM-HMW: dissolved organic matter high molecular weight; DOM-LMW: dissolved organic matter low molecular weight.

References

Albers, C.S., Kattnera, B.G., and Hagenb, W. (1996) The compositions of wax esters, triacylglycerols and phospholipids in Arctic and Antarctic copepods: evidence of energetic adaptations. Marine Chemistry 55(3-4):347-358.

Alperin, M.J., Martens, C.S., Albert, D.B., Suayah, I.B., Benninger, L.K., Blair, N.E., and Jahnke, R.A. (1999) Benthic fluxes and porewater concentration profiles of dissolved organic carbon in sediments from the North Carolina continental slope. Geochimica et Cosmochimica Acta 63:427-448.

Amon, R.M.W, and Benner, R, (1994) Rapid cycling of high molecular weight dissolved organic matter in the ocean. Nature 369:549-552.

Amon, R.M.W. and Benner, R. (1996) Bacterial utilization of different size classes of dissolved organic matter. Limnology and Oceanography 41:41–51.

Andrade, M.M.C., Pereira, N., and Antranikian, G. (1999) Extremely thermorphilic microorganisms and their polymer-hydrolytic enzymes. Revista de Microbiologia 30:287-298.

Atkinson, M.J and Smith, S.V. (1983) C:N:P Ratios of benthic marine plants. Limnology and Oceanography 28(3):568-574.

Armstrong, N.E. (1982) Responses of Texas estuaries to freshwater inflows. In: Estuarine Comparisons. Kennedy, V.S. (ed). Academic Press, New York, pp. 103-120.

Armstrong, N.E. (1987) The ecology of open-bay bottoms of Texas: a community profile. Biological Report 85(7.12). US Department of the interior fish and wildlife service. Research and development of national wetlands research center. Washington, DC. pp 1-12

Arnosti, C. (2004) Speed bumps in the carbon cycle: substrate structural effects on carbon cycling. Marine Chemistry 92:263-273.

Arnosti, C. and Holmer, M. (2003) Carbon cycling in a continental margin sediment: contrasts between organic matter characteristics and remineralization rates and pathways. Estuarine, Coastal and Shelf Science 58:197-208.

Arnosti, C. and Jorsensen, B.B. (2003) High activity and low temperature optima of extracellular enzymes in Arctic sediment: implications for carbon cycling by heterotrophic microbial communities. Marine Ecology Progress Series 249:15-24

Arnosti, C. and Repeta, D.J. (1994). Extracellular enzyme activity in anaerobic bacterial cultures: Evidence of pullulanase activity among mesophilic marine bacteria. Applied and Environmental Microbiology 60(3):840-846.

Arrieta, J.M. and Herndl, G. (2001) Assessing the diversity of marine bacterial b-glucosidases by capillary electrophoresis zymography. Applied and Environmental Microbiology 67(10):4896-4900

Azam, F., Fenchel, T., Field, J.G. Meyer-Reil, R.A. and Thingstad, F. (1983) The ecological role of water column microbes in the sea. Marine Ecology Progress Series 10:257-263.

Bacic, A., Harris, P.J., and Stone, B.A. (1988) Structure and function of plant cell walls. In: The biochemistry of plants, vol. 14. Carbohydrates, Preiss, ed. Academic Press. New York, p. 297-371

Berman, T. and Bronk, D.A. (2003) Dissolved organic nitrogen: a dynamic participant in aquatic systems. Aquatic Microbial Ecology 31:279-301

Boavida, M.J, and Wetzel, R.G. (1998) Inhibition of phosphatase activity by dissolved humic substances and hydrolytic reactivation by natural ultraviolet light. Freshwater Biology 40(2):285-293

Blackburn, T.H. (1983) The microbial nitrogen cycle. In: Microbial geochemistry. Krumbein W.E, (ed.) Blackwell Scientific Publications, Oxford, pp. 63- 89.

Blackburn, T.H. and Henriksen, K. (1983) Nitrogen cycling in differ ent types of sediment from Danish waters. Limnology and Oceanography 28:477-493.

Blackburn, T.H. (1991) Accumulation and regeneration: Processes at the benthic boundary layer. In: R.F.C. Mantoura, J.-M. Martin and R. Wollast, Editors, Ocean Margin Processes in Global Change, Wiley, New York, N.Y pp. 181–195.

Blackburn, T.H. (1991) Accumulation and regeneration: processes at the benthic boundary layer, In: ocean margin processes in global change. Mantoura, R.F.C., Martin, J.M., and Wollast, R. (eds.), pp.181-195.

Bell, J.E. and Bell, E.T. (1998) Proteins and enzymes. Prentice-Hall, Englewood Cliffs, NJ, 499pp.

Bender, M.L., Bergeron, R.J., and Komyama, K. (1984) The bioorganic chemistry of enzymatic catalysis. Wiley-Interscience, New York, 312pp.

Benner, R., Kaiser, K. (2003) Abundance of amino sugars and peptidoglycan in marine particulate and dissolved organic matter. Limnology and Oceanography 48:118-128

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

Bhatia, Y., Mishra, S., and Bissara, V.S. (2002) Microbial b-glucosidases: cloning, properties, and applications. Critical Reviews in Biotechnology 22(4):375-407

Bianchi, T.S., Dribb, J.E., and Findlay, S. (1993) Early diagenesis of plant pigments in Hudson River sediments. Estuarine and Coastal Shelf Science 36:517-527

Bianchi, T.S., Pennock, J.R., and Twiley, R.R. (1999) Biogeochemistry of the Gulf of Mexico estuaries. Bianchi, T.S., Pennock, J.R., Twiley, R.R. (eds.). John Wiley and Sons, Inc. New York, NY, pp. 29-81

Bianchi, T.S., Wysocki, L.A., Stewart, M., Filley, T.R., and Mckee, B.A. (2007) Temporal variability in terrestrially-derived sources of particulate organic carbon in the lower Mississippi River and its tributaries. Geochimica et Cosmochimica Acta 71(18):4425-4437

Boer, S.I., Arnosti, C., VanBeusekom, J.E.E., and Boetius, A. (2008) Temporal variations in microbial activities and carbon turnover in subtidal sandy sediments. Biogeosciences Discussions 5:4271-4313

Boetius, A. and Lotche, K. (1994) Regulation of microbial enzymatic degradation of organic matter in deep-sea sediments. Marine Ecology Progress Series 104:299-307.

Boetius, A. and Lochte, K. (1996) High proteolytic activities of deep-sea bacteria from oligotrophic polar sediments. Aquatic Microbial Ecology 48:269-276.

Boschker, H.T.S. and Cappenberg, T.E. (1998) Patterns of extracellular enzyme activities in litoral sediments of Lake Gooimeer, The Netherlands. FEMS Microbial Ecology 25:79-85.

Boudreau, B. (1997) Diagenetic models and their implementation: modeling transport and reactions in aquatic environments. Springer-Verlag, Berlin, Heidelberg, NY, 414pp.

Boynton, W.R. and Kemp, W.M. (1985) Nutrient regeneration oxygen consumption by sediments along an estuarine salinity gradient. Marine Ecology Progress Series 23:45-55.

Box, J.D. (1983) Investigation of the Folin-Ciocalteau phenol reagent for the determination of the polyphenolic substances in natural waters. Water Research 17:249-261.

Bruchert, V. and Arnosti, C. (2003) Anaerobic carbon transformation: experimental studies with flow-through cells. Marine Chemistry 80:171-183.

Burdige, D.J. and Gardner, K.G. (1998) Molecular weight distribution of dissolved organic carbon in marine sediment pore waters. Marine Chemistry 62:45-64.

