FACTORS AFFECTING CARBOHYDRATE PRODUCTION AND LOSS IN SALT MARSH SEDIMENTS OF GALVESTON BAY

A Thesis

by

CAROLYN ELAINE WILSON

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

MASTER OF SCIENCE

August 2009

Major Subject: Oceanography

FACTORS AFFECTING CARBOHYDRATE PRODUCTION AND LOSS IN SALT MARSH SEDIMENTS OF GALVESTON BAY

A Thesis

by

CAROLYN ELAINE WILSON

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

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Daniel C. O. Thornton
Committee Members,	Thomas Bianchi
	Stephen Davis
Head of Department,	Piers Chapman

August 2009

Major Subject: Oceanography

ABSTRACT

Factors Affecting Carbohydrate Production and Loss in Salt Marsh Sediments of Galveston Bay. (August 2009)

Carolyn Elaine Wilson, B.A., Austin College

Chair of Advisory Committee: Dr. Daniel C. O. Thornton

Benthic microalgae (BMA) living within the surface sediment of salt marshes are highly productive organisms that provide a significant proportion of organic carbon inputs into estuarine systems. BMA secrete extracellular carbohydrates in the form of low molecular weight carbohydrates and extracellular polymeric substances (EPS) as they migrate within the sediment. EPS plays an important role in the structure and function of BMA biofilms in shallow-water systems as EPS affects habitat structure, stabilizes the sediment, reduces sediment erosion, and is a carbon source for organisms.

This study looked at the effect of nutrients and carbohydrate additions on BMA biomass, bacterial biomass, carbohydrate production, and glycosidase activity in the surface 5 mm of intertidal sediment in a subtropical salt marsh (Galveston Bay, Texas). Nitrogen and phosphorus were added to cores collected from the salt marsh and incubated in the lab over four days. Very little change was seen in the biomass of the benthic microalgae or in the different carbohydrate fractions with the added nutrients. The mean chlorophyll *a* concentration was 13 ± 5 μg g^{-1} sediment, the mean saline extractable carbohydrate concentration was 237 ± 113 μg g^{-1} sediment, and the mean

EPS concentration was 48 ± 25 μg g^{-1} sediment. The chlorophyll *a* and saline extractable carbohydrate concentrations initially decreased over the first 24 hours, but then increased over the rest of the experiment, indicating a possible species compositional shift in the BMA. With no major response with nutrient additions, it is likely that a different environmental factor is limiting for the growth of the benthic microalgae, and therefore the production of sEPS, in this salt marsh.

A series of experiments was conducted *in situ* by adding glucose, alginic acid, and phosphorus to sediment within experimental plots. Samples were taken periodically over three to seven days to determine the biomass of the microbial community, enzyme activities and kinetics, and changes in the concentrations of several sediment carbohydrate pools. β-glucosidase activities (15 ± 3 nmol g^{-1} h^{-1}) were significantly higher than β-xylosidase (6 ± 2 nmol g^{-1} h^{-1}) and β-galactosidase (8 ± 2 nmol g^{-1} h^{-1}) activities within the sediment, and there was no suppression of β-glucosidase activity measured with the glucose addition. These data represent the first measurement of β-xylosidase and β-galactosidase activity in intertidal sediment dominated by BMA. Although preliminary experiments suggested a possible phosphorus limitation within the sediment, there was little change in the bacteria abundance or the benthic microalgae biomass when phosphorus was added *in situ*.

This study begins to illustrate the dynamics of carbohydrate production and loss in this salt marsh, and the ability for the microbial community in the salt marshes of Galveston Bay to adjust to the nutrient and carbohydrate treatments.

DEDICATION

I dedicate this work to my family for supporting me and encouraging me through this process and throughout my life.

ACKNOWLEDGEMENTS

I would like to thank my committee chair and advisor, Dr. Daniel C. O. Thornton, for his support and guidance through this degree, as well as my committee members, Dr. Thomas Bianchi and Dr. Stephen Davis, for their help and comments through this process.

I appreciate the help I received from Dr. Heath Mills by answering questions and allowing me to borrow a few items in his lab. I would like to highlight the sampling help I received from Lindsey Visser, Federico Alvarez, Charlie Rzadkowolski, and Jennifer Williams, who all were willing to spend a few hours in Galveston collecting sediment and water from the sampling site to my car. Thanks also go to Chris Paul and Stella Woodard for helping me handle specific issues that arose with my lab setup. I also want to thank the Geochemical Environmental Research Group (GERG) for allowing me to use their freeze dryer.

Special thanks go to everyone in the Department of Oceanography willing to listen to me and offering useful advice when problems and frustrations arose throughout this process.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Salt Marsh Mudflats

Intertidal mudflats that develop along coastal shorelines are ecologically significant areas. Mudflats are formed from sediment deposits of clay, silts and sand. The important physical properties of the sediment include size composition and morphology of the sediment particles, as well as the water content found within the sediment (Stal 2003). Important chemical properties of the sediment are the surface charge of the sediment particles and the ionic strength and composition of the pore water (Stal 2003). A diverse, microorganism-dominated community lives in mudflats and the activity of these organisms affects the physical (e.g. resistance to erosion) and chemical properties (e.g. gradients of oxygen and nutrients) of the habitat.

Salt marsh mudflats tend to consist mainly of microorganisms that are highly productive and help control the concentrations of organic and inorganic nutrients and their flux between the sediment and the overlying water (Underwood et al. 1998, Thornton et al. 2002, Thornton et al. 2007). These photoautotrophs can contribute up to 50% of the total photosynthetic production and up to 33% of carbon inputs into estuarine systems (Underwood & Paterson 2003). The other benthic organisms on the mudflats,

---

This thesis follows the style of Marine Ecology Progress Series.

such as polychaete worms, crabs, deposit feeders, etc are completely supported by the movement of organic material within the sediments on the mudflats (Lester & Gonzales 2002). Using carbon tracing experiments, carbohydrates produced by benthic microalgae are used by bacteria, infauna, and deposit feeders (Middelburg et al. 2000). Mudflats also provide coastal protection from storms and tidal surges. The biological processes that occur within the intertidal mudflats can have a stabilizing or destabilizing impact upon the sediments due to the movement of and interactions between the microorganisms and the infauna. Intertidal mudflats and salt marshes are currently threatened by anthropogenic pressures as well as other physical pressures like erosion from increasing tides (Defew et al. 2002).

Benthic Microalgae and EPS

The surfaces of mudflats are usually covered in microbial mats commonly dominated by photoautotrophs, such as benthic diatoms and certain species of cyanobacteria. These mats stabilize the sediment, which helps decrease erosive loss due to tidal forces and burrowing of other organisms (Defew et al. 2002, Hanlon et al. 2006). Microbial mats, sometimes defined as biofilms, are defined as a matrix of cells, sediment and mucilage that create a structure at the sediment surface (Underwood & Paterson 2003). It is this structure and the organisms within the structure that is the key to a mudflat's ability to provide vital ecosystem functions, such as coastal erosion protection, prevention of sediment desiccation, and carbon production (Underwood & Paterson

2003). Microbial mats also have the ability to withstand major environmental changes in temperature, wave action, and irradiance (Decho 2000).

Much of the photosynthetic algae in biofilms are cyanobacteria, and many species of cyanobactiera have the ability to fix atmospheric nitrogen (Stal 2003). These microbes can be found in nearly every illuminated area on earth and tend to function as pioneer species, allowing for other communities to colonize along with them. The benthic microalgae (BMA) community tends to be predominately diatoms and cyanobacteria. The diatom populations tend to be the dominating taxa within the biofilms (Stal 2003). Diatoms are nonflagellate, unicellular microalgae with cell walls made from silica. Both centric and pennate diatoms are found in sediments, but the pennate diatoms have the unique ability to move through the sediment. This mechanism is unique in microbial cells. The diatom cell has a slit in the surface of the silica cell wall, called a raphe, where carbohydrate polymers are secreted (Underwood & Paterson 2003). Diatoms tend to migrate vertically in response to tidal cycles and light availability. They move closer to the surface during periods of tidal emersion and during daylight hours, and this allows them to move closer to the surface when necessary for photosynthesis after periods of deposition or sediment mixing (Stal 2003, Underwood et al. 2004). This vertical movement is usually within 2 mm from the sediment surface and this distance is an average achieved between 2 and 3 hours when moving at a speed of 0.2 $\mu m\ s^{-1}$ (Hopkins 1963, Paterson 1986).

The carbohydrate polymers secreted by benthic diatoms are often called extracellular polymeric substances (EPS), and this EPS contributes to the mucilage that

helps bind the sediments within the biofilm matrix (Hanlon et al. 2006). Approximately 95% of EPS is made of polysaccharides mixed with lipoproteins. The polysaccharides are composed of neutral sugars, uronic acids, sulfonated sugars, and keta-linked pyruvate groups (Sutherland 1999). Physically, EPS appears in a range of tightly connected gels to a more loosely connected gel to a dissolved carbon state. The larger, more tightly bound gels tend to surround the cell like a protective capsule (Decho 2000). EPS production in sediments has been widely studied from the pathways of production to the changes in production with environmental changes. The pathway of EPS production from photosynthetic carbon fixation to exudation takes between 0.5 to 2 hours, depending on the environmental factors like nutrient concentrations, temperature changes, and light availability (Haynes et al. 2007). EPS secretion by BMA tend to increase with higher concentrations of NH_4^+ compared to higher concentrations of NO_3^- because the energy not used to reduce the nitrate is transferred for carbon production (Underwood & Paterson 2003). There is also an increase in EPS concentrations with changes in light that stimulates the movement of the BMA (Underwood & Paterson 2003).

Much of the environmental importance of photosynthetic biofilms is due to the EPS mucilage within the biofilm. EPS and extracellular low molecular weight carbohydrates act as a carbon source for microbes and deposit feeders, as well as reducing water loss during low tide near the sediment surface (Duyl et al. 1999, Decho 2000, Underwood & Paterson 2003). EPS helps stabilize the sediment, which reduces erosion in salt marshes (van Duyl et al. 1999, Decho 2000, van Duyl et al. 2000, Stal

2003, Underwood & Paterson 2003). Most previous work has focused on processes that affect the production of EPS (Madsen et al. 1993, de Brouwer & Stahl 2001, Thornton et al. 2002, Underwood & Paterson 2003, Underwood et al. 2004, Hanlon et al. 2006). Recently, researchers have started to focus on the losses of EPS from the ecosystem due tidal movements, grazing by other organisms, and hydrolysis of the polymers by exoenzymes secreted by heterotrophic bacteria, and how these losses work in balance with EPS production (Hanlon et al. 2006, Haynes et al. 2007). Understanding this balance between EPS production and loss will lead to a better understanding of the overall function these photosynthetic biofilms provide to the mudflat ecosystem.

Extracellular Enzymes

Extracellular enzymes, also known as exoenzymes, function by breaking down plant and microbial cell walls, as well as other macromolecules, like EPS, for microbial assimilation (Sinsabaugh et al. 2008). A wide variety of exoenzymes are used to target different substrates in order to recycle the dissolved organic matter found in the ecosystem (Mulholland et al. 2003). Different bacteria species are known to release different enzymes that target specific substrates, and researchers have used activity measurements to look into changes in substrate concentrations in order to identify ecological changes in microbial environments.

Originally, exoenzyme research in marine systems focused on activity rates in the water column. Hoppe (1983) designed the technique most commonly used for measuring enzyme activity rates in marine systems. A specific fluorescent-tagged

substrate is added to a sample at a pre-determined concentration in order to saturate the sample with that substrate. A spectrofluorometer is used to measure the change in fluorescence over a set period of time due to the enzyme splitting the substrate from the fluorescent tag. As more substrates are split from the fluorescent tag, the measured fluorescence increases. Thus, fluorescence is proportional to the activity of the enzyme cleaving the tagged substrate, allowing for an activity rate to be calculated. This method determines the potential maximum rate of activity for the targeted enzyme and the remineralization rate of natural substrates involved in specific processes (Hoppe 1983). Hoppe (1983) determined that enzyme activities measured using this method show a close relationship to processes of substrate turnover seen in nature and this method is a useful tool to estimate the supply of low molecular weight substances that can be used by the microbial community.

One of the EPS losses mentioned above was through hydrolysis by exoenzymes secreted by bacteria. Bacteria are only capable of taking up carbohydrates smaller than 600 Da through their cell walls (Boetius & Lochte 1996), and thus the exoenzymes are necessary for bacteria to utilize the large pool of EPS and oligosaccharides produced by BMA. Exoenzyme activity tends to be the initial response of the bacterial community to environmental changes around them, thus this is often seen as the limiting step for the break down and recycling of organic matter (Misic & Harriague 2007). The connection between carbon cycling and bacteria exoenzymes demonstrates the coupling between BMA productivity and bacterial biomass. Middleburg, et al. (2000) established this connection through C-tracer experiments. These tracer studies showed the transfer of

photosynthetic carbon moving from the BMA to the bacteria within a matter of hours. This experiment indicated rapid bacterial utilization of carbon sources that likely included EPS (Middelburg et al. 2000, Haynes et al. 2007). Haynes et al. (2007) tried to focus on the coupling between bacteria and BMA by measuring enzyme activities and found a shift in the bacterial community driven by substrate enrichment of structural polysaccharides similar to EPS. Arnosti et al. (2005) revealed no correlation between enzyme activities and temperature in the water column in areas around the world suggesting that diversity in the bacteria community drive the abundance of exoenzymes and the enzyme activities more than environmental parameters in different marine systems.

In the past, it has been assumed that exoenzyme hydrolysis is the rate limiting step for bacterial uptake of small carbohydrate fractions in the water column, but measurements taken in the Chesapeake Bay have revealed high concentrations of low molecular weight carbohydrates in comparison with the total carbohydrate concentration indicating that the enzyme activities are much higher than the rate of utilization by the bacteria (Steen et al.2008). With an abundance of usable carbohydrates in the system, this suggests the possible loss from the sediment or burial within the sediment of these carbohydrates.

Nutrient Limitation and Enrichment Effects on Sediment Biofilms

Primary production by the BMA is dependent on light and nutrient availability, and recent research has shown strong effects of nutrient enrichment on the species

composition and diversity within the sediments (Hillebrand et al. 2000). Changes in nutrient concentrations can also dictate the dominant photosynthetic microalgal species in the biofilm as well. When an area is phosphorus enriched, cyanobacteria generally will have the greatest biomass within a mat because the cyanobacteria have the ability to fix nitrogen into a usable organic form once the available nitrogen has been used up (Pinckney et al. 1995). Whereas, a marked increase in nitrogen and phosphorus available to these microbial mats creates competition and the diatoms become the most abundant community in the mat at the expense of cyanobacteria (Pinckney et al. 1995). Nutrient pressure also affects the grazing of benthic microalgae by the meiofauna and other benthic organisms. Past research has seen an increase in grazing rates with an overall increase in benthic microalgae biomass, and this grazing actually increases diversity within the microbial mat due to selective grazing of the dominant species in the mat (Hillebrand et al. 2000). Thus the highest diversity is established at high resource levels. Increasing the nutrient concentration could also change the bacterial composition within the sediments. The bacteria are essential because much of the inorganic nutrients entering the mudflats are transformed by the bacteria in the sediments for use by other organisms, for burial within the sediments, or for export from the sediments (Thornton et al. 2007). Different bacteria perform different functions, therefore the addition of one nutrient over another could change the bacterial community drastically. Recent work in other salt marsh sediments have shown that even though the plant community may be nitrogen limited, the bacterial community may actually be phosphorus limited with a secondary limitation of labile carbon (Sundareshwar et al. 2003). Exoenzymes have

been used to identify the limiting nutrient in systems as well. A measured increase in alkaline phosphatase activity indicates a deficiency of phosphorus (Sala et al. 2001, Romani et al. 2004, Misic & Harriague 2007).