Burdige, D.J. Berelson, W.M., Coale, K.H., McManus, J., and Johnson, K.S. (1999) Fluxes of dissolved organic carbon from California continental margin sediments. Geochimica et Cosmochimica Acta 63:1507-1515.

Burdige, D.J., Gardner, K.G., and Skoog, A. (2000) Dissolved and particulate carbohydrates in contrasting marine sediments. Geochimica et Cosmochimica Acta 64:1029-1041.

Burdige, D.J. (2001) Dissolve organic matter in Chesapeake Bay sediment pore waters. In: E. Canuel and T. Bianchi (eds.), Organic Geochemical Tracers in Estuaries. Organic Geochemistry 32:487-505.

Burns, A. and Ryder, D.S. (2001) Response of bacterial extracellular enzymes to inundation of floodplain sediments. Freshwater Biology 46:1299-1307.

Buttler, J.H.A. and Ladd, J.N. (1971) Importance of molecular weight of humic and fulvic acids in determining their effects on protease activity. Soil Biology and Biochemistry3:249-257

Cabecadas, L., Broqueira, M.J., and Cabecadas, G. (2004) Phytoplankton spring bloom in the Tagus coastal waters: hydrological and chemical conditions. Aquatic Ecology 33(3): 243-250.

Cammen, L. (1991) Annual bacterial production in relation to benthic microalgal production and sediment oxygen uptake in the intertidal sandflat and an intertidal mud-flat. Marine Ecology Progress Series 71:13-21.

Calvert, S.E. and Pedersen, T.F. (1992) Organic carbon accumulation and preservation in marine sediments: how important is anoxia? In: Productivity, accumulation, and preservation of organic matter in recent and ancient sediments. J.K. Whelan and J.W. Farrington (eds.), Columbia University Press, New York, NY. pp. 231-263.

Canfield, D. E., Jørgensen, B. B., Fossing, H., Glud, R., Gundersen, J., Ramsing, N. B., Thamdrup, B., Hansen, J. W., Nielsen, L. P., and Hall, P. O. J. (1993) Pathways of organic carbon oxidation in three continental margin sediments. Marine Geology 113: 27-40.

Canfield, D.E. (1994) Factors influencing organic carbon preservation in marine sediments: controls on carbon preservation. Chemical Geology 114(3-4):315-329

Canuel, E.A. and Martens, C.S. (1996) Reactivity of recently deposited oarganic matter: degradation of lipid compounds near the sediment water interface. Geochimica et Cosmochimica Acta 60:1793-1806.

Carlson, D. J., and Mayer, L. M. (1983) Relative influences of riverine and macroalgal phenolic materials on UV absorbance in temperate coastal waters. Canadian Journal of Fisheries and Aquatic Sciences 40:1258-1263.

Chaiken, I., Rose, S., and Karlsson, R. (1992) Analysis of macromolecular interactions using immobilized ligands. Analytical Biochemistry 201:197-210

Chen, X., Lohrenz, S.E., and Wiesenburg, D.A. (2000) Distribution and controlling mechanisms of primary production on the Louisiana-Texas continental shelf. Journal of Marine Systems 25(2):179-207

Cho, B. C. and Azam. F. (1988). Major role of bacteria in biogeochemical fluxes in the ocean's interior. Nature 332:441-443.

Choi, B., Zocchi, G., Canale,S., Wu, Y., Chan, S., and Perry, J.L. (2005) Artificial allosteric control of maltose binding protein. Physical Review Letters 94(3):1-4.

Christian, R. and Karl, D.M. (1995) Bacterial ectoenzymes in marine waters: activity rates and temperature responses in three oceanographic provinces. Limnology and Oceanography 40(6):1042-1049.

Chrost, R.J. (1989) Characterization and signature of β-glucosidase activity in lake water. Limnology and Oceanography 34:660-672.

Chrost, R.J. (1990) Microbial ectoenzymes in aquatic environments. In: Aquatic microbial ecology: biochemical and molecular approaches. J. Overbeck and R.J. Chrost (eds.), Springer-Verlarg, Berlin, Germany, pp. 47-78

Chrost, R.J. (1991) Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: Microbial Enzymes in Aquatic Environments. Chrost R.J. (ed.), Springer-Verlag, New York, NY, pp. 29-54.

Chrost, R.J. and Overbeck, J. (1990) Substrate-ectoenzyme interaction: significance of b-glucosidase activity for glucose metabolism by aquatic bacteria. Archiv fur Hydrobiologie Beiheft Ergebnisse der Limnologie 34:93-98

Colberg, P.J. (1988) Anaerobic microbial degradation of cellulose, lignin, oligolignols, and monoaromatic derivatives. In: A.J.B. Zehnder (ed.), Biology of anaerobic microorganisms. Wiley-Interscience, pp. 333-372.

Cotner, J.B., Johengen, T.H., and Biddanda, B.A. (2000) Intense winter heterotrophic production stimulated by benthic resuspension. Limnology and Oceanography 45:1672-1676.

Coveney, M.F. and Wetzel, R.G., 1992. , Effects of nutrients on specific growth rate of bacterioplankton in oligotrophic lake water cultures. Applied and Environmental Microbiology 58(1):150–156.

Cowan, J.L. and Boynton, W.R. (1996) Sediment water oxygen and nutrients exchanges along the longitudinal axis of Chesapeake Bay: seasonal patterns, controlling factors and ecological differences. Estuaries 19:562-580.

Cowie, G.L., and Hedges, J.I. (1992) Sources and reactivities of amino acids in a coastal marine sediment. Limnology and Oceanography 37(4):703-724.

Crump, B.C., Hopkinson, C.S., Sogin, M.L., and Hobbie, J.E (2004) Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. Applied and Environmental Microbiology 70(3): 1494–1505.

Cummins, S.P. and Black, G.W. (1999) Polymer hydrolysis in a cold climate. Extremophiles 3:81-87.

Dalsgaard, J., John, M., Kattner, G., Muller-Navarra, and D., Hagen, W. (2003) Fatty acid trophic markers in the pelagic marine environment. Advances Marine Biology 46:225-340.

D'Andrea, , A.F., Aller, R.C., and Lopez, G.R. (2002) Organic matter flux and reactivity on a South Carolina sandflat: the impacts of porewater advection and macrobiological structures. Limnology and Oceanography 47:1056-1070

Danovaro, R., Manini, E., and Fabiano, M. (2001) Exoenzymatic activity and organic matter composition in sediments of the Northern Adriatic Sea: response to a river plume. Microbial Ecology 44;235:251.

D'Elia, C.F., Sanders, J.G., and Boyton, W.R. (1986) Nutrient enrichment studies in a coastal plain estuary: phytoplankton growth in large-scale, continuous cultures. Canadian Journal of Fisheries and Aquatic Sciences 43:397-406.

de Leeuw, J.W., and Largeau, C. (1993) A review of macromolecular organic compounds that comprise living organisms and their role in kerogen, coal, and petroleum formation. In: Engel, M.H., Macko, S.A. (Eds.), Organic Geochemistry, Plenum, New York, pp. 23–72.

Deming, J.W., and Barros, J.A. (1993) The early diagenesis of organic matter: bacterial activity. In: Organic geochemistry, Engel, M.H., Mako, S.A. (eds.), vol. 6, Topics in Geobiology. Plenum Press, New York, p. 119-144.