Study Objectives

This study had two main foci. First, this research determined changes in benthic microalgal biomass and changes in several carbohydrate fractions in sediment with the addition of nitrogen and phosphorus to the sediment. Secondly, the research investigated bacterial exoenzyme activity and the hydrolysis of complex carbohydrates into commonly found monosaccharides – glucose, xylose, and galactose – and if these activity rates change with nutrient enrichment and organic matter enrichment. While there has been research focused on changes on benthic microalgal biomass and carbohydrate concentrations, much of this research has focused on salt marshes located in temperate areas (Sündback & Graneli 1988, Underwood et al. 1998, van Duyl et al. 2000, Defew et al. 2002, Thornton et al. 2002, Underwood et al. 2004, Abdullahi et al. 2006, Costa et al. 2007, Thornton et al. 2007), whereas Galveston Bay is a subtropical environment. Plus, one issue effecting the bay is the influence of non-point source nutrient additions to the estuary ecosystem (Lester & Gonzales 2002). Also, there has been some controversy in the literature on whether glucose additions affect the activity rate of β-glucosidase, the exoenzyme that hydrolyzes polymers into glucose molecules (Boetius & Lochte 1996, van Duyl et al. 1999, Haynes et al. 2007). Very little research has considered exoenzymes that hydrolyze polymers into smaller carbohydrate fractions

by targeting certain monosaccharides. In this study β-xylosidase and β-galactosidase activities were measured as enzymes that break down polymers containing xylose and galactose, respectively. This study measured changes in microalgal carbohydrate production and loss, particularly through bacterial hydrolysis, with increased availability of inorganic nutrients and/or organic matter, in order to predict how these changes will affect the role of the carbohydrates within the microbial mats at the surface of salt marsh sediments. My main hypotheses for these experiments were:

1. The growth of the BMA is generally nitrogen limited on East Beach in Galveston Bay, so an addition of nitrogen will increase the BMA biomass and the EPS concentration within the sediment.

   Rationale: Nitrogen is generally the limiting inorganic nutrient in marine systems (Graneli & Sündback 1985, Sündback & Graneli 1988, Sündback & Snoeijs 1991, Pinckney et al. 1995, Underwood et al. 1998, Hillebrand et al. 2000, Thornton et al. 2002, Underwood & Paterson 2003, Underwood et al. 2004, Haese et al. 2007, Thornton et al. 2007, Pinckney & Lee 2008). An alleviation of the nutrient limitation will lead to an increase in the BMA biomass, which will also result in higher EPS concentration in the sediment since the BMA are the primary source of EPS.
2. The activity of the glycosidic exoenzyme β-glucosidase will be significantly higher in the sediment than β-xylosidase and β-galactosidase.

Rationale: Glucose is the major component of sediment polysaccharides; therefore, there is more substrate available to glucosidase enzymes.

3. The addition of different carbohydrate fractions to the sediment will change the glycosidic exoenzyme activity. Specifically, an addition of glucose will result in an inhibition of β-glucosidase activity, but an addition of alginic acid will not result a change in the glycosidase activity.

   Rationale: In an environment where glucose is readily available and a highly desired food source, it would be a waste of energy and resources for microorganisms in intertidal sediments to express β-glucosidases. Alginic acid is a polymer of uronic acids (β-1,4-linked d-mannuronic acid and β-1,4-linked l-glucouronic acid) (FAO 1997), and therefore, it should not be hydrolyzed by β-glucosidase, β-xylosidase, and β-galactosidase.

4. The addition of the limiting inorganic nutrient will result in an increase in exoenzyme activity of the glycosides (β-glucosidase, β-xylosidase, and β-galactosidase) that affect the hydrolysis of EPS and other polysaccharides.

   Rationale: With the addition of the limiting inorganic nutrient, there should be an increase in the BMA biomass, which leads to an increase in EPS. Therefore, the higher polysaccharide concentrations would necessitate more exoenzymes to be released in order to hydrolyze these

polysaccharides and support the organic carbon requirements of a greater biomass of microorganisms.

This research will provide more insight into the role that BMA production and exoenzymes have in regulating sediment carbohydrate concentrations with additions of nutrients and carbohydrates to the sediment surface. For example, EPS has been cited (van Duyl et al. 1999, Decho 2000, van Duyl et al. 2000, Defew et al. 2002, Stal 2003, Underwood & Paterson 2003, Hanlon et al. 2006) as playing a significant role in binding together the sediment in surface biofilms. Change in sediment EPS concentrations in response to inorganic nutrient enrichment could affect the resistance of sediment to erosion. Moreover, EPS is also a carbon source for other organisms living near the sediment surface, such as bacteria, who release the exoenzymes to hydrolyze the EPS. Yet, the BMA continue producing EPS, which will balance out these losses. These experiments will show if there are any changes to this balance of carbohydrate production and loss via hydrolysis due to increases in inorganic nutrient and carbohydrate concentrations introduced to the sediment.

CHAPTER II

EFFECT OF NUTRIENT ENRICHMENT ON BENTHIC MICROALGAL BIOMASS AND CARBOHYDRATE DYNAMICS

One of the major issues identified for Galveston Bay that needs to be regularly monitored is the non-point source nutrient and pollution inputs in to the bay (Lester & Gonzales 2002). This influx of additional nutrients could have a significant impact on the BMA biomass and the EPS concentration within the sediment, thus affecting the mudflat ecosystem. This experiment investigated the changes in benthic microalgal biomass with the enrichment of nitrogen and phosphorus concentrations. The hypothesis addressed with this experiment is that nitrogen is the limiting inorganic nutrient for the mudflat ecosystem near Galveston Bay and with this increase in nutrients, there would be an increase in the BMA biomass and therefore, the EPS concentration within the sediment.

Methods

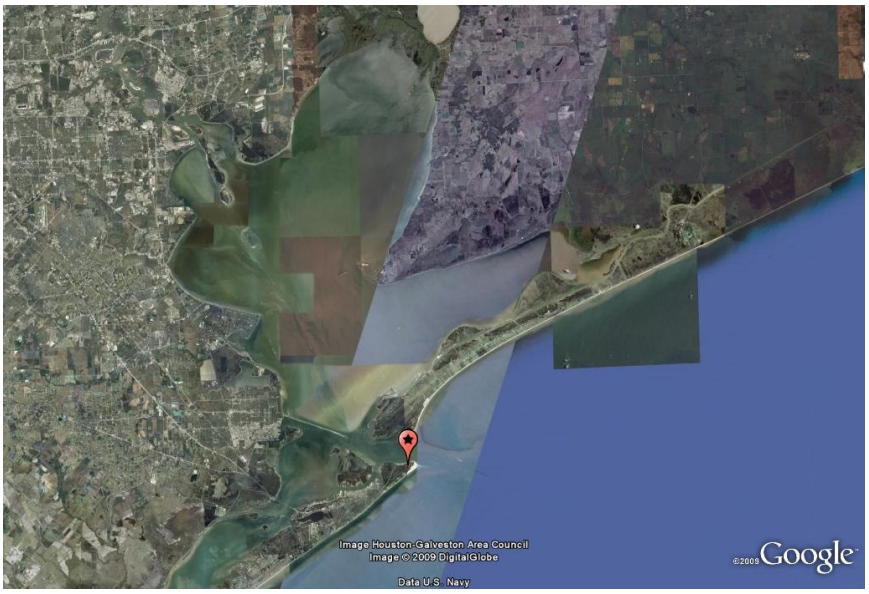
Sample Site

An intertidal salt marsh near East Beach on Galveston Island (Texas) was chosen for this study. The area, seen in the satellite image in Figure 1, is predominately mudflats and sandflats with patches of salt marsh plants and small stands of mangroves. The area experiences a regular tidal influence from the channel connecting Galveston Bay to the Gulf of Mexico. Figure 2 is an image of the immediate surroundings of the sampling site. This site was selected because it was less likely to be impacted by people and the flow of runoff into the bay.

Lab Experimental Design

During low tide 32 cores were taken from the sample site near the water's edge on September 3, 2007. The cores were collected with PVC pipe approximately 10 cm in diameter and cut to 10 cm in length. Once the sediment cores were removed using the PVC pipe, the cores were capped with a PVC cap at one end of the core. Approximately 90 L of water was collected from the sample site with plastic carboys. Three sediment samples were taken with a 20 mL syringe that had been converted into a push corer with a diameter of 1.7 cm and a depth of 0.5 cm. Three water samples were collected and filtered using a sterile 0.2 μm filter in order to measure the ambient nutrient concentrations. Water temperature and salinity measurements were taken at the sample site before leaving the site.

A



B

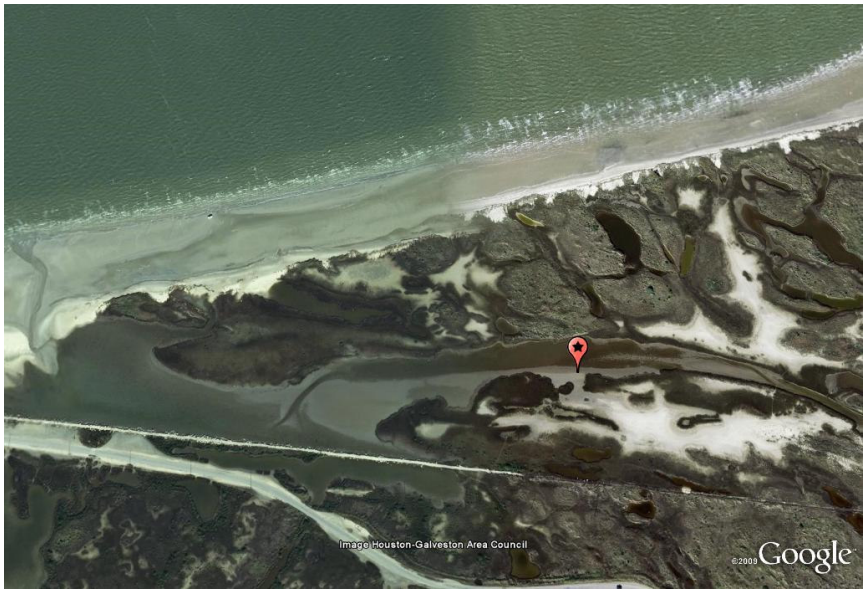


Figure 1: Satellite view of sample area on Galveston Island (Texas). (A) Sample site in relation to Galveston Bay and the entrance into the Gulf of Mexico. (B) Close up of the salt marsh containing the sample site.

Figure 2: Close-up photograph of sample area, Experiment #1. This photograph was taken on the intertidal mudflat used for sampling while facing toward the channel into the bay from the Gulf of Mexico.

Figure 3: Set-up for Experiment #1. Two tubs containing 4 cores each were used for each treatment – control, nitrogen addition, phosphorus addition, and nitrogen + phosphorus addition. Temperature and salinity were monitored in order to keep consistent with *in situ* measured temperature and salinity.

The cores were brought back to the lab at Texas A&M University in College Station, Texas for the experiment. The cores were split into four groups with 8 cores per group. Each set of 8 cores were treated with a different nutrient addition treatment – nitrogen addition, phosphorus addition, nitrogen + phosphorus addition, and a control of no addition. The cores were placed in plastic tubs with 10 L of water so that the cores within the tubs were completely covered. Four cores fit into one tub, therefore, there were two tubs randomly designated for each treatment. The tubs were placed in a small pool filled with reverse osmosis (RO) water that was circulated through a VWR Digital Temperature Controller to maintain the water within the tubs at the *in situ* temperature of 31.5 ^{o}C. Ten lamps containing 100 watt daylight florescence light bulbs were hung over the cores in the tubs. These lights were connected to timers allowing them to run for 12 hours each day providing 175-200 μmoles photons s^{-1}. Tubing was strung within each tub with air blowing through to continually mix the water within the tubs. (Fig. 3) The cores were left to settle down for approximately 12 hours before the first nutrient addition. The experiment started with the first addition of nutrients into the overlying water. Five mL of a concentrated solution of ammonia chloride was added to the tubs containing the cores designated for the nitrogen addition in order to produce a final concentration of 20 μM of nitrogen in the overlying water. Five mL of a concentrated solution of sodium phosphate was added to the tubs containing cores designated for the phosphorus addition in order to have a final concentration of 5μM of phosphorus in the overlying water. These nutrient concentrations were chosen to be high concentrations for the Galveston Bay area based on previously recorded measurements (Zimmerman &

Benner 1994, Santschi 1995, Lester & Gonzales 2002) in order to simulate nutrient enrichment that could naturally occur from non-point source runoff introduced by the Trinity and San Jacinto rivers. Both nutrient additions were added to the tubs containing the cores designated for the nitrogen and phosphorus additions. Immediately after the nutrients were added, one core from each treatment was sacrificed and sampled. Five sediment samples from each core were taken using the push corer (1.7 cm diameter and a sampling depth of 0.5 cm) for chlorophyll and carbohydrate analysis, placed into scintillation vials and frozen at -20 ^{o}C in the dark. Three 20 mL water samples were taken from the overlying water in each tub, filtered through a sterile 0.2 μm filter, and frozen for nutrient analysis. Every 12 hours, nutrients were added into the overlying water and one core from each treatment was sacrificed, allowing for the experiment to last 3.5 days. The light, salinity, and temperature were monitored and maintained throughout the experiment. Ultra high purity (UHP) water was added daily to the tubs in order to maintain the water level without changing the salinity. Once the lab experiment was complete, the sediment samples were freeze dried in the dark. The freeze dried sediment was crushed and large bits of vegetation were removed in preparation for the chlorophyll and carbohydrate analyses.

Chlorophyll Analysis

Chlorophyll *a* was analyzed spectrophotometrically based on a procedure modified by Stahl et al. (1984) from a procedure designed by Lorenzen (1967), which uses methanol to extract chlorophyll *a* from the sediment sample. Approximately 200

mg of freeze dried sediment from each sample was weighed into a 15 mL centrifuge tube. Two ml of 100% methanol was added to each tube. The methanol contained a small amount of magnesium carbonate to prevent the acidification of the sediment sample. The samples were quickly mixed and stored in the dark for 24 hours at 4°C. After 24 hours, the samples were quickly mixed again then centrifuged (Eppendorf centrifuge 5804R) for 15 minutes at 1789 x *g* and at a temperature of 4°C. One mL of each sample was pipetted into a polystyrene semi-micro cuvette and measured in a spectrophotometer (Shimadzu 1240 UV-mini spectrophotometer) at 665 and 750 nm. The second wavelength is used as a turbidity correction. A drop of 10% HCl was added to each cuvette to acidify each sample. After 5 minutes, the cuvettes were read again at 665 and 750 nm. The acidification allows for the correction of phaeopigments to be made to the chlorophyll measurements. A blank of methanol was used to zero the spectrophotometer at each wavelength. Using these absorbance measurements, chlorophyll *a* concentrations were calculated using equations developed by Stahl et al. (1984) and converted to $\mu g\ g^{-1}$ sediment.