Duddrigde, J.E. and Wainwright, M. (1982) Enzyme activity and kinetics in substrate-amended river. Water Research 16:239-334

Dugas, H. (1996) Bioorganic chemistry: a chemical approach to enzyme action. (3rd ed). Springer Advanced Texts in Chemistry, New York, 700pp.

Eftink, M.R., and Ghiron, M.A. (1981) Fluorescence Quenching Studies with Proteins Analytical Biochemistry 114:199-227.

Eglinton, G. and Logan, G. (1991) Molecular preservation. Philosophical Transactions of the Royal Society of London, B. 333:315-328.

Emerson, S and Hedges, J.I. (2003) Sediment diagenesis and benthic fluxes. In: Treatise on geochemistry, Holland, H.D. and Turekian, K.K. (eds.), Elsevier–Pergamon, Oxford, pp. 293–319.

Emery, K.O. (1968). Relict sediments on continental shelves of the world. American Association of Petroleum Geologists Bulletin 52:445-464.

Enríquez, S., Duarte, C.M. and Sand-Jensen, K. (1993) , Patterns in decomposition rates among photosynthetic organisms: the importance of detritus C:N:P content. Oecologia 94:457-471.

Engle, V.D., Krutz, J.C., Smith, L.M., Chancy, C. and Bougeois, P. (2007) A classification of U.S. estuaries based on physical and hydrological attributes. Environmental Monitoring Assessment 129:397-412.

Epstein, S.S. (1997) Microbial food webs in marine sediments. II. Seasonal changes in trophic interactions in a sandy tidal flat community. Microbial Ecology 34;199-209.

Espeland, E.M. and Wetzel, R.G. (2001) Effects of photosynthesis on bacterial phosphatase production in biofilms. Microbial Ecology 42(4):524-530

Fabiano, M. and Danovaro, R. (1994) Composition of organic matter in sediments facing a river estuary (Tyrrhenian Sea): relationship with bacteria and microphytobenthic biomass. Hydrobiologia 277:71-81.

Fabiano, M. and Danovaro, R. (1998) Enzymatic activity, bacterial distribution, and organic matter composition in sediments of the Ross Sea (Antarctica). Applied and Environmental Microbiology 64:3838-3845

Fenchel, T. and Findlay, B.J. (1995) Ecology and evolution in anoxic worlds. Oxford University Press, New York, NY.

Fenchel, T., King, G.M., and Blackburn, T.H. (1998) Bacterial biogeochemistry: the ecophysiology of mineral cycling. Academic Press, San Diego, CA, pp. 1-59.

Ferreira, P., Medina, M., Guillén, F., Martínez, M.J.H., van Berkel, W.J., and Martínez, A.T. (2005) Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols. Biochemistry Journal 389(3):371-378.

Fisher, R.R., Carlson, P.R., and Barber, R.T. (1982) Sediment nutrient regeneration in three North Carolina estuaries. Estuarine, Coastal, and Shelf Science 14:101-116.

Flaten, G.A.F., Castberg, T., Tanaka, T., and Thingstad, T.F. (2003) Interpretation of nutrient-enrichment bioassay by looking at sub-populations in a marine bacterial community. Aquatic Microbial Ecology 33:11-18

Flint, R.W. (1984) Phytoplankton production in the Corpus Christi bay estuary. Contributions in Marine Sciences 27:63-83

Flint, R.W., Powell, G.L., and Kalke, R.D. (1986) Ecological effects from the balance between new and recycled nitrogen in Texas coastal waters. Estuaries 9(4A):284-294.

Foremman, C.M. Franchini, P. and Sinsabaugh, R.L. (1998) The trophic dynamics of riverine bacterioplankton: relationship among substrate availability, ectoenzymes kinetics, and growth. Limnology and Oceanography 43(6): 1344-1352.

Fourqurean, J.W. and Schrlau, J.E. (2003) Changes in nutrient content and stable isotope ratios of C and N during decomposition of seagrasses and mangrove leaves along a nutrient availability gradient in Florida Bay, USA. Chemistry and Ecology 19:373-390.

Foster, S., Huettel, M., and Ziebs, W. (1996) Impact of boundary layer flow velocity on oxygen utilization in coastal sediments. Marine Ecology Progress Series 143:173-185

Freudenthal, T., Wagner, T., Wenzhoffer, F., Zabel, M., and Wefer, G. (2001) Early diagenesis of organic matter from sediments of the eastern subtropical Atlantic: evidence of stable nitrogen and carbon isotopes. Geochimica et Cosmochimica Acta 65:1795-1808.

Froelich, P.N., Bender, M.L., Luedtke, N.A. (1982) The marine phosphorus cycle. American Journal of Science 282:474-511

Fry, B., Scalan, R.S., and Parker, P. (1977) Stable carbon isotope evidence for two sources of organic matter in coastal sediments: seagrasses and plankton. Geochimica et Cosmochimica Acta 41:1875-1877.

Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996) Census and consensus in bacterial ecosystems: the LuxR-LuxL family of quorum sensing. Annual Reviews in Microbiology 50:727-751

Gajewski, A.J. and Chrost, R.J., and Siuda, W. (1993) Bacterial lipolytic activity in an eutrophic lake. Archives of Hydrobiology 128:107-126

Gajewski, A.J., Chrost, R.J. (1995) Production and enzymatic decomposition of organic matter by microplankton in a eutrophic lake. Journal of Plankton Research 17(4):709-728

Gajewski, A.J., Kirschner, A.K.T., and Velimirov, B. (1997). Bacterial lipolytic activity in a hypertrophic dead arm of the river Danube in Vienna. Hydrobiologia 334:1-10.

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Clevland, C., Green, P., Holland, E., Karl, D.M., Michaels, A.F., Porter, J.H. Townsend, A., and Vorosmarthy, C. (2004) Nitrogen cycles: past, present, and future. Biogeochemistry 70:153-226.

Gardner, W.S., Cotner, J.B., and Herche, L. (1993) Chromatographic measurement of nitrogen mineralization rates in marine coastal waters. Marine Ecology Progress Series 93:65-73.

Gardner, W.S., Benner, R., Amon, R., Cotner, J., Cavaletto, J., and Johnson, J. (1996) Effects of high molecular weight dissolved organic matter on the nitrogen dynamics on the Mississippi River plume. Marine Ecology Progress Series 133:287-297.

Gardner, W.S., McCarthy, M.J., An, S., Sodolev, D., Sell, S., and Brock, D. (2006) Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnology and Oceanography 51(1):2558-2568

Goel, R., Mino, T., Satoh, H., and Matsuo, T. (1997) Effects of electron acceptor conditions on hydrolytic enzymes synthesis in bacterial cultures. Water Research 31(10):2597-2603

Goldman, J. C., Caron, D. A., and Dennett, M. R. (1987). Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnology and Oceanography 32: 1239-1252.

Gonzales, T and Baudouy-Robert, J. (1996) Bacterial aminopeptidases: Properties and functions. FEMS Microbiology Reviews 18(3):19-344.

Goto, N., Mitamura, O., and Terai,H. (2001) Biodegradation of photosynthetically produced extracellular organic carbon from intertidal benthic algae. Journal of Experimental Marine Biology and Ecology 257:73-86

Gottschalk, R.P. (1985) Regulation of bacterial metabolism. In: Bacterial Metabolism, second ed. Springer-Verlag, New York, pp. 178-207.

Gruber, D.F., Simjouw, P., Seitzinger, S.P, and Taghon, G.L. (2006) Dynamics and characterization of refractory dissolved organic matter produced by a pure bacterial culture in an experimental predator-prey system. Applied and Environmental Microbiology 72(6):4184-4191.

Gul, S., Sreedharan, S.K., and Brocklehurst (1998) Enzyme assays essential data, Rickwood, D. and Hames, B.D. (Eds.), John Wiley and Sons, Chichester, UK. pp. 8-14

Hames, D.B. and Hooper, N.M. (2000) Biochemisty. 2nd edition, Garland Science/BIOS Scientific Publishers Limted, Oxon, UK, p. 69-90

Hansen, L.S. and Blackburn, T.H. (2006) Mineralization budgets in sediment microcosms: effect of the infauna and anoxic conditions. FEMS Microbiology Letters 102(1):33-43

Harris, D., Horwath, W.R., and Kesse, V. (2001) Acid fumigation of soils to remove carbonates prior to total organic carbon or carbon-13 isotopic analysis. Soil Science Society of America Journal 65(6):1853-1856.

Haslam, E. (1998) Practical polyphenolics from structure to molecular recognition and physiological action. Cambridge University Press. Cambridge, UK, p.84-174

Hedges, J.I., 1988. Polymerization of humic substances in natural environments. In: Humic substances and their role in the environment, Frimmel, F.C., and Christman, R.C. (Eds.), Wiley, Chichester, pp. 45-58.

Hedges, J.I. and Stern, J.H. (1984) Carbon and nitrogen determination of carbonate containing solids. Limnology and Oceanography 29:657-663

Hedges, J.I., Clark, W.A., and Cowie, G.L. (1988) Organic matter sources to the water column and surficial sediments of a marine bay. Limnology and Oceanography 33(5):1116-1132.

Hedges, J.I. and Keil, R.G. (1995) Sedimentary organic matter preservation: an assessment adds speculative synthesis. Marine Chemistry 49:81-115.

Hedges, J.I., Hu, F.S., Devol, A.H., Hartnett, H.E., Tsamakis, E. and Keil, R.G. (1999) Sedimentary organic matter preservation: a test of selective degradation under oxic conditions. American Journal of Science 299:529-555.

Helland, S.J. (2003) Free amino acid and protein content in the planktonic copepod *Temora longicornis* compared to *Artemia franciscana*. Aquaculture 215(1-4):213-228.

Henrichs, S.M. (1992) Early diagenesis of organic matter in marine sediments: progress and perplexity. Marine Chemistry39:119-149.

Henrichs, S.M. and Reeburgh, W.S. (1987) Anaerobic mineralization of marine sediment organic matter: rates and the role of anaerobic processes in the oceanic carbon economy. Geomicrobiology Journal 5:191-237.

Henrichs, S.M., Farrington, J.W., and Lee, C. (1994) Peru upwelling region sediments near 15S. 1. Dissolved free and total hydrolysable amino acids. Limnology and Oceanography 29:20-34.

Henriksen, K., Kemp, W.M. (1988) Nitrification in estuarine and coastal marine sediments. In: Nitrogen cycling in marine environments. Blackburn, T.H., and Sorensen, J. (eds.), John Wiley, New York, NY, pp.207-249.

Hess, V.L. and Szabo, A. (1979) Ligand binding to macromolecules: allosteric and sequential models of cooperativity. Journal of Chemical Education 56:289-293

Hill, B.H., Elonen, C.M., Jicha, T.M., Cotter, A.M., Trebitz, A.S., and Danz, N.P. (2006) Sediment microbial enzyme activity as an indicator of nutrient limitation in Great Lakes coastal wetlands. Freshwater Biology 51(9):1670-1683

Hodell, D.A., and Schelske, C.L. (1998) Production, sedimentation, and isotopic composition of organic matter in Lake Ontario. Limnology and Oceanography 43(2):200-214.

Hoffman, M. and Decho, A.W. (1999) Extracellular enzymes within microbial biofilms and the role of the extracellular polymer matrix. In: Microbial Extracellular Polymer Substances. Wingender, J., Neu, T. and Flemming, H.-C., (eds.), Springer, Berlin, pp. 219-227

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

Hoppe, H.G. (1991) Microbial extracellular enzyme activity: a new key parameter in aquatic ecology. In: Microbial Enzymes in Aquatic Environments. Chrost R.J. (ed.), Springer-Verlag, New York, NY, pp 60-83.

Hoppe, H.G., Gocke, K., and Kuparinen, J. (1990) Effect of H_2S on heterotrophic substrate uptake, extracellular enzyme activity and growth of brackish water bacteria. Marine Ecology Progress Series 64:157-167

Howarth, R.W., Billen, G., Swaney, D., Townsend, A., Iaworski, N., Lajtha, K., Downing, R., Elmgren, N., Caraco, T., Jordan, T., Berendse, F., Freney, J., Dudenyarov, V., Murdoch, P., and Zhao-liang, Z. (1996) Regional nitrogen budgets and riverine N and P fluxes for the human influences. Biogeochemistry 35:181-226.

Huang, C.T., Xu, K.D., McFeters, G.A., and Steward, P.S. (1998) Spatial patterns of alkaline phosphatese expression within bacterial colonies and biofilms in response to phosphate starvation. Applied Environmental Microbiology 64:1526-1531

Huettel, M. and Gust, G. (1992) Impact of bioroughness on interfacial solute exchange in permeable sediments. Marine Ecology Progress Series 89(23): 253-267.

Huettel, M. and Rusch, A. (2000) Transport and degradation of phytoplankton in permeable sediment. Limnology and Oceanography 45:534-549.

Jacobson, M.E., Mackin, J.E., and Capone, D.G. (1987) Ammonium production in sediments inhibited with molybdate: implications for sources of ammonium in anoxic marine sediments. Applied Environmental Microbiology 53(10):2435-2439

Jaffe, D.A. (2000) The nitrogen cycle. In: Earth system science – from bogeochemical cycles to global change. Jacobson, M.C., Charlson, R.J., Rhode, H., and Orians, G.H. Academic Press, New York, NY. p. 263-284.

Jahnke, R.A. and Jahnke, D.B. (2000) Rates of C,N,P, and Si recycling and denitrification at the US mid-Atlantic continental slope depocenter. Deep-Sea Research 47:1405-1428.

Jahnke, R.A., Richards, M., Nelson, J., Robertson, C., Rao, A., Jahnke, D. (2005) Organic matter remineralization and porewater exchange rates in permeable South Atlantic Bight continental shelf sediments. Continental Shelf Research 25(12-13): 1433-14-52

Jorgensen, B.B. (1983) Processes at the sediment-water interface. In: The major biogeochemical cycles and their interactions. Bolin, B Cook, RB (eds), SCOPE 21. John Wiley and Sons, Chichester, pp. 477-509.

Jorgensen, N.O.G., Kroen, N., Coffin, R.B., and Hoch, M.P. (1999) Relations between bacterial nitrogen metabolism and growth efficiency in an estuarine and an open-water ecosystem. Aquatic Microbial Ecology 18:247-261.

Karlsson, R. (1994) Real-time competitive kinetic analysis of interactions between low-molecular-weight ligands in solution and surface-immobilized receptors. Analytical Biochemistry 221(1):142-151

Kamer, M. and Rassoulzadegan, F. (1995) Extracellular enzyme activity: indications for high short-term variability in a coastal marine ecosystem. Microbial Ecology 30(2):143-156

Kemp, W.M. and Boynton W.R. (1984) Spatial and temporal coupling of nutrient inputs to estuarine primary production: the role of particulate transport and decomposition. Bulletin Marine Science 35:522–535.

Keil, R.G., Montluçon, D.B., Prahl, F.G. and Hedges, J.I. (1994) Sorptive preservation of labile organic matter in marine sediments. Nature 370:549-552.