Carbohydrate Analysis

Different carbohydrate fractions were extracted and analyzed spectrophotometrically using a procedure modified from frequently used methods (Underwood et al. 1995, Hanlon et al. 2006, Thornton & Visser 2009). Approximately 100 mg from each sample of freeze dried sediment were placed in 15 mL centrifuge tubes with 4 mL of 25 ‰ (w/v) NaCl solution. The centrifuge tubes were placed on an

orbital shaker and shaken for 30 minutes at room temperature and then centrifuged for 15 minutes at 1789 x *g* at a temperature of 4 ^{o}C. The supernatant contained the saline-extractable carbohydrate fraction. This fraction is mainly extracellular dissolved and colloidal carbohydrates containing saline-extractable EPS and low molecular weight carbohydrates. A volume of 0.8 mL of the supernatant was removed and placed in a Pyrex boiling tube. The carbohydrate concentration for this fraction was analyzed using the phenol-sulfuric acid assay designed by Dubois et al. (1956). A volume of 0.4 mL of 5 % (w/v) phenol and 2 mL concentrated sulphuric acid from a rapid dispensing bottle were added to each sample. The boiling tubes were shaken and allowed to cool for 30 minutes. One ml from each boiling tube was placed in a cuvette, and the absorbance was measured at 485 nm. The spectrophotometer was zeroed against a cuvette of UHP water.

Approximately 200 mg of freeze dried sediment (the exact weight was recorded) from each sample was measured out into 15 mL centrifuge tubes in order to extract saline-extractable EPS. The sEPS was extracted with 4 mL of 25 ‰ (w/v) NaCl solution by gently shaking for 30 minutes on an orbital shaker table. After centrifugation, 1.5 mL of the supernatant was pipetted into another 15 mL centrifuge tube with 3.5 mL of cold (4 ^{o}C) reagent alcohol. The tubes were placed in the dark for 24 hours at 4 ^{o}C. The tubes were centrifuged and the supernatant was discarded. A small amount of white precipitate containing the sEPS was located at the bottom of the centrifuge tubes. One

mL of UHP water was added to each tube and the tube was vigorously mixed for approximately 5 minutes in order to dissolve the precipitate. A volume of 0.8 mL from each centrifuge tube was placed into boiling tubes and the carbohydrates were analyzed using the phenol-sulfuric acid assay.

To measure the total carbohydrate concentration within each sediment sample, 25 mg of freeze dried sediment was added directly into the boiling tubes and analyzed using the phenol-sulfuric acid assay. After the boiling tubes cooled, the contents were transferred into centrifuge tubes and centrifuged before transferring the sample into the cuvettes for the absorbance measurements.

The phenol-sulfuric acid assay was calibrated using D-glucose dissolved in UHP water as the standard over the range 0 to 100 μg ml^{-1} (Fig. 4). Carbohydrate content of the sediment was expressed as glucose equivalents and normalized to dry weight of the sediment.

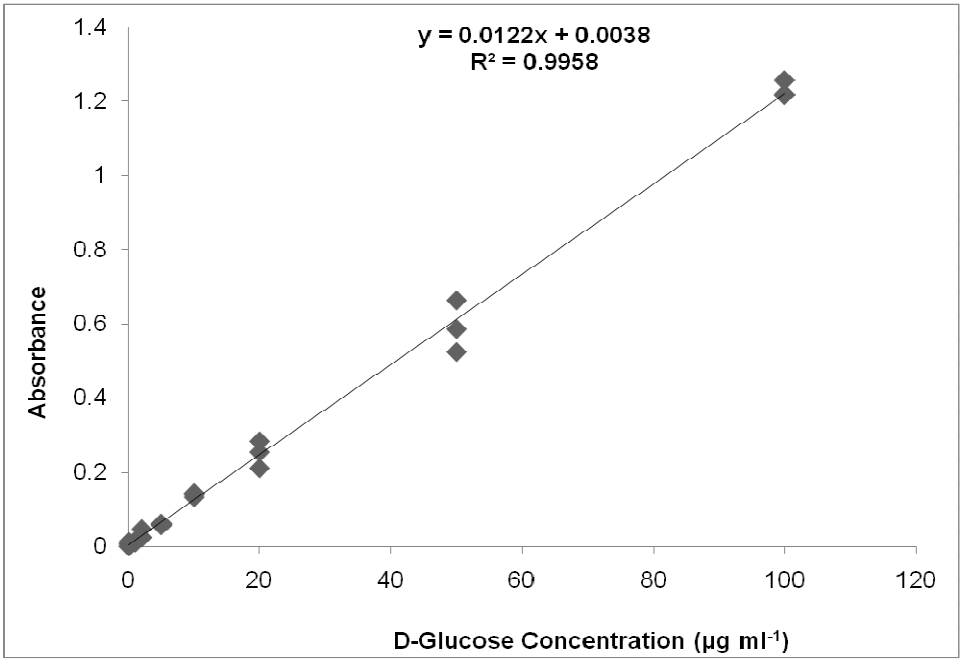


Figure 4: Carbohydrate calibration for Experiment #1.

Nutrient Analysis

One sample of frozen 0.2 μm filtered overlying water for each treatment at each time period was sent to GERG (Geochemical Environmental Research Group, Texas A&M University) in order to measure the nitrate, ammonium, phosphate, nitrate and silicate concentrations using a Technicon II Autoanalyzer with procedures based on and modified from Aminot and Kerovel (1982), Armstrong et al. (1967), Bernhardt and Wilhelms (1967), and Harwood and Kuhn (1970).

Statistical Analysis

Data was analyzed using Sigmastat 3.1 (Systat Software, Inc.). Analysis of variance (ANOVA) statistical tests were conducted on the chlorophyll and carbohydrate data sets that met the assumptions of normality and equality of variance. Data that did not meet these assumptions was log(n+1) transformed before analysis. Post hoc Tukey tests were conducted along with the ANOVAs. Regression analyses were calculated to on the data to identify relationships between the chlorophyll and carbohydrate data.

Results

Nutrient Analysis

The *in situ* nutrient concentrations of the water nearest the sample site were 7.7 μmoles L^{-1} ammonium, 1.8 μmoles L^{-1} nitrate, 1.8 μmoles L^{-1} phosphate, and 41.5 μmoles L^{-1} silicate. Figure 5 shows the ammonium, phosphate, nitrate and silicate concentrations over the duration of the experiment. The black line on the ammonium and phosphate graphs (Fig. 5A and 5B) display the potential concentrations after the nutrient were added to the tubs assuming none of the additions were consumed or produced by processes within the tub. The ammonium concentration remained lower than 10 μM, except for the nitrogen treatment peaking at 12.5 μM after 60 hours into the incubation. Phosphate concentrations within each treatment increased over the incubation period, but the largest increase occurred in the treatments of phosphorus and nitrogen + phosphorus with peak concentrations of 14.7 μM and 14.5 μM respectively after 84 hours. Neither the ammonium or phosphate concentrations were near as high as the expected concentrations. Nitrate concentrations (Fig 5C) saw very little change over the course of the experiment within the nutrient enriched treatments. The control treatment increased over the first 24 hours and then decreased to a concentration near the initial concentrations of nitrate. Silicate concentrations (Fig. 5D) decreased through the

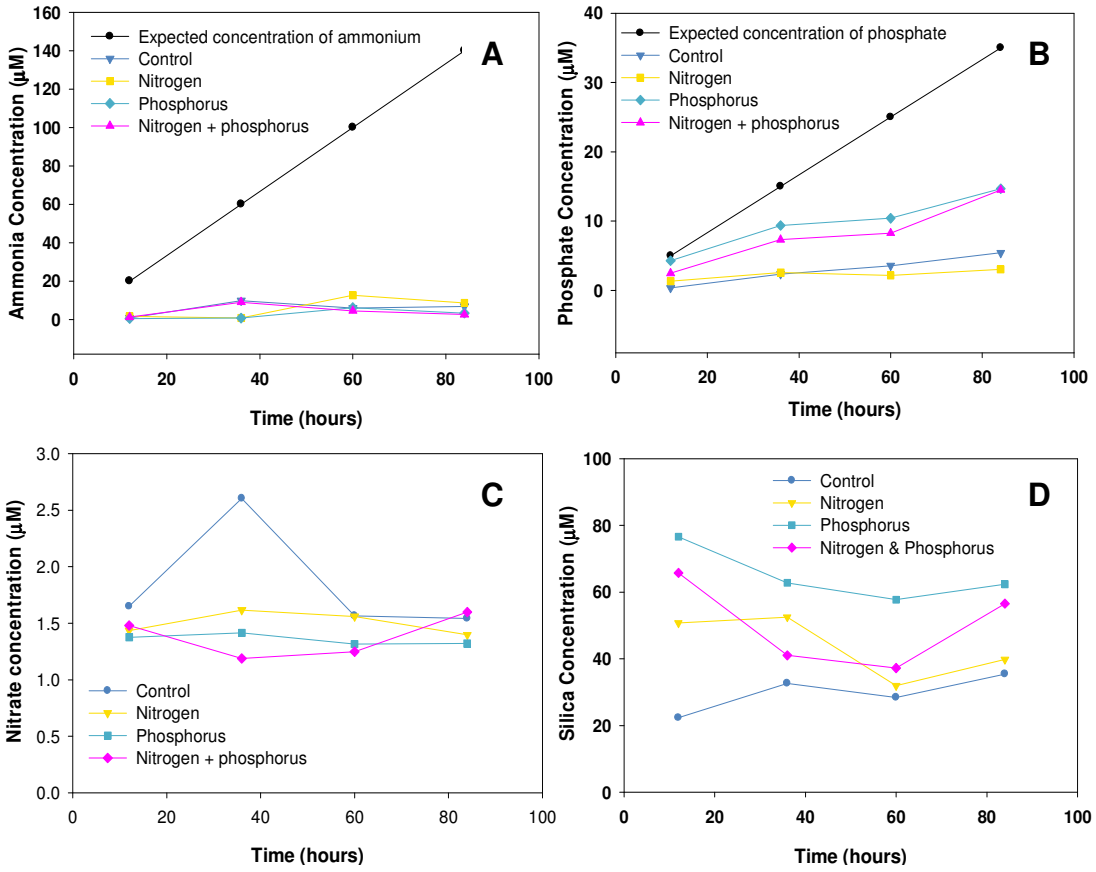


Figure 5: Nutrient concentrations for Experiment #1. Measurements were from the overlying water from each treatment over the course of the experiment. Both A and B have a line displaying the nutrient concentrations if none of the additions were changed by processes within tubs. (A) ammonia concentration (B) phosphate concentration (C) nitrate concentration (D) silicate concentration. Data points show concentrations of one sample from each treatment.

course of the incubations with the nitrogen and the phosphorus treatments, increased with the control treatment, and initially decreased, then increased in the nitrogen + phosphorus treatment. A decrease in silicate indicates a possible increase in BMA biomass as the diatoms use the silicate for their frustules.

BMA Biomass

Figure 6 shows the change in chlorophyll *a* concentrations with each treatment over the course of the incubations. Chlorophyll *a* is generally used an indicator for BMA biomass within the sediment. Over the course of this experiment, there was no significant difference between the treatments added to the cores, but there were two significant trends that occurred. There was a significant difference in chlorophyll *a* concentrations with sampling time over the course of the experiment ($F_{7, 128} = 3.087$, $p < 0.01$) Using post-hoc pair wise tests, a significant decrease ($p < 0.05$) in BMA biomass was seen from the beginning of the experiment to 24 hours into the experiment either indicating a die off of the BMA or increased herbivory from the meiofauna within the sediment. All treatments saw an increase in BMA biomass after 24 hours, but the increase seen in the nitrogen + phosphorus addition was significant ($p < 0.05$). The chlorophyll *a* concentration in this treatment increased from 9.7 $\mu g\ g^{-1}$ sediment ± 3.8 (mean ± standard deviation) to 19.4 $\mu g\ g^{-1}$ sediment ± 4.8 after 72 hours.

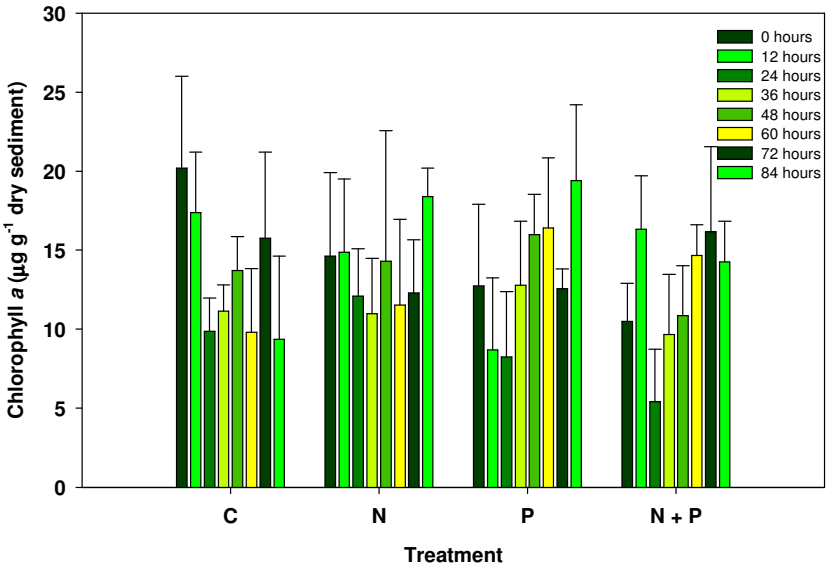


Figure 6: Chlorophyll concentrations for Experiment #1. Concentrations were from within the sediment over time with each nutrient addition. The treatments were control (C), nitrogen addition (N), phosphorus addition (P), and nitrogen + phosphorus (N + P). No nutrients were added to the control cores. Bars show mean + SD (n = 5).

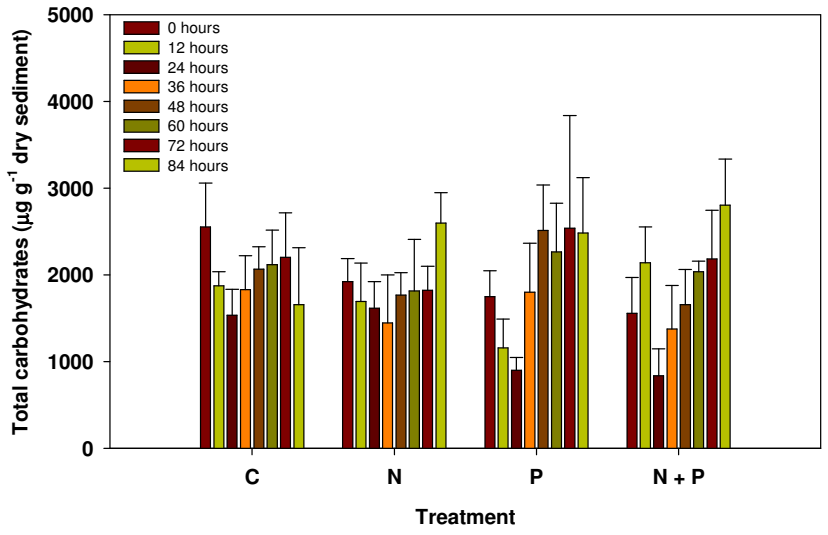


Figure 7: Total carbohydrate concentrations for Experiment #1. Concentrations were from within sediment over time with each nutrient addition. The treatments were control (C), nitrogen addition (N), phosphorus addition (P), nitrogen + phosphorus addition (N + P). No nutrients were added to the control cores. Bars show mean + SD (n = 5).