Kleeberg, A. (2002) Phosphorus sedimentation in seasonal anoxic Lake Scharmutzel, NE Germany. Hydrobiologia 472(1-3):53-65

King, G.M. (1986) Characterization of β-glucosidase activity in intertidal marine sediments. Applied and Environmental Microbiology 51:373-380.

King, G.M. (1991) Measurement of acetate concentrations in marine porewaters by using an enzymatic approach. Applied Environmental Microbiology 57(12):3476-3481

Kirchman, D.L. (1990) Limitation of bacterial growth by dissolved organic matter in the subarctic Pacific. Marine Ecology Progress Series 62:47-54.

Klok, J., Blass, M., Cox, H.C. de Leeuw, J.W. Rijpstra, W.I.C., and Schenck, P.A. (1984) Qualitative and quantitative characterization of the total organic matter in a recent marine sediment (Part II). Organic Geochemistry 6:265-278.

Kovarova-Kovar, K. and Egli, T. (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. Microbial Molecular Biology Reviews 62:646-666.

Kristensen, E., and Blackburn, T.H. (1987) Fate of organic carbon and nitrogen in experimental marine sediment systems: influence of bioturbation and anoxia. Journal of Marine Research 45(1):231-257.

Kristensen, E. (1988) Benthic fauna and biogeochemical processes in marine sediments: microbial activities and fluxes. In: Blackburn, T.H. and Sorensen, J. (eds.) Nitrogen Cycling in Coastal Marine Environments. John Willey and Sons, New York, NY, pp. 275-299

Kristensen, E. and Holmer, M. (2001) Decomposition of plant materials in marine sediment exposed to different electron acceptors (O_2, NO_3^-, and SO_4^{-2}), with

emphasis on substrate origin, degradation kinetics, and the role of bioturbation. Geochimica et Cosmochimica Acta 65(3):419-433.

Kristensen, E., Jensen, M.H., and Jensen, K.M (1997) Temporal variations in microbenthic metabolism and inorganic nitrogen fluxes in sandy and muddy sediments of a tidally dominated bay in the northern Wadden Sea, Helgoland. Journal of Marine Research 51(3): 295-320.

Kristensen, E. and Hansen, K. (1995) Decay of plant detritus in organic-poor marine sediment: production rates and stoichiometry of dissolved C and N compounds. Journal of Marine Research 53:675-702.

Kristensen, E. and Saiyed, I.A. and Devol, A.H. (1995) Aerobic and anaerobic decomposition of organic matter in marine sediment: which is faster? Limnology and Oceanography 40(8):1430-1437.

Ladd, J.N. and Butler, J.H.A. (1975) Humus-enzyme systems and synthetic organic polymer-enzyme analogs. In: Soil biology, Vol 4, Paul, E.A. and McLaren, A.D. (eds.), Marcel Dekker, New York, NY, p. 143-194

Landen, A. and Hall, P.O.J. (1998) Seasonal variation of dissolved and adsorbed amino acids and ammonium in near-shore marine sediment. Marine Progress Ecology Series 170:67-84.

Lee, C and Wakeham, S.G. (1989) Organic matter in seawater: biogeochemical processes. In: chemical oceanography. Riley, J.P. (ed.) Vol 9. Academic Press, Ny, p. 1-51

Lee, C. (1992) Controls on organic carbon preservation: the use of stratified water bodies to compare intrinsic rates of decomposition in oxic and anoxic systems. Cosmochimica et Geochimica Acta 56: 3323–3335.

Lerman, A. (1979) Geochemical processes, water and sediment environments. John Wiley and Sons, New York, NY, 481 p.

Liaw and Penner (1990) Substrate-velocity relationships for the *Trichoderma viride* cellulase-catalyzed hydrolysis of cellulose. Applied and Environmental Microbiology 56(8):2311-2318.

Liu, D., Wong, P.T.S., and Dutka, B.J. (1973) Determination of carbohydrates in lake sediment by a modified phenol-sulfuric acid method. Water Research 7:741-746

Liu, Y., Lam, M.C., and. Fang, H.H.P. (2001) Adsorption of heavy metals by EPS of activated sludge. Water Science and Technology 43(6):59–66.

Lopez, N.I., Duarte, C.M., Vakkesinos, F., Romero, J., Alcovero, T. (1995) Bacterial activity in NW Mediterranean seagrass (Posidonia oceanica) sediments. Journal of Experimental Marine Biology and Ecology 187:39-49

López, N.I., Duarte, C.M., Vallespinos, F., Romero, J., and Alcoverro, T. (1998) Effects of nutrient additions on bacterial activity in seagrass (*Posidonia oceanica*) sediments. Journal of Experimental Marine Biology and Ecology 224:155–166.

Lochte, K., Boetius, A., and Petry, C. (1999) Microbial food webs under severy nutrient limitations: life in the deep sea. Microbial Biosynthesis: new frontiers. Preceedings of the 8th international symposium on microbial ecology. Bell, C.R., Brylinsky, M., Johnson-green, P. (eds), Atlantic Canada Society for Microbial Ecology, Canada.

Lucas, F.S., Bertru G., and Höfle, M.G. (2003) Characterization of free-living and attached bacteria in sediments colonized by Hediste diversicolor. Aquatic Microbial Ecology 32:165–174.

Lundblad, R. L. (2007) Biochemistry and molecular biology compendium. Academic Press, NY. p. 331-349

Ma, L. and Kantrowitz, E.R. (1994) Mutations at histidine 412 alter zinc binding and eliminate transferase activity in *Escherichia coli* alkaline phosphatase. Journal of Biological Chemistry 269(50):31614-31619

MacIntyre, H.L., Geider, R.J. and Miller, D.C (1996) Microphytobenthos: the ecological role of the "secret garden" of unvegetated, shallow-water marine habitats. I. distribution, abundance and primary production. Selected papers from the first annual marine and estuarine shallow water science and management conference, Estuaries 19(2A): 186-201.

MacIntyre, H.L., and Cullen, J.J. 91996) Primary production by suspended and benthic microalgae in a turbid estuary: time-scales of variability in San Antonio Bay, Texas. Marine Ecology Progress Series 145:245-268

McQueen, P.W., Wordsworth, P.M., Daly, J.M., and Rickard, P.A.D. (2005) Product inhibition of Trichodermareesei and Cellulosomas glycosidase activities. Biotechnology Letters 5(10):689-692

Madigan, M.T., Martinko, J.M., and Parker, J. (2002) Brock biology of microorganisms, 10th edition. Prentice Hall, Upper Saddle River, NJ. 1385p.

Mallet, C. and Debroas, D. (2000) Relations between organic matter and bacterial proteolytic activity in sediment surface layers of a eutrophic lake. Archives of Hydrobiology 149(2):327-335

Marangoni, A.G. (2003) Enzyme Kinetics: A modern approach. John Wiley & Sons, Inc., New York, NY, 244 p.

Marinelli, R.L., Boudreau (1996) An experimental and modeling study of pH and related solutes in an irrigated, anoxic coastal sediment. Journal of Marine Research 54:939-966

Marinelli, R.L., Jahnke, R.A., Craven, D.B., Nelson, J.R., and Eckman, J.E. (1998) Sediment nutrient dynamics on the South Atlantic Bight continental shelf. Limnology and Oceanography 43:1305-1320

Martens, C.S. and Klump, J.V. (1984) Biogeochemical cycling in a an organic-rich marine basin. An organic carbon budget for sediments dominated by sulfate reduction and methanogenesis. Geochimica et Cosmochimica Acta 48:1987-2004

Matsui, M., Jonathan, H., Walling, L., andWalling, L.L. (2006) Leucine aminopeptidases: diversity in structure and function. Biological Chemistry 387:1535-1544

Mayer, L.M. (1989) Extracellular proteolytic enzyme activity in sediments of an intertidal mudflat. Limnology and Oceanography 34(6):973-981.