Carbohydrates

Figure 7 shows the change in the total carbohydrate concentration over time with treatment. There was no significant difference between the treatments added to the sediment cores, but a couple of significant differences within the treatments with time did occur ($F_{21, 127} = 4.176$, $p < 0.001$). Using post-hoc pair wise tests, an initial significant decrease ($p < 0.001$) was seen over the first 24 hours within the phosphorus treatment to a concentration of 902 ± 144 μg g^{-1} sediment. Over the following 24 hours, there was a significant increase ($p < 0.001$) in the total carbohydrate concentration for the phosphorus treatment of 2511 ± 528 μg g^{-1} sediment, and the concentration remained there for the remainder of the experiment. The same decreasing, then increasing trend was seen in the nitrogen + phosphorus treatment. Over the first 24 hours, the total carbohydrate concentration decreased ($p < 0.05$) to 838 ± 308 μg g^{-1} sediment, and the total carbohydrate concentration increased significantly ($p < 0.001$) from the first 24 hours to the end of the experiment to a concentration of 2803 ± 529 μg g^{-1} sediment.

Figure 8 shows the change in the saline-extractable carbohydrate concentrations within the sediment over time with treatment. Again, there was no significant difference between the treatments for the concentration of saline-extractable carbohydrates. The same trends of an initial decrease in concentration followed by an increase in concentration were seen with this carbohydrate fraction that was seen with the total carbohydrate concentration ($F_{21, 126} = 4.037$, $p < 0.001$). Using post-hoc pair wise tests, for both the phosphorus treatment and the nitrogen + phosphorus treatment, a significant increase ($p < 0.05$) was only seen from between the first 24 hours and the final sampling

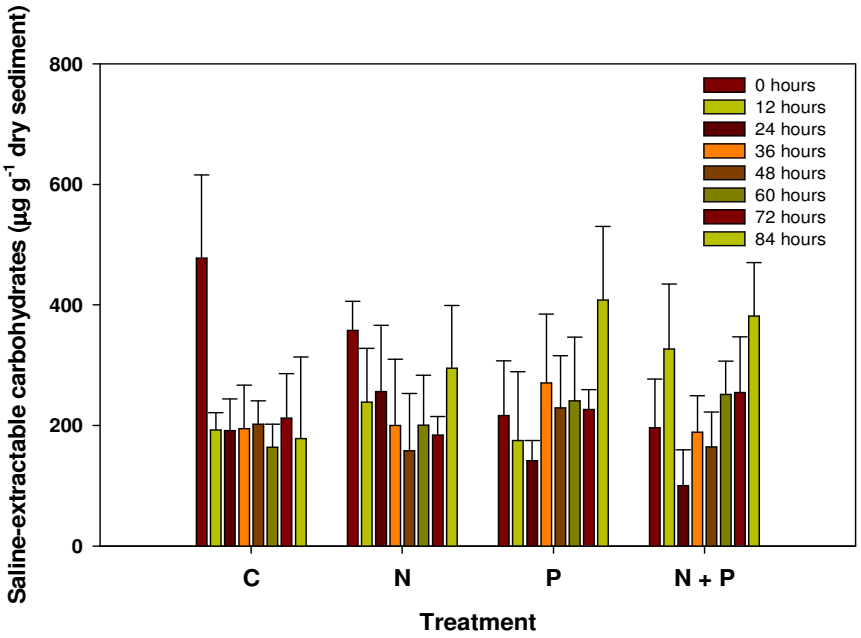


Figure 8: Saline-extractable carbohydrate concentrations for Experiment #1. Concentrations were from within sediment over time with each nutrient addition. The treatments were control (C), nitrogen addition (N), phosphorus (P), and nitrogen + phosphorus addition (N + P). No nutrients were added to the control cores. Bars show mean + SD (n = 5).

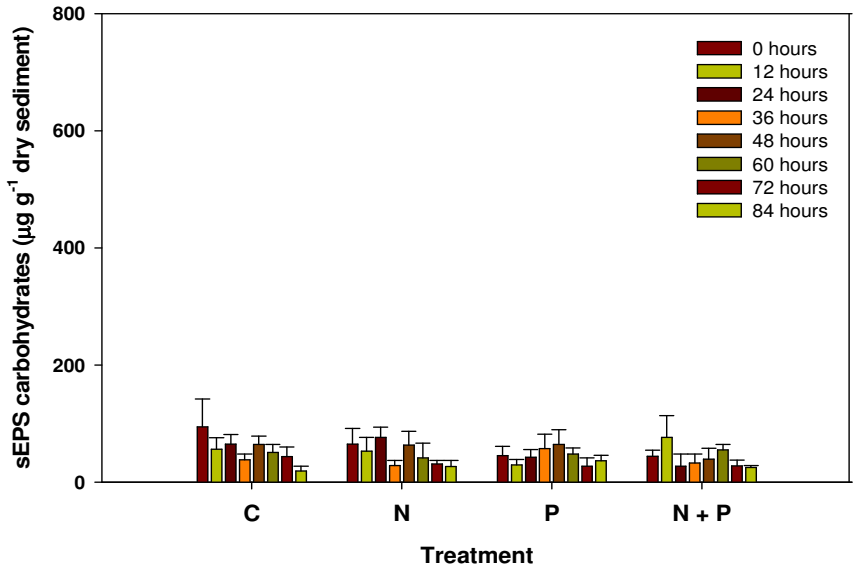


Figure 9: sEPS carbohydrate concentrations for Experiment #1. Concentrations were from within sediment over time with each nutrient addition. The treatments were control (C), nitrogen addition (N), phosphorus addition (P), and nitrogen + phosphorus addition (N + P). No nutrients were added to the control cores. Bars show mean + SD (n = 5).

time of 84 hours, with final concentrations of 408 ± 122 and 382 ±88 μg g^{-1} sediment, respectively.

Figure 9 shows the change in the sEPS carbohydrate concentrations within the sediment over time with treatment. There was a significant difference among the treatments seen in the control treatment ($F_{3, 126} = 3.905$ $p < 0.01$), which was significantly higher ($p < 0.05$) in concentration than the nitrogen + phosphorus treatment, particularly over the first 24 hours of the experiment. No other major trends were seen over the course of the experiment for the sEPS carbohydrate concentrations.

Figure 10 shows the linear correlations between chlorophyll *a* concentrations and saline-extractable carbohydrate and sEPS carbohydrate concentrations. The data were pooled for the whole experiment, irrespective of treatment and sampling date. While both regressions were significant ($p < 0.001$), the correlation between the chlorophyll *a* concentrations and the saline-extractable carbohydrate concentrations, seen in Fig. 6A, was stronger ($r^2 = 0.453$), than the correlation between the chlorophyll *a* concentrations and the sEPS carbohydrate concentrations ($r^2 = 0.140$).

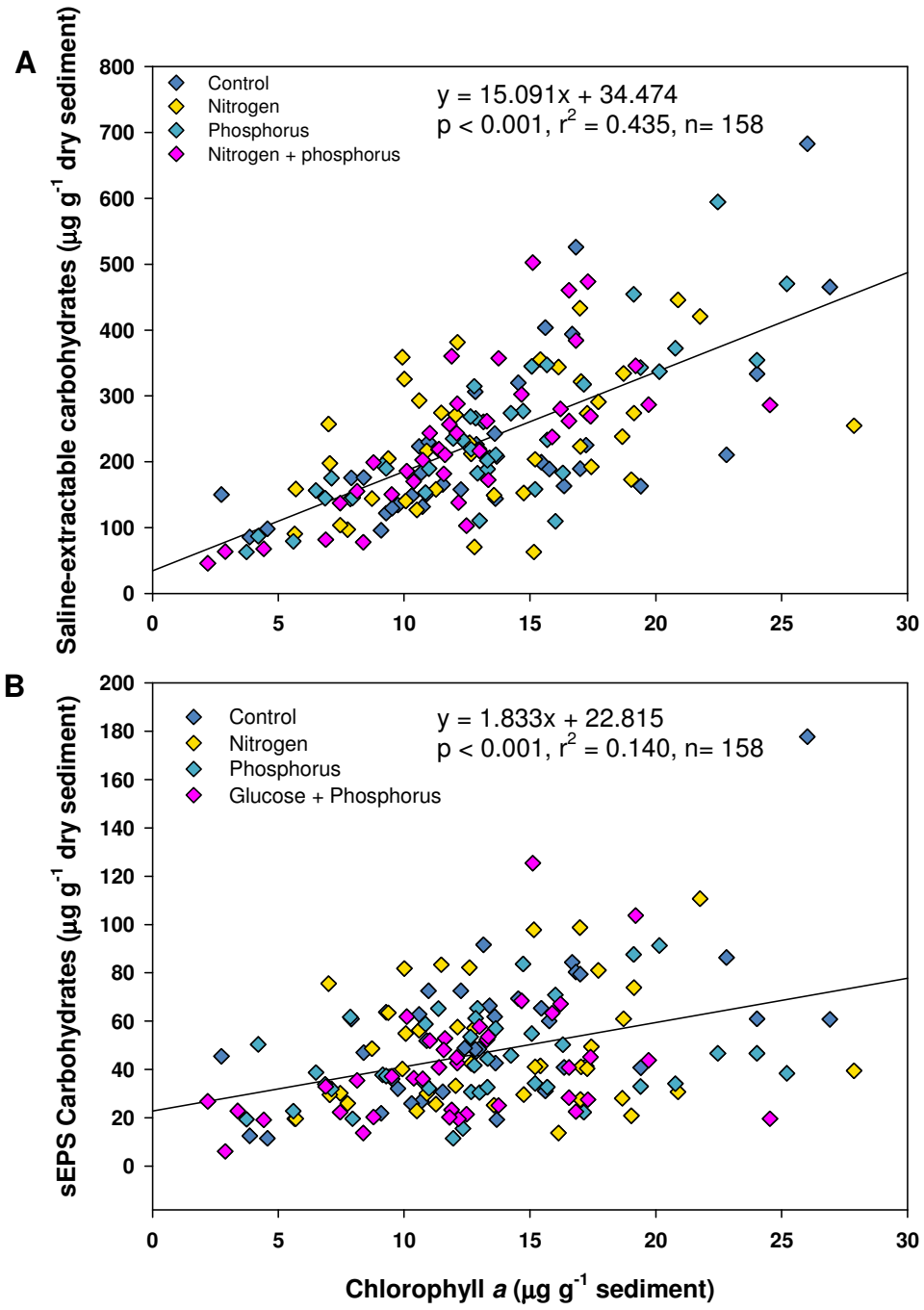


Figure 10: Chlorophyll and carbohydrate relationships for Experiment #1. Relationships are between chlorophyll *a* with saline-extractable carbohydrate concentrations and sEPS carbohydrate concentrations. (A) Saline-extractable carbohydrates (B) sEPS carbohydrates.

Discussion

Influence of Nutrients on BMA Biomass

The nutrients were added to the sediment cores in an effort to simulate high nutrient supply and to determine whether nitrogen or phosphorus was the inorganic nutrients limiting the biomass of the BMA at the site. Chlorophyll *a*, along with the saline-extractable carbohydrate fraction, was used as a proxy to determine growth in the BMA. The nutrient additions did not affect very much change in the BMA biomass throughout the experiment. Overall, the chlorophyll *a* concentration, the total carbohydrate concentration, and the saline-extractable carbohydrate concentration displayed a decrease over the initial 24 hours, followed by an increase in concentration. This looks to be an indication of a die off of the BMA possibly from increased grazing pressure or a shift in the species composition of the photosynthetic microalgae. Neither nitrogen nor phosphorus could be identified as the limiting inorganic nutrient with this experiment and there was little change in the BMA biomass and EPS concentrations. Therefore, the hypothesis for this experiment can be rejected.

A BMA response to nutrient additions has not always been seen in past research as well. Neither Underwood et al. (1998) or Stutes et al. (2006) saw a strong response in the BMA biomass and primary production with nutrient enrichment. High variability in biomass can be common amongst intertidal sediment diatoms due to a variety of factors, such as erosion, tidal exposure, sediment deposition, sediment desiccation, temperature, grazing pressure, and interspecific competition between populations

(Admiraal et al 1984. Underwood et al. 1998). Pinckney and Lee (2008) found that the subtidal BMA biomass across Galveston Bay can be variable with the season and spatially throughout the bay. Light availability has also been cited as the possible limiting factor in estuarine systems preventing an effect from nutrient limitation to appear (Stutes et al. 2006, Hanlon et al. 2006). At temperate latitudes, the sediment can experience irradiances of over 2000 μmol m^{-2} s^{-1} during the day, especially during periods of tidal emersion (Perkins et al. 2001), and the sediment cores in this experiment only received approximately 200 μmol m^{-2} s^{-1} for 12 hours periods daily through the lighting system set up in the lab. While Perkins et al. (2001) did not see the highest rates of carbohydrate production when the irradiance was at its highest, they did see increased rates of low molecular weight carbohydrates as the irradiance approached 600 μmol m^{-2} s^{-1} and increased rates of sEPS as the irradiance approached 460 μmol m^{-2} s^{-1}. These irradiances were still much higher than the irradiance provided to the cores in this experiment, thus it is likely the BMA within the cores in this experiment may have been light limited, but the BMA's response to light can be complex. Lower levels of irradiance over a long period of time, as seen in this experiment, can provide a similar response in the BMA as compared to shorter bursts of high levels of irradiances seen in natural systems. In other studies, even when biomass and productivity increases were measurably observed with nutrient additions, hypotheses were still suggested about other environmental factors that could directly or indirectly affect these increases regardless of the nutrient additions (Armitage et al. 2006, Graneli & Sündback, 1985).

Although the nitrogen + phosphorus treatment was not significantly different from the other treatments in chlorophyll *a* and carbohydrate concentrations, this treatment did show significant increases and decreases in the chlorophyll *a*, saline-extractable carbohydrate, and total carbohydrate concentrations. Pinckney et al. (1995) noticed enhanced diatom growth in their treatment of nitrogen + phosphorus added to sediment located in an estuary in North Carolina, indicating a possible co-limitation at high nitrogen levels. No measurements of dissolved inorganic nitrogen concentrations within the sediment porewater were taken, however, the ammonium concentration did not increase in the overlying water in any of the treatments over the course of the experiment, meaning the ammonium additions to the water were regularly utilized by microorganisms in the overlying water or transformed throughout the experiment. Ammonium can be transformed into nitrate through nitrification or into nitrogen gas though the process of anammox (Dong et al. 2000). There was very little change seen in the nitrate concentrations in the nutrient enriched treatments over the course of the experiment in the overlying water suggesting that any ammonium transformed into nitrate was used up in that form as well. Zimmerman and Benner (1994) measured ammonium and phosphate fluxes from the sediment across Galveston Bay in spring and summer months. They saw a flux from the sediment to the water column of approximately 2μM m^{-2} h^{-1} of phosphate and approximately 22 μM m^{-2} h^{-1} ammonium. If flux measurements were taken for this experiment, it may have helped identify the movement of the added nutrients.

There was a positive correlation between chlorophyll *a* concentration and saline-extractable carbohydrate concentration during this experiment. There tends to be a close positive relationship between the BMA biomass and the saline-extractable carbohydrate concentration (Underwood & Smith 1998, Thornton et al. 2002, Hanlon et al. 2006). This correlation was not seen between chlorophyll *a* and sEPS concentrations. This production of low-molecular weight carbohydrates is often closely associated with photosynthesis (Bellinger et al. 2005).