Mayer, L.M. (1986) Measurement of hydrolytic activity and incorporation of dissolved organic substrates by microorganisms in marine sediments. Marine Ecology Progress Series 31:143-149.

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

Mayer, L.M. (1999) Extent of coverage of mineral surfaces by organic matter in marine sediments. Geochimica et Cosmochimica Acta 63:207-215.

Mayer, L.M., and Rice, D.L. (1992) Early diagenesis of proteins: a seasonal study. Limnology and Oceanography 37:280-295.

Meyers, P.A. and Ishiwatari, R. (1993) Lacustrine organic geochemistry – an overview of indicators of organic matter sources and diagenesis in lake sediments. Organic Geochemistry 20:867-900

Meyer-Reil, L.A. (1983) Benthic response to sedimentation events during autumn to spring at shallow water station in the Western ght.II. Analysis of benthic bacterial populations. Marine Biology 77:247-256.

Meyer-Reil, L.A. (1986) Measurement of hydrolytic activity and incorporation of dissolved organic substrates by microorganisms in marine sediments. Marine Ecology Progress Series 31:143-149.

Meyer-Reil, L.A. (1987) Seasonal and spatial distribution of extracellular enzymatic activities and microbial incorporation of dissolved organic substrates in marine sediments. Applied and Environmental Microbiology 53:1748-1755.

Meyer-Reil, L.A. (1991) Ecological aspects of enzyme activity in marine sediments. In: Enzymes in Aquatic Environments. Chrost, R.J. (ed.), Springer-Verlag, New York, NY, pp. 84-95.

Middelburg, J.J., Soetaert, K., and Herman, P.M.J. (1997) Deep Sea Research 44:327-334

Morrison, J.F. and Walsh, C.T. (1988) The behavior and significance of slow-binding enzyme inhibitors. Advanced Enzymology Related Areas Molecular Biology 61:201-301

Mudryk, Z. and Podgorska, B. (2005) Generic composition and respiratory activity of heterotrophic bacteria of marine sandy beach (South Baltic Sea). Polish Journal of Ecology 53:97-103

Munster, U. and Chrost, R.J. (1990) Origin, composition, and microbial utilization of dissolved organic matter. In: Aquatic Microbial Ecology, Chrost, R.J. (ed.), Springer-Verlag, New York, NY, pp. 8-37.

Nannipieri, P, Ceccanti, B., Conti, C., and Bianchi, D. (1982) Hydrolases extracted from soil: their properties and activities. Soil Biology and Biochemistry 14:257-263

Nannipieri, P., kandeler, E., and Ruggiero, P. (2002) Enzyme activities and microbiological and biochemical processes in soil. in: Enzymes in the environment: activity ecology and applications. Burns, R.G. and Dick, R.P. (eds.) p. 1-33

Nichols, M.N. and Biggs, R.B. (1985) Estuaries. In: Coastal sedimentary environments (Davis, R.A, ed.). Spring-Verlag, New York, pp. 77-186

Nielsen, S.L., Sand-Jensen, K., Borum, J., and Geertz-Hansen (2002) Phytoplankton, nutrients, and transparency in Danish coastal waters. Estuaries 25:930-937

Nixon, S.W. (1981) Remineralization and nutrient cycling in coastal marine ecosystems. In: Neilson, B.J. and Cronin, L.E. (eds.) Estuaries and Nutrients, Humana Press, Clifton, NJ, pp. 111-138

Nixon, S.W., Ammerman, J.W., Atkinson, L.P., Berounsky, V.M., Billen, G., Boicourt, W.C., Boyon, W.R., Church, T.M., Ditoro, D.M,, Elmgren, R., Garber, J.H., Giblin, A.E., Jahnke, R.A., Owens, N.J.P., Pilson, M.E.Q., and Seitzinger, S.P. (1996) The fate of nitrogen and phosphorus at the land-sea margin of the North Atlantic Ocean. Biogeochemistry 35:141-180.

Nuwer, M.J. and Keil, R.G. (2005) Sedimentary organic matter geochemistry of Clayoquot Sound, Vancouver Island, British Columbia. Limnology and Ocenography 50(4):1119-1128.

Ogawa, H., Amagai, Y., Koike, I., Kaiser, K., and Berner, R. (2001) Production of refractory dissolved organic matter by bacteria. Science 292:917-920.

Oh, H., Hoff, J.E., Armstrong, G.S., and Haff, L.A. (1980) Hydrophobic interaction in tannin-protein complexes. Journal of Agricultural and Food Chemistry 28:394-398

Overbeck, J. (1991). Early studies on ecto- and extracellular enzymes in aquatic environments. In: Microbial Enzymes in Aquatic Environments. Chrost, R. J. (ed.), Springer-Verlag, New York, NY, pp: 29-54.

Paerl, H.W., Dennis, R.L., and Whitall, D.R. (2002) Atmospheric deposition of nitrogen: implications for nutrient over-enrichment of coastal waters. Estuaries 25:677-693.

Page, M.I. and Williams, A. (1987) Enzyme mechanisms. Lodon, England. Royal Society of London, 550p.

Panagiotopolus, C. and Sempere, R. (2005) Molecular distribution of carbohydrates in large marine particles. Marine Chemistry 95:31-49

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

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

Parsons, T.R., Matia, Y., and Lalli, C. (1984) A manual of chemical and biological methods for seawater analysis. Pergamon, New York, NY, 173pp.

Parr, S.R. (1983) Some kinetic properties of the β-D-glucosidase (cellobiase) in a commercial cellulose product from *Pennicillium funiculosum* and its relevance in the hydrolysis of cellulose. Enzyme Microbiology Technology 5:457:462.

Pearl, H.W., Dyble, J., Twomey, L., Pinkney, J.L., Nelson, J., and Kerkhof, L. (2002) Characterizing man-made and natural modifications of microbial diversity and activity in coastal ecosystems. Antonie Van Leeuwehoek 81(1-4):487-507

Podgorska, B. and Mudryk, Z.J. (2003) Distribution and enzymatic activity of heterotrophic bacteria decomposing selected macromolecular compounds in a Baltic Sea sandy beach. Estuarine, Coastal, and Shelf Science 56:539-546.

Poremba, K., and Hoppe, H. (1995) Spatial variation of benthic microbial production and hydrolytic enzymatic activity down the continental slope of the Celtic Sea. Marine Ecology Progress Series 118:237- 245.

Porter, T.E., Owens, M.S., and Cornwell, J.C. (2006) Effect of sediment manipulation on the biochemistry of experimental sediment systems. Journal of Coastal Research 22(6):1539-1551

Priest, F.G. (1984) Extracellular enzymes. Van Nostrand Reinold Co. Ltd., Wokingham, UK, p. 17-79.

Priest, F.G. (1992) Synthesis and secretion of extracellular enzymes in bacteria. In: Microbial degradation of natural products. Winkelman, G. (ed.), VCH, New York, p. 1-26.

Premuzic, E.T., Benkovitz, C.M., Gaffney, J.S. and Walsh, J.J. (1982) The nature and distribution of organic matter in the surface sediments of world oceans and seas. Organic Geochemistry 4:63–77.