Methodological Improvements

A few issues arose that might have affected the results of the nutrient additions. The lab setup may not have been representational of *in situ* conditions due to certain limitations. Light availability has been cited previously as the overall limitation in growth, instead of the nutrient concentrations, for estuarine sediments (Graneli & Sündback 1985, Hanlon et al, 2006, Stutes et al. 2006), and it is possible that the sediment cores were not receiving enough light in the lab setup. The travel time between Galveston and College Station may have also been a factor affecting the microalgal growth in the sediment cores. Also while transporting the cores, they experienced physical disturbance due to the shaking and vibrations from the car, which can agitate the BMA and the biofilm structures within the sediment. The cores were allowed to settle for 12 hours before starting the experiment, but the microbial community may have needed more time to re-establish before beginning the experiment. Sündback and Snoeijs (1991) noted that in their sediment cores, space for colonization

may have been a limitation for growth for their experiment. This could also have been an issue with the cores for this experiment. The overlying water in the tubs was not filtered before placed in the tubs with the cores. Any biological activity in the water could have affected the amount of nutrients entering into the sediments after the additions.

In order to continue this experiment in the future, these issues would need to be addressed. If this experiment was run multiple times across the seasonal changes, the limiting nutrient might be more easily identified. Evidence of nutrient limitation can be seen seasonally due to changes in other environmental factors like light availability and temperature. It would also be useful to monitor the meiofaunal and bacterial biomass using microscopy in order to address any trophic interactions that could affect the BMA biomass with nutrient additions. It would also be useful to identify the species composition of the photosynthetic microorganisms in order to recognize any compositional shifts due to the nutrient enrichment. Species composition could be determined either through direct cell counts (Sündback & Snoeijs 1991, Underwood et al 1998, Hillebrand et al. 2002) or using high performance liquid chromatography (HPLC) to determine concentrations chlorophyll *a*, as well as chlorophyll *b*, fucoxanthin, and zeaxanthin (Pinckney et al. 1995, Armitage et al. 2006, Pinckney & Lee 2008). These different photosynthetic pigments are indicators of different assemblages of photosynthetic microorganisms within the sediment. A better understanding of the trophic interactions within the sediment could lead to an environmental cue to eutrophic conditions.

CHAPTER III

EXOENZYME ACTIVITY IN SALT MARSH SEDIMENTS

Because EPS and the other carbohydrate fractions are ecologically important for the mudflat ecosystem, it was important to continue focusing on these carbon fractions. The perspective was switched from measuring carbohydrates in order to infer the production by the BMA with nutrient enrichment to measuring the hydrolysis of these carbohydrates with nutrient and carbohydrate enrichment. The second set of experiments focused on the effectiveness of certain exoenzymes -- β-glucosidase, β-xylosidase, and β-galactosidase -- which hydrolyze oligo-and polysaccharides like EPS, in un-amended sediments and sediments to which carbohydrates (glucose or alginic acid) or phosphorus had been added *in situ*. There were three hypotheses set forth for this set of experiments. First, the glycosidic activity of the exoenzyme β-glucosidase will be higher than β-xylosidase and β-galactosidase. Second, an addition of carbohydrates will change the enzyme activity of the same three exoenzymes. Third, an addition of the limiting inorganic nutrient will increase the BMA biomass and the EPS concentration, which will lead to an increase in the activities of β-glucosidase, β-xylosidase, and β-galactosidase.

Methods

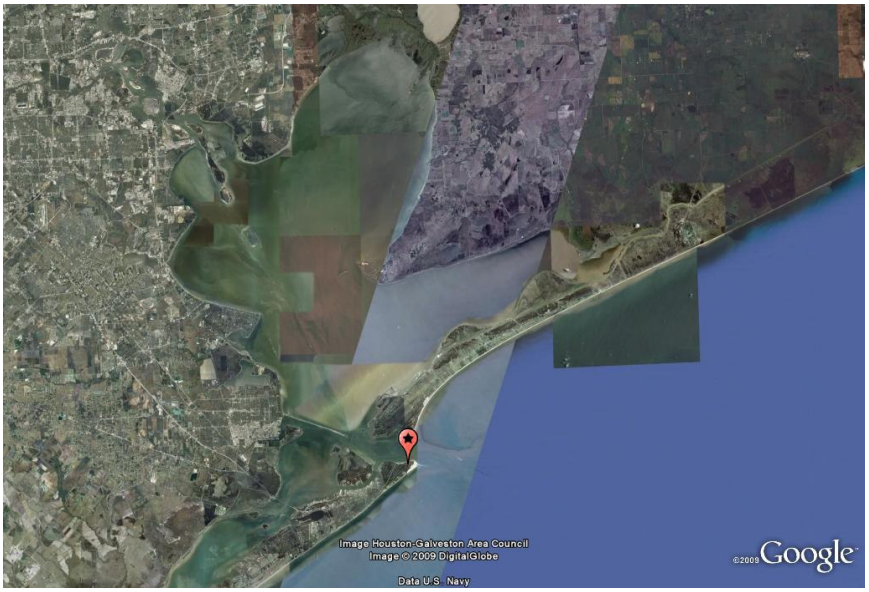
Sample Site

An intertidal salt marsh near East Beach on Galveston Island (Texas) was chosen as a convenient location for this study. The area, seen in the satellite image (Fig. 11), is predominately mudflats and sandflats with patches of salt marsh plants and small stands of mangroves. The salt marsh experiences a regular tidal influence from the channel connecting Galveston Bay to the Gulf of Mexico. Figure 12 is an image of the immediate surroundings of the sampling site.

Sediment Sample Preservation Methods

Before beginning the full experiments, a preliminary experiment was conducted to decide upon a preservation strategy for the sediment samples used to measure enzyme activity. Enzyme activity can change rapidly with the change in substrate availability

A



B

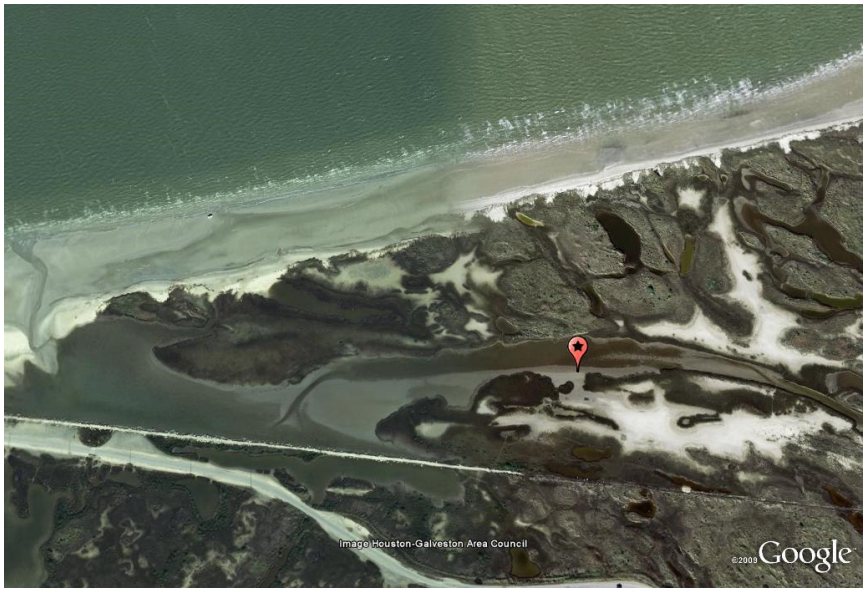


Figure 11: Satellite images of sample area on Galveston Island (Texas). (A) Sample site in relation to Galveston Bay and the entrance into the Gulf of Mexico. (B) Close up of the salt marsh containing the sample site.

Figure 12: Close-up photograph of sample area, Experiment #2. This photograph was taken on the intertidal mudflat used for sampling while facing toward the channel into the bay from the Gulf of Mexico.

and temperature, thus it was necessary to find a method for transporting these samples and preserving them in order to keep the measured enzyme rate in the lab similar to what it would be if measured immediately after sampling. Past researchers using similar methods have used sodium azide to kill the organisms within the sediment (Hanlon et al. 2006), while other researchers kept their samples cold before measuring enzyme rates (Boetius & Lochte 1994, Penton & Newman 2007). For the preservation test, scintillation vials were set up to collect six sediment samples (cores, 1.7 cm in diameter and 0.5 cm deep) for each preservation strategy – sodium azide at room temperature, sodium azide at 4^{o}C, formalin at room temperature, formalin at 4^{o}C, no preservative at room temperature, and no preservative at 4^{o}C. Each vial contained 15 mL of Harrison's artificial seawater at a salinity of 20 ppt based on average salinity measurements previously measured at the sample site. Harrison's artificial seawater was created as a salt base, thus no inorganic nutrients were added to the samples with this solution. The recipe for Harrison's was modified by Berges et al. (2001) from a recipe created by Harrison et al. (1980). The liquid was necessary to create a slurry for the enzyme activity analysis and to add preservative to those vials that required it.

Preliminary Slurry Experiment to Determine Nutrient Additions

A preliminary experiment was also conducted to determine which nutrients and carbohydrates might elicit a response in the enzyme activity in the sediment from my sample site. The treatments for the nutrient and carbohydrate preliminary slurry

experiments were added nitrogen, added phosphorus, added nitrogen + phosphorus, added glucose, and added alginic acid. For this experiment, six samples (cores, 1.7 cm in diameter and 0.5 cm in depth) were collected for each treatment. Prior to sampling, the treatments were added to 15 mL of Harrison's in each scintillation vial. 20 μM ammonia chloride was added to the vials for nitrogen additions and 5 μM sodium phosphate was added to the vials for phosphorus additions. One hundred μg g^{-1} alginic acid was added to the vials for the alginic acid additions, and 300 μg g^{-1} glucose was added to the vials for the glucose additions. The sediment weight was estimated based on the average weight of the sediment in the small cores collected from the sample site from previous visits. The nutrient concentrations were based on previously recorded measurements (Santschi 1995, Lester & Gonzales 2002) in order to simulate nutrient enrichment that could naturally occur from non-point source runoff introduced by the Trinity and San Jacinto rivers. The carbohydrate additions were based on naturally occurring sediment concentrations collected previously (Thornton & Visser 2009).

Small sediment cores using a push corer (1.7 cm in diameter, depth of 0.5 cm) were placed within each vial and all the vials returned to the lab. Maximum enzyme rates were measured using a potentially saturating concentration of 500 μM of β-glucosidase. This concentration was decided on based on practice kinetic runs with β-glucosidase preformed on sediment samples collected near the sample site. Measurements were taken on return from Galveston, and then 1 day, 2 days, 4 days, and 11 days after the samples were collected. The procedure used to measure enzyme activities is detailed below.

In Situ *Experiment Design*

Experiment #2.1 focused on the carbohydrate additions. Before heading to the sample site, 0.2% (w/v) sodium azide was mixed with artificial seawater and 15 mL were added to scintillation vials in preparation for the sediment collection and preservation for measuring enzyme activity. The bottoms were removed from polyethylene sandwich boxes to create rectangular plot enclosures. The plot enclosures were created to contain the treatment added to the sediment and to define the area where samples were collected. The enclosures had a lip that was raised approximately 1 cm above the sediment and extended approximately 2 cm into the sediment. The area of the sediment surface within the plot enclosures was approximately 257 cm^2. Each plot contained a volume of approximately 128 cm^3 in the upper 5 mm, which was the depth sampled. It was assumed that any additions made to the sediment would remain in the upper 5 mm and be diluted in this sediment volume. This volume was used to calculate the concentration of carbohydrate additions for this experiment. Glucose was chosen as an addition to simulate an increased concentration in low-molecular weight carbohydrates and alginic acid was chosen as an addition of a polysaccharide with similar properties to EPS. The final sediment content of the glucose and alginic acid additions were based on the naturally occurring sediment content of saline-extractable carbohydrate (300 $\mu g\ g^{-1}$ sediment) and EPS carbohydrate (100 $\mu g\ g^{-1}$ sediment) sediment contents (Thornton & Visser 2009). In order to induce a response in enzyme activity with the addition, carbohydrates were added at twice the naturally occurring

sediment content. Carbohydrates were added in a 50 mL solution that was poured on top of the sediment to produce an addition of 600 μg g^{-1} of glucose and 200 μg g^{-1} of alginic acid within the top 5 mm of the sediment. These liquid treatment additions were created in the lab by dissolving the added carbohydrate in Harrison's artificial seawater at a salinity of 20 ppt. Because artificial seawater was being added to the sediment, a third treatment for this experiment was an addition of just the seawater to see if it affects the sediment. The fourth treatment was a dry control where no treatment was added to the surface of the sediment.

The sampling occurred from October 29, 2008 to November 4, 2008. On arriving at the sample site on Galveston Island, the plot enclosures were assigned a treatment – 4 plot enclosures per treatments – and randomly laid down and staked in place in 2 rows of 8 enclosures within the intertidal zone. A push corer was used to collect sediment cores (1.7 cm in diameter and 0.5 cm in depth) for carbohydrate and

chlorophyll *a* analysis. A 100-1000 μL pipette tip, with most of the tip cut off, was used to collect small sediment samples (0.7 cm in diameter and 0.5 cm in depth) for bacterial counts. Before adding the treatments to the sediments, initial samples were collected – 1 core per plot for chlorophyll and carbohydrate analysis, 1 core per plot preserved in sodium azide for exoenzyme analysis, and 1 small core per plot preserved in 4% (v/v) formalin for bacteria counts. All samples were immediately put on ice in the dark. The treatment was then carefully poured on top of the sediment in the appropriate plots so that a layer approximately 2 mm deep of liquid slowly percolated into the sediment. Temperature measurements were taken from the sediment and the closest area of standing water and salinity measurements were taken from that same area of standing water before leaving the site. Samples were again taken during the same low tide period approximately 2 hours after adding the treatment. Samples were then to be taken after 1 day, 2 days, 5 days, and 7 days later. Figure 13 is an image of the *in situ* set up.

Figure 13: Set-up for Experiment #2. Photograph of the plot enclosures placed on the sediment. Four plots for each treatment were randomly placed in to the sediment into 2 rows of 8 plot enclosures.

Experiment #2.2 focused on glucose additions again, as well as phosphorus additions. The glucose concentration was kept that same as in Experiment #2.1. The phosphorus concentration needed to be high enough in order to induce a response and so it was not the limiting nutrient, thus an intended sediment concentration of 10 μM within the sediment was decided upon. The glucose and phosphorus liquid additions were made up the lab the same way as before with artificial seawater at a salinity of 20 ppt. Five treatments were prepared – glucose addition, phosphorus addition, glucose + phosphorus addition, a control of saline addition, and a control of no addition. Sodium azide was not used as a preservative for this experiment because despite precautions, there were concerns about potential exposure of researchers in the lab to the highly toxic preservative. Instead the samples were placed on ice and kept cold immediately after collection.

This Experiment ran from November 15 to 16, 2008. The experiment time period was shortened because based on previous experiments, any response from the additions would be visible over the first 24 hours. Twenty plot enclosures were placed in the sediment, with 4 replicates of each treatment. The plots were laid down in 2 rows with 10 in each row on the intertidal sediment, and the plots were randomly assigned a treatment to reduce artifacts that may have resulted from grouping the replicates together. Samples were collected prior to adding the treatment. Along with the sediment samples for chlorophyll and carbohydrates, exoenzyme analysis and bacteria counts, one more push core sample was collected to measure the available phosphate concentration within the pore water. The treatments were added to the appropriate plots

creating a layer of liquid above the sediment surface. Another set of samples were collected approximately 2 hours later and then again 24 hours later. All the samples were kept on ice until returning to the lab.

After returning to the lab, all the sediment cores for chlorophyll *a*, carbohydrate, and phosphate concentration analysis were kept in the dark at -20 ^{o}C. All the samples for enzyme analysis and the bacteria counts were kept in the dark at 4 ^{o}C. All the sediment core samples collected for both Experiments #2.1 and 2.2 for chlorophyll and carbohydrate analysis were freeze dried overnight in the dark at GERG.

Exoenzyme Analysis

The exoenzyme analysis was started as soon as the samples arrived at the lab. The procedure to measure the exoenzyme activity rates was modified based on the method of Marx et al. (2001), which was modified from a procedure designed by Hoppe (1983). The substrates, 4-methylumbelliferone-β-D-glucoside, 4-methylumbelliferone-β-D-xyloside, and 4-methylumbelliferone-β-D-galactoside, were acquired from Sigma-Aldrich Co. Ltd. The substrates were predissolved in 1 mL of ethylene glycol monomethylether, also known as methylcellosolve (Hoppe 1983). The substrate solutions were made up to 50 mL of 1000 μM with sterile buffer solution. Two other working solutions of 100 μM and 10 μM were made up by dilution of the 1000 μM substrate solution with sterile buffer. The buffer was 2-(N-Morpholino)ethanesulfonic acid (MES) obtained from Sigma-Aldrich Co. Ltd. The buffer was made up to a concentration of 0.1M and autoclaved to sterilize. The standard, 4-methylumbelliferone,

was also acquired form Sigma-Aldrich Co. Ltd. This standard was made up to 100 mL of a 10 mM stock solution with methanol. This solution was diluted to a concentration of 1 μM with sterile buffer solution.

Polystyrene 96-well plates were used for the enzyme activity measurements. Two different plate set ups were used depending on whether enzyme activity (defined as the rate of enzyme activity that occurs at a saturating concentration of substrate) was being measured or if enzyme kinetics were being measured. To measure maximum rates, the first plate included samples from the first sampling time period, as well as two controls and two standards. Each subsequent plate contained samples from the other collection times until all samples had been analyzed. Figure 14 is a schematic diagram of the plate set up to measure maximum rates for Experiment #2.1. Each well used for the analysis contained 20 μL of the appropriate sediment slurry sample added after the sample was homogenized by shaking. Within each well, the appropriate amounts of buffer and the florescent-tagged substrate were added to the 20 μL of sample sediment slurry already in the well so that the substrate concentration within the well was saturating. The total volume within each well was always 200 μL. Test runs with sediment collected from the same sampling area indicated a saturating substrate concentration of approximately 500 μM, thus this was the concentration used for Experiment #2.1. To achieve this concentration, 80 μL of buffer and 100 μL of the 1000 μM solution of the fluorescent-tagged substrate were added to the well with the sediment sample. There were three replicates of each sample run on the plate.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control, Sample 1			Control, Sample 2			Control, Sample 3			Control, Sample 4		
B	Saline Control, Sample 1			Saline Control, Sample 2			Saline Control, Sample 3			Saline Control, Sample 4		
C	Glucose, Sample 1			Glucose, Sample 2			Glucose, Sample 3			Glucose, Sample 4		
D	Alginic Acid, Sample 1			Alginic Acid, Sample 2			Alginic Acid, Sample 3			Alginic Acid, Sample 4		
E	Heat Treated Sediment			Sterile UHP Water								
F												
G	MUF Standard with Heat Treated Sediment											
H	MUF Standard with Sterile UHP Water											

MUF (µl)	60	10	20	30	40	50	60	70
Buffer (µl)	180	170	160	150	140	130	120	110

B

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Control, Sample 1			Control, Sample 2			Control, Sample 3			Control, Sample 4			Time 1
B	Saline Control, Sample 1			Saline Control, Sample 2			Saline Control, Sample 3			Saline Control, Sample 4			
C	Glucose, Sample 1			Glucose, Sample 2			Glucose, Sample 3			Glucose, Sample 4			
D	Alginic Acid, Sample 1			Alginic Acid, Sample 2			Alginic Acid, Sample 3			Alginic Acid, Sample 4			
E	Control, Sample 1			Control, Sample 2			Control, Sample 3			Control, Sample 4			Time 2
F	Saline Control, Sample 1			Saline Control, Sample 2			Saline Control, Sample 3			Saline Control, Sample 4			
G	Glucose, Sample 1			Glucose, Sample 2			Glucose, Sample 3			Glucose, Sample 4			
H	Alginic Acid, Sample 1			Alginic Acid, Sample 2			Alginic Acid, Sample 3			Alginic Acid, Sample 4			

Sediment Sample: 20 µl, Buffer: 80 µl, and Fluorescent-Tagged Substrate: 100 µl of 1000 µM stock solution

Figure 14: Enzyme activity schematic for Experiment #2.1. Diagram of 96-well plate set-up for the first run for exoenzyme activity analysis for Experiment #2.1. (A) Diagram of first plate run with standards and controls. (B) Diagram of each plate made after the first plate. The grayed out cells were unused during this analysis run. The substrate concentration in each well was 500 µM.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control, Sample 1			Control, Sample 2			Control, Sample 3			Control, Sample 4		
B	Saline Control, Sample 1			Saline Control, Sample 2			Saline Control, Sample 3			Saline Control, Sample 4		
C	Glucose, Sample 1			Glucose, Sample 2			Glucose, Sample 3			Glucose, Sample 4		
D	Phosphate, Sample 1			Phosphate, Sample 2			Phosphate, Sample 3			Phosphate, Sample 4		
E	Gluc & Phos, Sample 1			Gluc & Phos, Sample 2			Gluc & Phos, Sample 3			Gluc & Phos, Sample 4		
F	Heat Treated Sediment			Sterile UHP Water								
G	MUF Standard with Heat Treated Sediment											
H	MUF Standard with Sterile UHP Water											

MUF (µl)	60	10	20	30	40	50	60	70
Buffer (µl)	180	170	160	150	140	130	120	110

B

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Control, Sample 1			Control, Sample 2			Control, Sample 3			Control, Sample 4			Time 1
B	Saline Control, Sample 1			Saline Control, Sample 2			Saline Control, Sample 3			Saline Control, Sample 4			
C	Glucose, Sample 1			Glucose, Sample 2			Glucose, Sample 3			Glucose, Sample 4			
D	Phosphate, Sample 1			Phosphate, Sample 2			Phosphate, Sample 3			Phosphate, Sample 4			
E	Gluc & Phos, Sample 1			Gluc & Phos, Sample 2			Gluc & Phos, Sample 3			Gluc & Phos, Sample 4			
F	Control, Sample 1			Control, Sample 2			Control, Sample 3			Control, Sample 4			Time 2
G	Saline Control, Sample 1			Saline Control, Sample 2			Saline Control, Sample 3			Saline Control, Sample 4			
H	Glucose, Sample 1			Glucose, Sample 2			Glucose, Sample 3			Glucose, Sample 4			

Sediment Sample: 20 µl, Buffer: 0 µl, and Fluorescent-Tagged Substrate: 180 µl of 1000 µM stock solution

Figure 15: Enzyme schematic for Experiment #2.2. Diagram of 96-well plate set-up for the first run for exoenzyme activity analysis for Experiment #2.2. (A) Diagram of first plate run with standards and controls. (B) Diagram of each plate made after the first plate. The grayed out cells were unused during this analysis run. The substrate concentration in each well is 900 µM.

After running all the sediment samples for Experiment #2.1, the kinetics curves (described below) indicated that a higher maximum substrate concentration may be necessary to saturate the enzyme in order to induce potential maximum rates. For Experiment #2.2, the maximum substrate concentration was increased to 900 μM, which was achieved in the wells by adding 180 μL of the 1000 μM substrate solution to the 20 μL of sediment slurry in each well. Figure 15 shows a diagram of the plate setup to measure the maximum rates for Experiment #2.2.

To measure enzyme kinetics, varying substrate concentrations were achieved in the plate wells by adding 20 μL of the sediment sample slurry and varying the amount of buffer and the 10, 100, and 1000 μM working stock solutions of substrate added to the wells in order to produce a range of substrate concentrations. The substrate concentrations in the wells increased over eight wells with concentrations of 2, 8, 20, 50, 80, 200, 300, and 500 μM for Experiment #2.1. The plate set up to measure enzyme kinetics for Experiment #2.1 and #2.2 is shown in Figure 16. Because these plates were so time consuming to set up and needed to be analyzed as quickly as possible, each sample was run once with only the substrate β-glucosidase for Experiment #2.1. Also, the samples analyzed for kinetics activity all came from day 5. The first plate contained the first 6 sediment samples, plus two controls and two standards. Each subsequent plate contained the following samples until all samples from day 5 had been analyzed.

A

	1	2	3	4	5	6	7	8	9	10	11	12	MUF (µl)	Buffer (µl)
Buffer (µl)	140	20	140	80	20	140	120	80						
Substrate (µl)	40	160	40	100	160	40	60	100						
Substrate Conc. (µM)	2	8	20	50	80	200	300	500						
A	Sample #1										Standard with Heat Treated Sediment	Standard with Autoclaved Water	0	180
B	Sample #2												20	160
C	Sample #3												30	150
D	Sample #4												40	140
E	Sample #5												50	130
F	Sample #6												60	120
G	Autoclaved Water												70	110
H	Heat Treated Sediment												80	100

B

	1	2	3	4	5	6	7	8	9	10	11	12	MUF (µl)	Buffer (µl)
Buffer (µl)	140	20	140	80	20	140	120	80	40	0				
Substrate (µl)	40	160	40	100	160	40	60	100	140	180				
Substrate Conc. (µM)	2	8	20	50	80	200	300	500	700	900				
A	Sample #1										Standard with Heat Treated Sediment	Standard with Autoclaved Water	0	180
B	Sample #2												20	160
C	Sample #3												30	150
D	Sample #4												40	140
E	Sample #5												50	130
F	Sample #6												60	120
G	Autoclaved Water												70	110
H	Heat Treated Sediment												80	100

Figure 16: Enzyme kinetics schematic for Experiment #2. Diagrams of the 96-well plate setups for the kinetics runs. The gray highlighted cells were unused cells on the plate. The blue cells represent volumes from the 10 µM substrate solution. The green cells represent volumes from the 100 µM substrate solution. The green cells represent volumes from the 1000 µM substrate solution. (A) Plate diagram for Experiment #2.1 (B) Plate diagram for Experiment #2.2

After completing Experiment #2.1, re-evaluating this procedure, and shortening the sample collection period for Experiment #2.2, it became feasible to run kinetics on all the samples collected with all three exoenzymes – β-glucosidase, β-xylosidase, and β-galactosidase. The range of substrate concentrations to create the kinetics curve was also increased to include concentrations of 700 and 900 μM.

The controls used for the enzyme activity analyses were autoclaved UHP water and heat-treated sediment from the sample site. To prepare the control sediment, the samples was placed in a microwave for 15 seconds on high power. Both were treated to eliminate any enzymes or organisms that affect the enzyme concentration. The UHP control allowed for detection of any fluorescence changes without the influence of the sediment sample. Twenty μL from both controls were placed in the appropriate wells and the volumes of buffer and substrate added were the same as for the sediment samples, keeping the volume of the well at 200 μL. The sterile water and heat-treated sediment controls were also used as the 20 μL sample for the standard runs. The appropriate amounts of buffer and standard were added to the wells to get the final amounts of 0, 20, 30, 40, 50, 60, and 70 pmol of 4-MUB in seven wells.

The fluorescence intensity was measured using the Spectramax Gemini EM, a computer-controlled microplate fluorimeter, and the data was recorded using SoftMax Pro 4.8 software. With this instrument, the temperature could be controlled and measurements could be taken at equal intervals over a set time period. The fluorimeter was set at 25 ^{o}C and the time period was 1.5 hours. The plate was shaken for 5 seconds before the first reading and 3 seconds before each subsequent reading. The fluorescence

was measured once every minute for the 1.5 hour time period. A test run was completed with the fluorimeter with sediment from the sample site and using β-glucosidase in order to evaluate the fluorescent signal at different wavelengths to identify the target emission and excitation wavelengths. The excitation wavelength was 346 nm and the emission wavelength was 460 nm.

Chlorophyll Analysis

Chlorophyll *a* was analyzed spectrophotometrically based on a procedure modified by Stahl et al. (1984) from a procedure designed by Lorenzen (1967), which uses methanol to extract chlorophyll *a* from the sediment sample. Approximately 200 mg of freeze dried sediment from each sample was weighed into a 15 mL centrifuge tube. Two mL of 100% methanol was added to each tube. The methanol contained a small amount of magnesium carbonate to prevent the acidification of the sediment sample. The samples were quickly mixed and stored in the dark for 24 hours at 4 ^{o}C. After 24 hours, the samples were quickly mixed again then centrifuged (Eppendorf centrifuge 5804R) for 15 minutes at 1789 x *g* and at a temperature of 4 ^{o}C. One ml of each sample was pipetted into a polystyrene semi-micro cuvette and measured in a spectrophotometer (Shimadzu 1240 UV-mini spectrophotometer) at 665 and 750 nm. The second wavelength is used as a turbidity correction. A drop of 10% HCl was added to each cuvette to acidify each sample. After 5 minutes, the cuvettes were read again at 665 and 750 nm. The acidification allows for the correction of phaeopigments to be made to the chlorophyll measurements. A blank of methanol was used to zero the

spectrophotometer at each wavelength. Using these absorbance measurements, chlorophyll *a* concentrations were calculated using equations developed by Stahl et al. (1984) and converted to μg g^{-1} sediment.

Carbohydrate Analysis

Different carbohydrate fractions were extracted and analyzed spectrophotometrically using a procedure modified from frequently used methods (Underwood et al. 1995, Hanlon et al. 2006, Thornton & Visser 2009). Approximately 100 mg from each sample of freeze dried sediment were placed in 15 mL centrifuge tubes with 4 mL of 25 ‰ (w/v) NaCl solution. The centrifuge tubes were placed on an orbital shaker and shaken for 30 minutes at room temperature and then centrifuged for 15 minutes at 1789 x *g* at a temperature of 4°C. The supernatant contained the saline-extractable carbohydrate fraction. This fraction is mainly extracellular dissolved and colloidal carbohydrates containing saline-extractable EPS and low molecular weight carbohydrates. A volume of 0.8 mL of the supernatant was removed and placed in a Pyrex boiling tube. The carbohydrate concentration for this fraction was analyzed using the phenol-sulfuric acid assay designed by Dubois et al. (1956). A volume of 0.4 mL of 5 % (w/v) phenol and 2 ml concentrated sulfuric acid from a rapid dispensing bottle were added to each sample. The boiling tubes were shaken and allowed to cool for 30 minutes. 1 mL from each boiling tube was placed in a cuvette, and the absorbance was measured at 485 nm. The spectrophotometer was zeroed against a cuvette of UHP water.

Approximately 200 mg of freeze dried sediment (the exact weight was recorded) from each sample was measured out into 15 mL centrifuge tubes in order to extract saline-extractable EPS. The sEPS was extracted with 4 ml of 25 ‰ (w/v) NaCl solution by gently shaking for 30 minutes on an orbital shaker table. After centrifugation, 1.5 mL of the supernatant was pipetted into another 15 mL centrifuge tube with 3.5 mL of cold (4 ^{o}C) reagent alcohol. The tubes were placed in the dark for 24 hours at 4 ^{o}C. The tubes were centrifuged and the supernatant was discarded. A small amount of white precipitate containing the sEPS was located at the bottom of the centrifuge tubes. One ml of UHP water was added to each tube and the tube was vigorously mixed for approximately 5 minutes in order to dissolve the precipitate. A volume of 0.8 mL from each centrifuge tube was placed into boiling tubes and the carbohydrates were analyzed using the phenol-sulfuric acid assay.