Quay, P.D., Wildur, D.O., Richey, J.E., Hedges, J.I., Devol, A.H. and Victoria, R. (1992) Carbon cycling in the Amazon River: implications from the 13C composition of particles and solutes. Limnology and Oceanography 37:857-871

Ransom, B., Kim, D., Kastner, M., and Wainwright, S. (1998) Organic matter preservation on continental slopes: importance of mineralogy and surface area. Geochimica et Cosmochimica Acta 62(8):1329-1345

Rasheed, M., Badran, M.I., and Huettel, M. (2003) Influence of sediment permeability and mineral composition on organic matter degradation in three sediments from the Gulf of Aqaba, Red Sea. Estuarine, Coastal, and Shelf Science 57(1-2):369-384.

Rath, J., Schiller, C., and Herndl, G.J. (1993) Ectoenzymatic activity and bacterial dynamics along a trophic gradient in the Caribbean Sea. Marine Ecology Progress Series 102:89-96

Read, S.M. and Northcote, D.H. (1981) Minimization of variation in the response to different proteins of the Coomassie Blue-G dye-binding assay for protein. Analytical Biochemistry 116:53-64

Reichardt, W. (1986) Enzymatic potential for decomposition of detrital biopolymers in sediments from Kiel Bay. Ophelia 26:239-384.

Reimers, C., Stecher, H.A., III, Taghon, G.L., Fuller, C.M., Huettel, M., Rusch, A. Ryckelynck, N. and Wild, C. (2004) In Situ measurement of advective solute transport in permeable shelf sands. Continental Shelf Research 24:183-201.

Riggs, Riggs, S.R., Snyder, S.W., Hine, A.C., and Mearns, D.L. (1996) Hardbottom morphology and relationship to the geologic framework: Mid-Atlantic continental shelf. Journal of Sedimentary Research, Section B: Stratigraphy and Global Studies 66(4):830-846.

Robenson, J.L. and Tilton, R.D. (1995) Effect of concentration quenching on fluorescence recovery after photobleaching measurements. Biophysical Journal 68:2145-2155.

Rocha, C. (1998) Rhythmic ammonium regeneration and flushing in intertidal sediments of the Sado estuary. Limnology and Oceanography 43:823-831

Roden, E.E., Tuttle, J.H., Boynton, W.R. and Kemp, W.M. (1985) Carbon cycling in mesohaline Chesapeake bay sediments. 1: POC decomposition rates and mineralization pathways. Journal of Marine Research 53:799-819

Romani, A.M. and Sabater, S. (2000) Influences of algal biomass on extracellular enzyme activity in river biofilms. Microbial Ecology 40:16-24

Rowe, G.T., Kaegi, M.E.C., Morse, J.W., Boland, G.S., and Briones, E.G.E. (2002) Sediment community metabolism associated with continental shelf hypoxia, Northern Gulf of Mexico. Estuaries 25(6A):1097–1106.

Rusch, A., Forster, S. and Huettel, M. (2001) Bacteria, diatoms and detritus in an intertidal sandflat subject to advective transport across the water-sediment interface. Biogeochemistry 55(1): 1-27.

Rush, A., Huettel, M. (2000) Advective particle transport into permeable sediments – evidence from experiments in an intertidal sandflat. Limnology and Oceanography 45:525-533

Rush, A., Huettel, M., Reimers, C.E., Taghon, G.L., and Fuller, C.M. (2003) Activity and distribution of bacterial populations in Middle Atlantic Bight shelf sands. FEMS Microbiology Ecology 44:89-100

Rysgaard, S., Christensen, P.B., and Nielsen, L.P. (1995) Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Marine Ecology Progress Series 126:111-121.

Seigel, I.H. (1975) Enzyme kinetics. John Wiley and Sons, New York, NY, 608p.

Seiter, K., Hense, C., Schroter, J., and Zabel, M. (2004) Organic carbon content in surface sediments: defining regional provinces. Deep Sea Research Part I: Oceanographic Research Papers 51(12):2001-2026.

Schalemberg, M., Kalff, J., and Rasmussen, J. (1989) Solutions to problems in enumerating sediment bacteria by direct counts. Applied Environmental Microbiology 55:1214-1219.

Schmid, M.C., Petersen-Risgaard, N., Vossenberg, J., Kruypers, M.M.M., Lavik, G., Petersen, J., Hulth, S., Thandrup, B., Canfield, D., Dalsgaard, T., Rysgaard, S., Sejr, M.K., Camp, H.J.M., and Jetten, M.S.M. (2007) Anaerobic ammonium-oxiding bacteria in marine environments: widespread occurrence but low diversity. Environmetal Microbiology 9(6):1476-1484.

Seibert, K.J., Troukhanova, N.V., an Lynn, P.Y. (1996) Nature of polyphenol-protein interactions. Journal of Agricultural and Food Chemistry 44(1):8085

Seitzinger, S.P. (1988) Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. Limnology and Oceanography 33:702-724.

Serrano, L. and Boon, P.I. (1991) Effect of polyphenolic compounds on alkaline phosphatase activity: its implication for phosphorus regeneration in Australian freshwaters. Archives of Hydrobiology 123(1):1-19

Shum, K.T. and Sundby, B. (1996) Organic matter processing in continental shelf sediments- the subtidal pump revisited. Marine Chemistry 53(1-2):81-87

Sirova, D., Vrba, J., and Rejmankova, E. (2006) Extracellular enzyme activities in benthic cyanobacterial mats: comparison between nutrient-enriched and control sites in marshes of northern Belize. Aquatic Microbial Ecology 44(1):11-20

Smith, C.A. and Wood, E.J. (1991) Biological molecules – molecular and cell biochemistry. Chapman and Hall, New York, NY, p.105-123

Spencer, C.M., Cai, Y., Martin, R., Gaffney, S.H., Goulding, P.N. Magnolate, D., Lilley, T.H., and Haslam, E. (1988) Polyphenol complexation – some thoughts and observations. Phytochemistry 27:2397-2409

Staats, N., Stal, L.J., and Mur, L.R. (2000) Exopolysaccharide production by the epipelic diatom Cylindrotheca closterium: effects of nutrient conditions. Journal of experimental Marine Biology and Oceanography 249:1-12

Steinberg, G, P. D., and Van Altena, I. (1992) Tolerance of marine invertebrate herbivores to brownalgal phlorotannins in temperate Australasia. Ecological Monographs 62:189 222.

Stern, J.L., Hagerman, A.E., Steinderg, P.D., and Winter, J.A. (1996) A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. Journal of Chemical Ecology 22(7):1273-1293

Suárez, I. and Marañón, E. (2003) Photosynthate allocation in a temperate sea over an annual cycle: the relationship between protein synthesis and phytoplankton physiological state. Journal of Sea Research 50(4):285-299.

Sun, M., Aller, R.C. and Lee, C. (1991) Early diagenesis of chlorophyll-a in Long Island Sound sediments: a measure of carbon flux and particle reworking. Journal of Marine Research 49:379-401.

Sun, M., Aller, R.C. and Lee, C., and Wakeham, S.G. (2002) Effects of oxygen and redox oscillations on degradation of cell-associated lipids in surface marine sediments. Geochimica et Cosmochimica Acta 66:203-212.

Sunback, K., Enoksson, V., Graneli, W. and Petterson, K. (1991) Influence of sublittoral microphytobenthos on the oxygen and nutrient flux between sediment and watet: a laboratory continuous flow study. Marine Ecology Progress Series 74:263-279

Tanoue, E. and Handa, N. (1980) Some characteristic features of the vertical profile of organic matter in recent sediment from the Bering Sea. Journal of Oceanographical Society of Japan 36:1-14.