To measure the total carbohydrate concentration within each sediment sample, 25 mg of freeze dried sediment was added directly into the boiling tubes and analyzed using the phenol-sulfuric acid assay. After the boiling tubes cooled, the contents were transferred into centrifuge tubes and centrifuged before transferring the sample into the cuvettes for the absorbance measurements.

For Experiment #2.2, two more carbohydrate fractions were extracted. The hot-water (HW) carbohydrate fraction contains mainly intercellular carbohydrates, such as glucan, and the hot-bicarbonate (HB) carbohydrate fraction contains the tightly bound and capsular EPS (Abdullahi et al. 2006, Hanlon et al. 2006). After completing the initial saline extraction procedure for the saline-extractable carbohydrates, 4 mL of reagent alcohol was added to each tube, quickly mixed, and sat for 5 minutes at room temperature in order to remove any chlorophyll *a* in the sediment, which could affect these carbohydrate fractions. The tubes were centrifuged for 15 minutes at 1789 x *g* at a temperature of 4 ^{o}C. The supernatant was discarded and the quick extraction with reagent alcohol was repeated. After discarding the supernatant a second time, 4 mL of UHP water was added to each centrifuge tube and quickly mixed. The tubes were placed in a water bath set at a temperature of 95 ^{o}C for 60 minutes for extraction. The tubes were centrifuged and 0.8 mL from each sample was placed in a boiling tube and

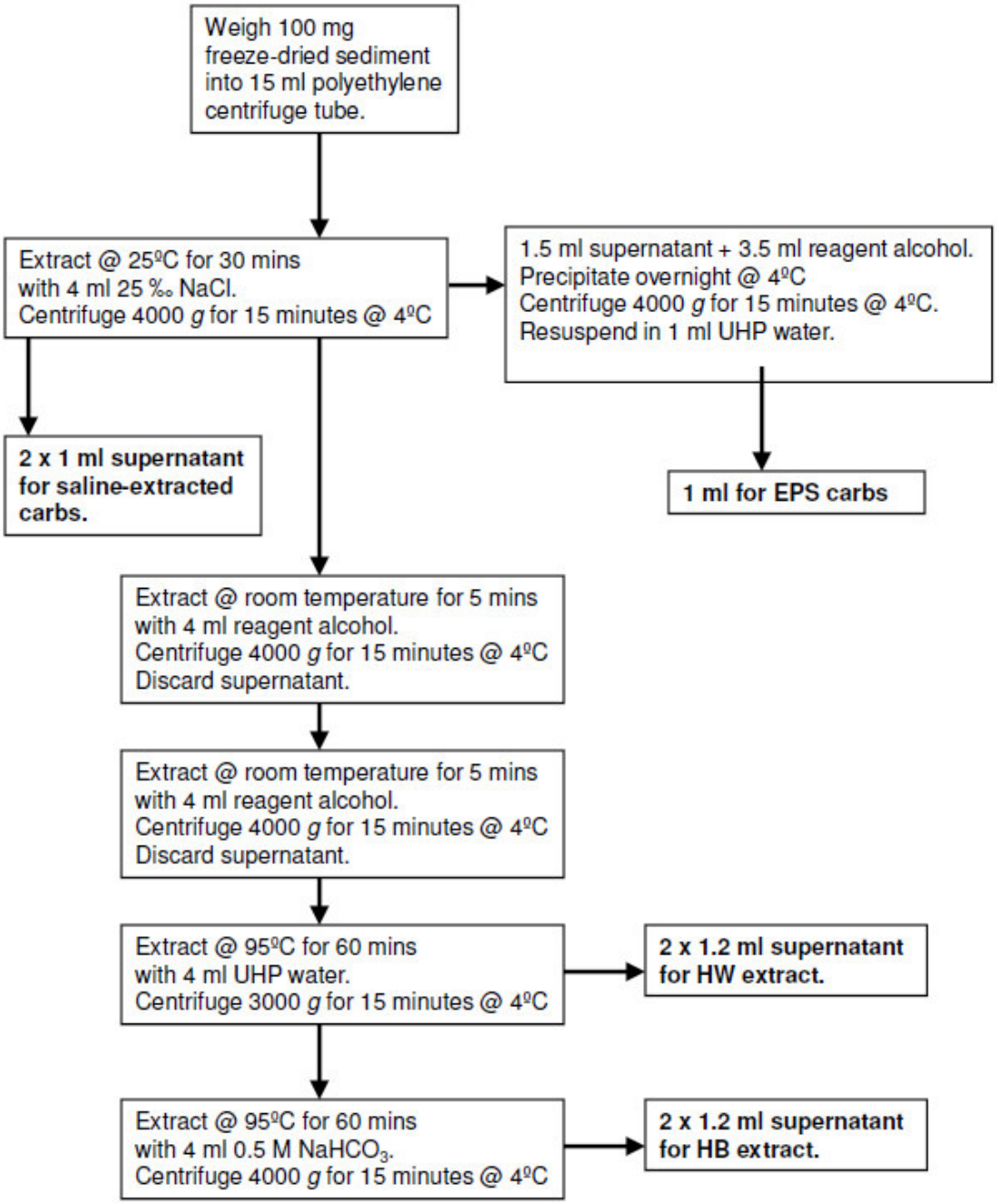


Figure 17: Flow chart of carbohydrate extraction procedure.

the hot-water carbohydrate concentration was analyzed using the phenol-sulfuric acid assay. After removing the remaining supernatant from the hot-water extraction, 4 mL of 0.5 M sodium bicarbonate was then added to the sediment samples, and the samples were quickly mixed. The tubes were placed in the water bath again for 60 minutes at a temperature of 95 ^{o}C. A volume of 0.8 mL for each sample was placed within boiling tubes and the hot-water extracted carbohydrate concentration was analyzed using the phenol-sulfuric acid assay. Figure 17 is a flow chart displaying the procedure for the carbohydrate analysis.

The phenol-sulfuric acid assay was calibrated using D-glucose dissolved in UHP water as the standard over the range 0 to 100 μg mL^{-1} (Fig. 18). Carbohydrate content of the sediment was expressed as glucose equivalents and normalized to dry weight of the sediment.

Bacteria Cell Counts

Sediment samples for bacteria counts were taken to in order to compare changes in the bacteria abundance with changes in enzyme activity. In a sterile flow hood, the sediment samples and filter-sterilized 9 mL of 0.01 M tetrasodium pyrophosphate-3%

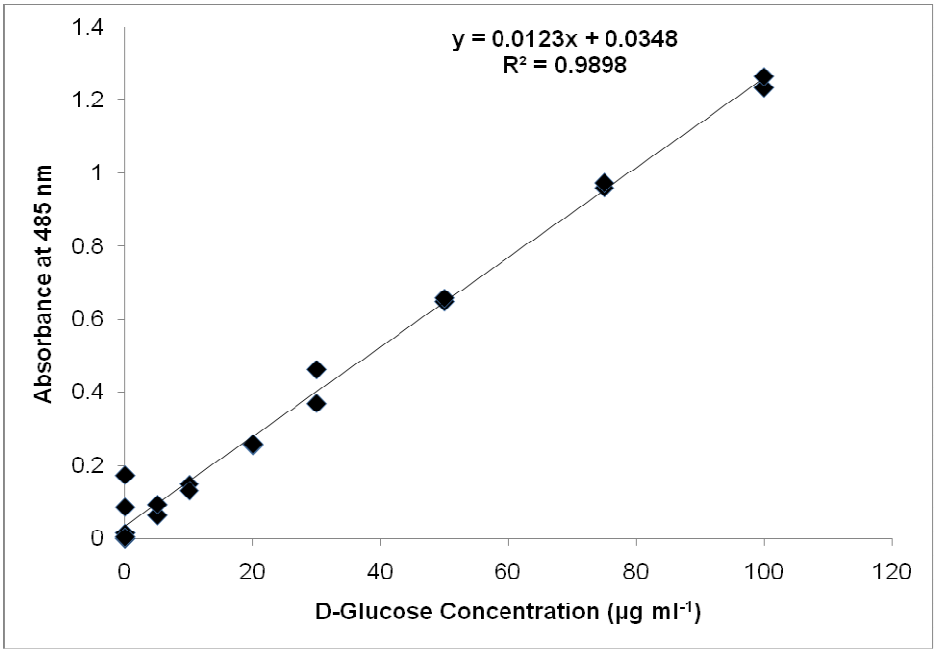


Figure 18: Carbohydrate calibration for Experiment #2.

NaCl solution (PPi-NaCl) (Tso & Taghon 1997) were mixed within a sterile 15 mL centrifuge tube. The centrifuge tubes were placed on an orbital shaker table for 30 minutes. After shaking the tubes, 100 µL from the diluted sediment sample and 900 µl of PPi-NaCl were added to two sterile 1.5 mL microcentrifuge tube for each sample. The samples were stained with 100 µL of 5 µg mL^{-1} solution of 4'6-diamidino-2-phenylindole dihydrochloride (DAPI) based on a procedure by Porter & Feig (1980). The stained samples were incubated in the dark for 1 hour at 4 ^{o}C. A sterile glass filter assembly was used to filter very slowly 1 mL of each stained sample and 1 additional mL of PPi-NaCl onto a black 25 mm diameter, 0.2 µm pore, polycarbonate filter (Nuclepore). A glass fiber (GF/C) filter was placed between the filter and the glass filtration system in order to help evenly distribute the sample over the polycarbonate filter. Filter blanks were made by staining and filtering 2 mL of PPi-NaCl, in order to count the ambient background numbers of bacteria, and these numbers were subtracted from the bacteria counts from the sediment samples. The filters were placed on a drop of immersion oil of a glass slide. Another drop of immersion oil was placed in between the filter and the cover slip. The slides were stored in the dark at 4 ^{o}C until counted.

Bacteria cells were counted using a Zeiss Axioplan 2 Imaging microscope using a 100x oil immersion objective. The bacteria fluoresced a blue-green color using the DAPI fluorescence filter on the microscope. The bacterial counts, in cells μg^{-1} sediment, were calculated using the counts of bacteria cells, the area of the filter counted, and the amount of sediment on the filter.

Phosphate Analysis

The frozen samples were brought to room temperature and weighed. Five mL of UHP water was added to each sample. The sample was mixed and transferred into a 15 mL centrifuge tube. The tubes were centrifuged at 1789 x *g* for 15 minutes at room temperature. Four mL of the supernatant was removed and placed within a second 15 mL centrifuge tube. Using a procedure designed by Murphy and Riley (1962), 0.4 mL of a reagent solution was added to each sample. The reagent solution was 100 mL of 10% (w/v) ammonium paramolybdate, 250 mL of 16% (v/v) sulfuric acid, 100 mL of 5.4% (w/v) ascorbic acid, and 50 mL of 0.14% (w/v) potassium antimonyl-tartrate solutions. After 30 minutes, the samples were measured in the spectrophotometer at 885 nm against UHP water.

The phosphate analysis was calibrated using potassium dihydrogen phosphate dissolved in UHP water over the range of 0-25 μg L^{-1}.

Statistical Analysis

Data was analyzed using Sigmastat 3.1 (Systat Software, Inc.). Analysis of variance (ANOVA) statistical tests were conducted on the chlorophyll, carbohydrate, enzyme, phosphorus and bacteria data sets that met the assumptions of normality and equality of variance. Data that did not meet these assumptions was log(n+1) transformed before analysis. Post-hoc Tukey tests were performed along with the ANOVAs. Regression analyses were calculated between the chlorophyll and the carbohydrate data to identify correlations within the data. Correlation analyses were carried between the V_{max} and K_m values and the carbohydrate fractions using the Pearson product moment correlation.

Results

Sediment Sample Preservation

For this preliminary experiment, it was important to find the best way to preserve the enzymes in the sediment, as well as prevent more enzymes to be released after sampling in an attempt to measure *in situ* enzyme activities in the lab a few hours after sampling. There was a significant difference with treatment ($F_{5, 150} = 24.373$, $p < 0.001$) and with time ($F_{4, 150} = 43.911$, $p < 0.001$). Based on post-hoc pair wise tests, the preservation strategy of using sodium azide and keeping the samples on ice produced β-glucosidase rates significantly higher ($p < 0.01$) than the other treatments, except for the strategy of using sodium azide and keeping the samples at room temperature (Fig. 19). This treatment was also the most stable across the time period with a significant difference appearing only with day two. Day two was an anomaly most likely due to a mistake in the procedure. The low activities suggest that no substrate was added to the samples. Also, the rates increased dramatically after 4 days, which means these samples should not be left for very long before measuring exoenzyme activity.

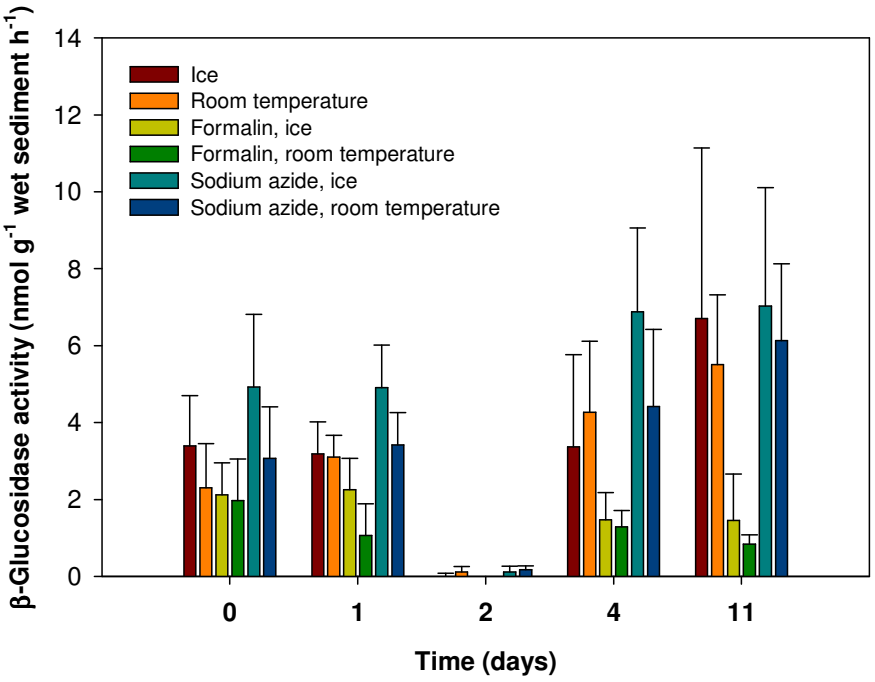


Figure 19: Preliminary sediment preservation experiment. Preliminary test of β-glucosidase enzyme activity over time with different sample preservation methods. Bars show mean + SD (n = 6)

Preliminary Slurry Experiment

There was a significant difference with treatment for the slurry experiment with nutrient treatments ($F_{4, 140} = 16.871$, $p < 0.001$). Figure 20 shows a significant increase ($p < 0.05$) in β-glucosidase activity with the phosphorus addition. The phosphorus addition also had significantly higher ($p < 0.01$) enzyme rates than the other treatment, except for the nitrogen addition treatment. There was also a significant difference with treatment for the slurry experiment with carbohydrate additions ($F_{4, 140} = 15.881$, $p < 0.001$). Figure 21 displays a significant increase ($p < 0.01$) in β-glucosidase activity with the alginic acid addition over seven days. The alginic acid treatment also had enzyme rates significantly higher ($p < 0.05$) than the other treatments. It is important to note that the error bars in all three of these figures are rather high masking any other changes with treatment or time. These results led to the decisions on treatments made for Experiment #2.1 and Experiment #2.2.