Teague, K., Madden, C., and Day, J. (1988) Sediment oxygen uptake and net sediment-water nutrient fluxes in a river dominated estuary. Estuaries 11:1-9.

Texas Department of Water Resources (1981) Nueces and Mission-Aransas estuaries: a study of the influence of freshwater inflows. Rpt #LP-108, Texas Department Water Resources, Austin, TX.

Tholosan, O. Lamy, F., Garcini, J., Polychronaki, T., and Bianchi, A. (1999) Biphasic extracellular proteolytic enzyme activity in benthic water and sediment in the northwestern Mediterranean Sea. Applied Environmental Microbiology 65:1619-1626.

Thurman, E.M. (1985) Organic geochemistry of natural waters. Martinus Nijhof/Dr. W. Junk Publishers, Boston, MA, p.363-440

Tissot, B.P. and Welte, D.H. (1978) Petroleum occurrence and formation. 2nd revised and enlarged edition, Spring-Verlag. New York, NY, p.

Trimmer,M., Risgaard-Petersen, N., Nicholls, J.C., and Engston, P. (2006) Direct measurement of anaerobic ammonium oxidation (anammox) and denitrification in intact sediment cores. Marine Ecology Progress Series 326:37-47

Tunner, R.E. and Rabalais, N.N. (1991) Changes in Mississippi river water quality this century: implications for coastal food webs. Bioscience 41:140-147.

Turley, C. M. and Lochte, K. (1990) Microbial response to the input of fresh detritus to the deep-sea bed. Paleogeography, Paleoclimatology, and Paleoecoloy 89:2-23

Tyson, R.V. (1995) Sedimentation rate, dilution, preservation and total organic carbon: some results of a modeling study. Organic Geochemistry 32(2):333-339.

Tyler, B. (1978) Regulation of the assimilation of nitrogen compounds Ann Rev. Biochemistry 47:1127-62.

Twilley, R.R., Cowan, J., Miller-Way, T., Montagna, P.A., and Mortazavi, B. (1999) Benthic nutrient fluxes in selected estuaries in the Gulf of Mexico. In biogeochemistry of Gulf of Mexico estuaries (eds: Biachi, T.S., Pennock, J.R. and Twilley, R.R.) John Willey, NY. pp. 163-209

Unanue, M.U., Ayo, B., Agis, M., Slezak, D., Herndl, G.J., and J. Iriberri (1999) Ectoenzymatic Activity and uptake of monomers in marine bacterioplankton described by a biphasic kinetic model. Microbial Ecology 37:36-48.

Underwood, G.J.C., Paterson, D.M., and Parkes, R.J. (1995) The measurement of microbial carbydrate exopolymers from intertidal sediments. Limnology and Oceanography 40(7):1243-1253

Valiela, I. (1984) Marine ecological processes. Springer-Verlag, New York, NY, 346 p.

Van Heemst, (1999) Multiple sources of alkylphenols produced upon pyrolysis of DOM, POM and recent sediments. Journal of Analytical and Applied Pyrolysis 52(2): 239-256.

Verma, L., Martin, J.P., Haider, K. (1975) Decomposition of carbon-14-labeled proteins, peptides, and amino acids; free and complexed with humic polymers. Soil Scientific Society of American Proceedings 39:279-284

Vetter, Y. and Deming, J.W. (1994). Extracellular enzyme activity in the Arctic northeast water polynya. Marine Ecology Progress Series 114:23-34.

Vetter, Y., Deming, J.W., Jumars, P.A., and Krieger-Brockett, B.B. (1998) A predictive model of bacterial foraging by means of freely released extracellular enzymes. Microbial Ecology 36:75-92.

Vorosmarty, C.J. and Loder, T.C. (1994) Spring-neap tidal contrasts and nutrient dynamics in a marsh-dominated estuary. Estuaries 17:537-551.

Vrba, J., Callieri, C., Bittl, T., Simek, K., Bertoni,R., Filandr, P., Harman, P., Hejzlar, J., Macek, M., and Nedoma, J. (2004) Are bacteria the major producers of extracellular glycolytic enzymes in aquatic environments? Inernational Reviews of Hydrobiology 89(01):102-117

Warnken, K.W. Gill, G.A., Santschi, P.H. and Griffin, L.L. (2000) Benthic exchange of nutrients in Galveston Bay, Texas. Estuaries 23:647-661.

Warnken, K.W., Santschi, P.H., Roberts, K.A., and Gill, G.A. (2008) The cycling and oxidation pathways of organic carbon in a shallow estuary along the Texas gulf coast. Estuarine, Coastal, and Shelf Science 76(1): 69-84

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

Wetzel, R.G. (1991) Extracellular enzymatic interactions: storage, redistribution, and interspecific communication. In: Chrost, R.J. Microbial Enzymes in aquatic environments. Spring-Verlag , New York. pp. 6-28.

Wetzel, R.G. (1999) Organic phosphorus mineralization in soils and sediments. In: phosphorus biogeochemistry of subtropical ecosystems (eds: Reddy, K.R., O'Connor, G.A., Schelske, C.L.), CRC Press, Boca Raton, FL. pp. 225-245.

Whitman, W.B. Coleman, D.C. Wiebe, J. (1998) Prokaryotes: the unseen majority. Proceeding of the National Science Academy 95(12):6578-6583.

Witte U., Wenzhofer, F., Sommer, S., Boetius, A., Heinz, P., Aberle, N., Sand, M., Cremer, A., Abraham, W.-R., Jørgensen, B.B., and Pfannkuche, O. (2003) In situ experimental evidence of the fate of a phytodetritus pulse at the abyssal sea floor. Nature 424:763-766.

Williams, C.J. and Jochem, F. (2006) Ectoenzymes kinetics in Florida Bay: implications for bacterial carbon source and nutrient status. Hydrobiologia 569:113-127

Wollast, R. (1991) The coastal organic carbon cycle: fluxes, sources, and sinks. In: Ocean margin processes in global change. Mantoura, R.F.C., Martin, J.M., Wollast, R. (eds.), John Wiley and Sons, New York, NY, p. 365-381.

Wollast, R. (1993) Interactions in estuaries and coastal waters. In: The major biogeochemical cycles and their interactions. Bolin, B. and Cook, R.B. (eds.), John Wiley, Chichester, UK, p. 385-409.

Zhang, Q., Zhang, J., Shen, J., Silva, A., Dorothy, A.D., and Colin J. B. (2006) A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. Journal of Applied Phycology 18: 445–450.

Ziebis, W., Foster, S., Huettel, M., and Jorgensen, B.B. (1996) Complex burrows of the mud shrimp *Callianassa truncate* and their geochemical impact in the sea bed. Nature 382:619-622

Vita

Afonso Souza was born in Belo Horizonte, MG, Brazil, son of Cesar Romeu de Sousa and Maria Luiza de Rezende de Souza. Prior to this doctoral degree, he obtained his Bachelor's degree in Biological Sciences at the Pontifex Catholic University of Minas Gerais and Master's degree in Biological Oceanography at The Florida State University in Tallahassee, FL. His research findings during his master studies improved the technique of epifluorescence microscopy to enumerate active prokaryotes marine sediments. The current doctoral program in Marine Science offered him the opportunity to teach undergraduate students the laboratory portion of an introductory course in oceanography. This was a valuable experience.

Permanent address: 1309 Lopezville Road, Socorro, NM. 87801

This dissertation was typed by Afonso Cesar Rezende Souza.