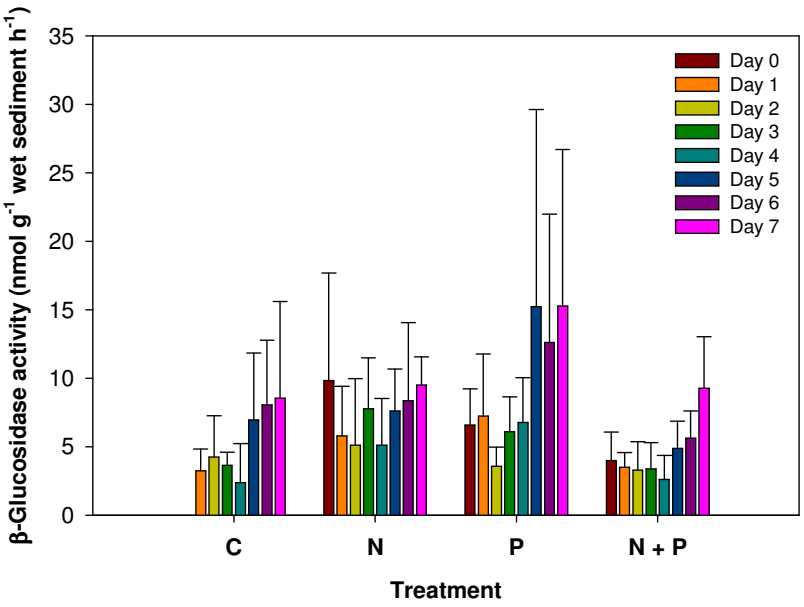


Figure 20: Preliminary slurry experiment with nutrients. β-glucosidase enzyme activity over time with different nutrient treatments. The treatments were control (C), nitrogen addition (N), phosphorus addition (P) and nitrogen + phosphorus addition (N + P). Bars show means + SD (n = 5)

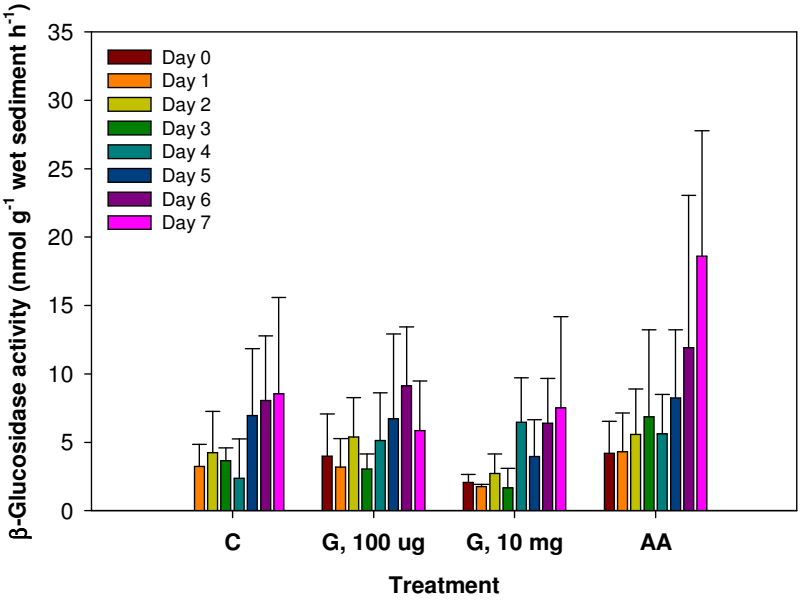


Figure 21: Preliminary slurry experiment with carbohydrate additions. β-glucosidase enzyme activity over time with different carbohydrate additions. The treatments were control (C), glucose addition of 100 μg (G, 100 ug), glucose addition of 10 mg (G, 10 mg), and alginic acid (AA). Bars show means + SD (n = 5)

Environmental Conditions

For Experiment #2.1, the sediment temperature ranged from 24.5 to 25 ^{o}C. The salinity of the water nearest the sample site was 26 to 30 ppt for the entire experiment. For Experiment #2.2, the sediment temperature ranged from 14.1 to 18.7 ^{o}C. During this sampling period, the air temperature was cooler than during Experiment #2.1, and the wind was stronger. The salinity of the water nearest the sample site ranged from 31 to 35 ppt.

BMA Biomass

Figure 22 shows the change in chlorophyll *a* concentration within the sediment over time with treatment for Experiment #2.1, which indicates the BMA biomass in the sediment. Over the time period, there was a significant difference between the treatments ($F_{3, 72} = 3.939$, $p < 0.05$), seen with significantly higher chlorophyll *a* concentrations in the control treatment compared to the alginic acid treatment ($p < 0.01$). The chlorophyll a concentration in the control treatment varied over the time period with an average concentration of 8.9 ± 2.0 μg g^{-1} sediment. The chlorophyll *a* concentration in the alginic acid treatment also varied over the time period with an average concentration of 7.5 ± 1.0 μg g^{-1} sediment.

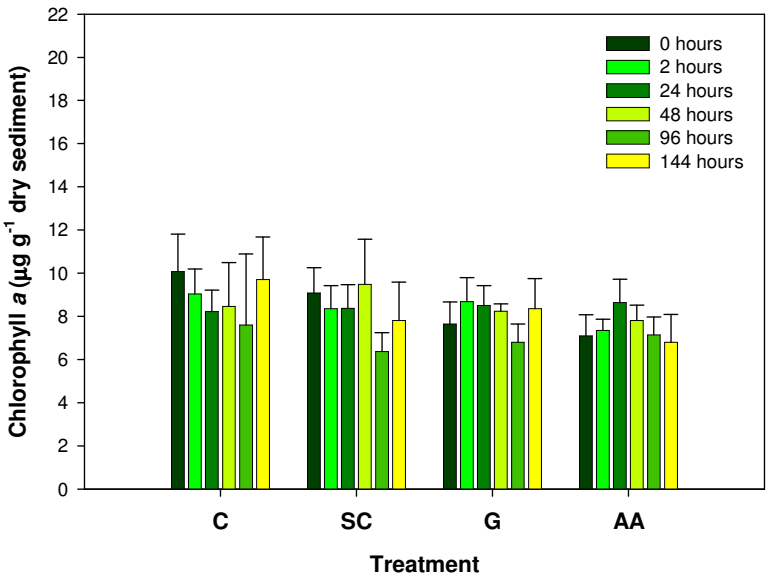


Figure 22: Chlorophyll concentrations for Experiment #2.1. Concentrations were from within the sediment over time with each treatment for Experiment #2.1. Treatments are control (C), saline control (SC), glucose addition (G), and alginic acid addition (AA). Bars show mean + SD (n=4).

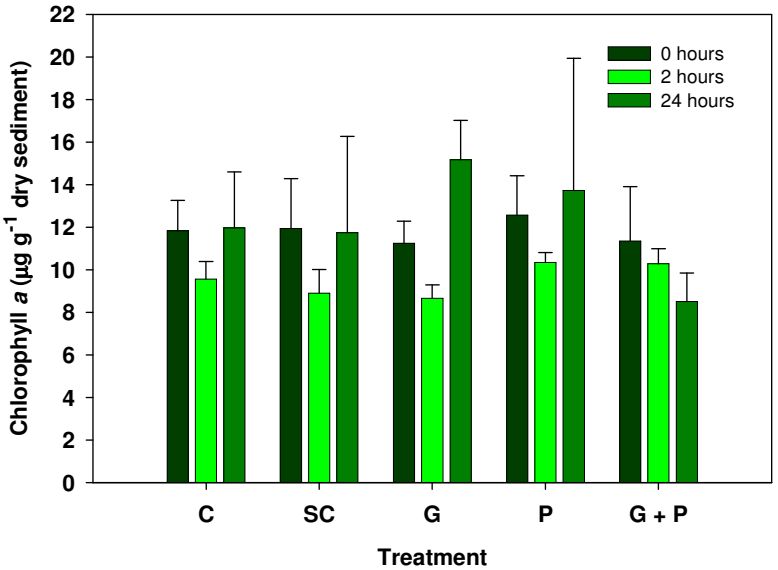


Figure 23: Chlorophyll concentrations for Experiment #2.2. Concentrations were from within the sediment over time with each treatment for Experiment #2.2. Treatments were control (C), saline control (SC), glucose addition (G), phosphorus addition (P), and nitrogen + phosphorus (N + P). Bars show mean + SD (n=4).

Figure 23 shows the change in chlorophyll *a* concentration for Experiment #2.2. There was no significant change between treatments, but there was a significant difference with time ($F_{2, 44} = 6.506$, $p < 0.01$). A trend appeared with a decrease in chlorophyll *a* concentration over the first 2 hours, followed by an increase after 24 hours in all treatments except the glucose and phosphorus treatment, but this trend was only significant for the glucose treatment. From 2 hours to 24 hours after the glucose addition, there was a significant increase in chlorophyll *a* concentration ($p < 0.01$) going from 8.65 ± 0.64 μg g^{-1} sediment to 15.17 ± 1.85 μg g^{-1} sediment, which was the highest mean over the other treatments.

Carbohydrates

Figure 24 shows the change in the total carbohydrate concentration, as well as the saline-extractable carbohydrate fraction and the sEPS carbohydrate fraction concentrations within the sediment over time with treatment for Experiment #2.1. There was no significant difference in the total carbohydrate concentrations between or within treatments over the experimental time period. Thus, when pooling all the data, the mean total carbohydrate concentration was 1553 ± 345 μg g^{-1} sediment. There was no significant difference in the saline-extractable carbohydrate concentrations between treatments. After pooling all the data, the mean saline-extractable carbohydrate concentration was 312 ± 84 μg g^{-1} sediment. There was a significant difference between treatments in the sEPS carbohydrate concentration ($F_{3, 72} = 6.964$, $p < 0.001$), seen as a significant difference ($p < 0.01$) between the saline control treatment and the control

treatment, as well as between the saline control treatment and the alginic acid treatment. Both differences were seen during the first 24 hours of the experiment. There was also a significant difference in a treatment with time ($F_{15, 72} = 7.903$, $p < 0.001$). Within the saline control treatment, there was a significant change ($p < 0.05$) in sEPS over the first 24 hours with the concentration decreasing from 61.2 ± 11.4 μg g^{-1} sediment to 19.0 ± 11.7 μg g^{-1} sediment, but then significantly jumped ($p < 0.05$) over the next 24 hours to 60.3 ± 10.5 μg g^{-1} sediment. The concentration remained near this concentration for the remainder for the experiment, which was close to the mean at the start of the experiment. The glucose treatment behaved a little differently. A significant increase ($p < 0.001$) was seen in the sEPS concentration over 2 hours from 13.6 ± 9.9 μg g^{-1} sediment to 66.1 ± 24.4 μg g^{-1} sediment, and the concentration remained approximately at this concentration for the remainder of the experiment. The alginic acid treatment behaved the same as the glucose treatment with a significant increase ($p < 0.01$) from the initial concentration of 31.8 ± 10.4 μg g^{-1} sediment to 76.9 ± 3.0 μg g^{-1} sediment. The sEPS concentration remained near this concentration for the rest of the experiment.

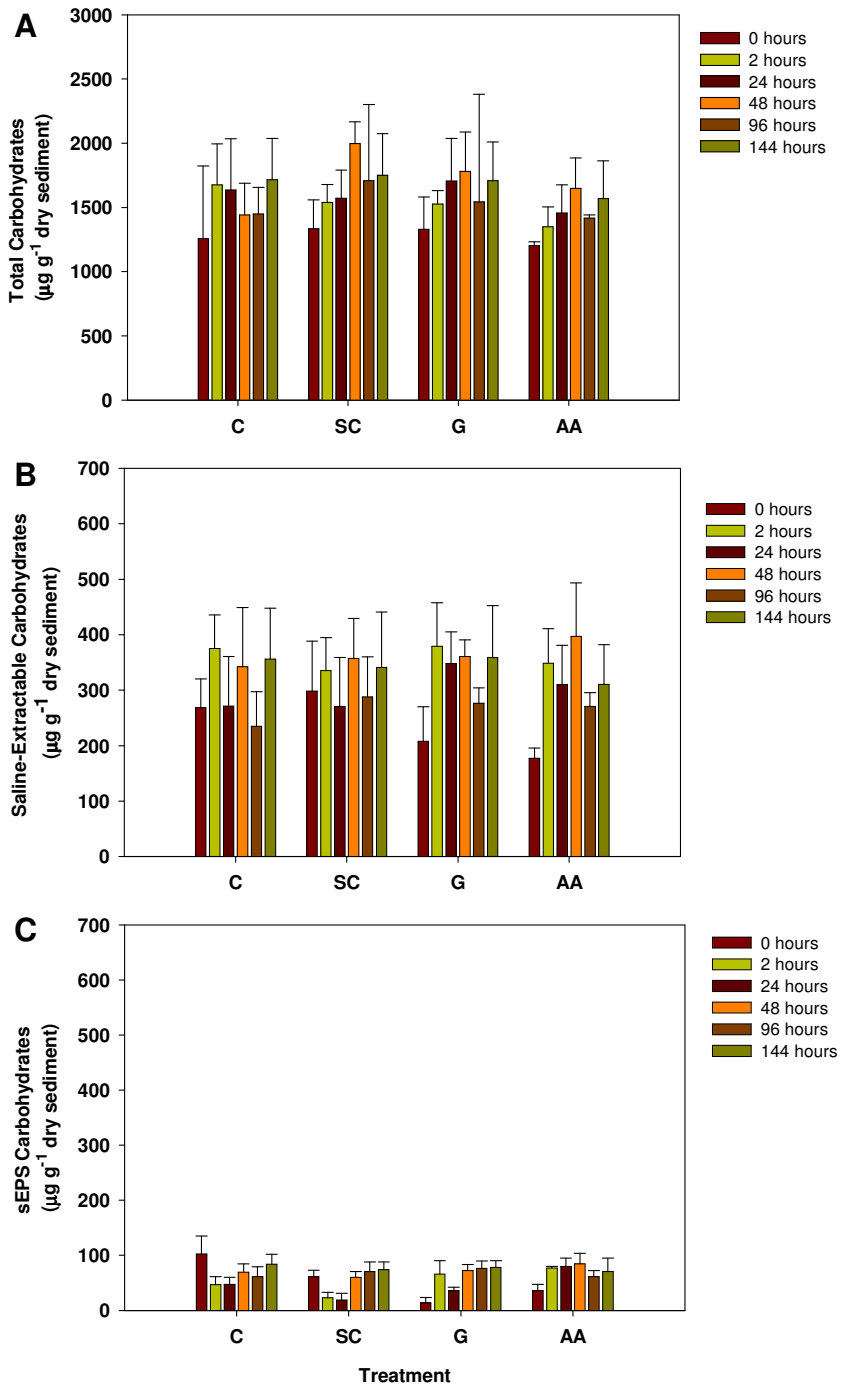


Figure 24: Carbohydrate concentrations for Experiment #2.1. Concentrations were from within the sediment over time for each treatment for Experiment #2.1. (A) total carbohydrate concentration (B) saline-extractable carbohydrate concentration (C) sEPS carbohydrate concentration. Treatments were control (C), saline control (SC), glucose addition (G), alginic acid addition (AA). Bars represent mean + SD (n = 4).

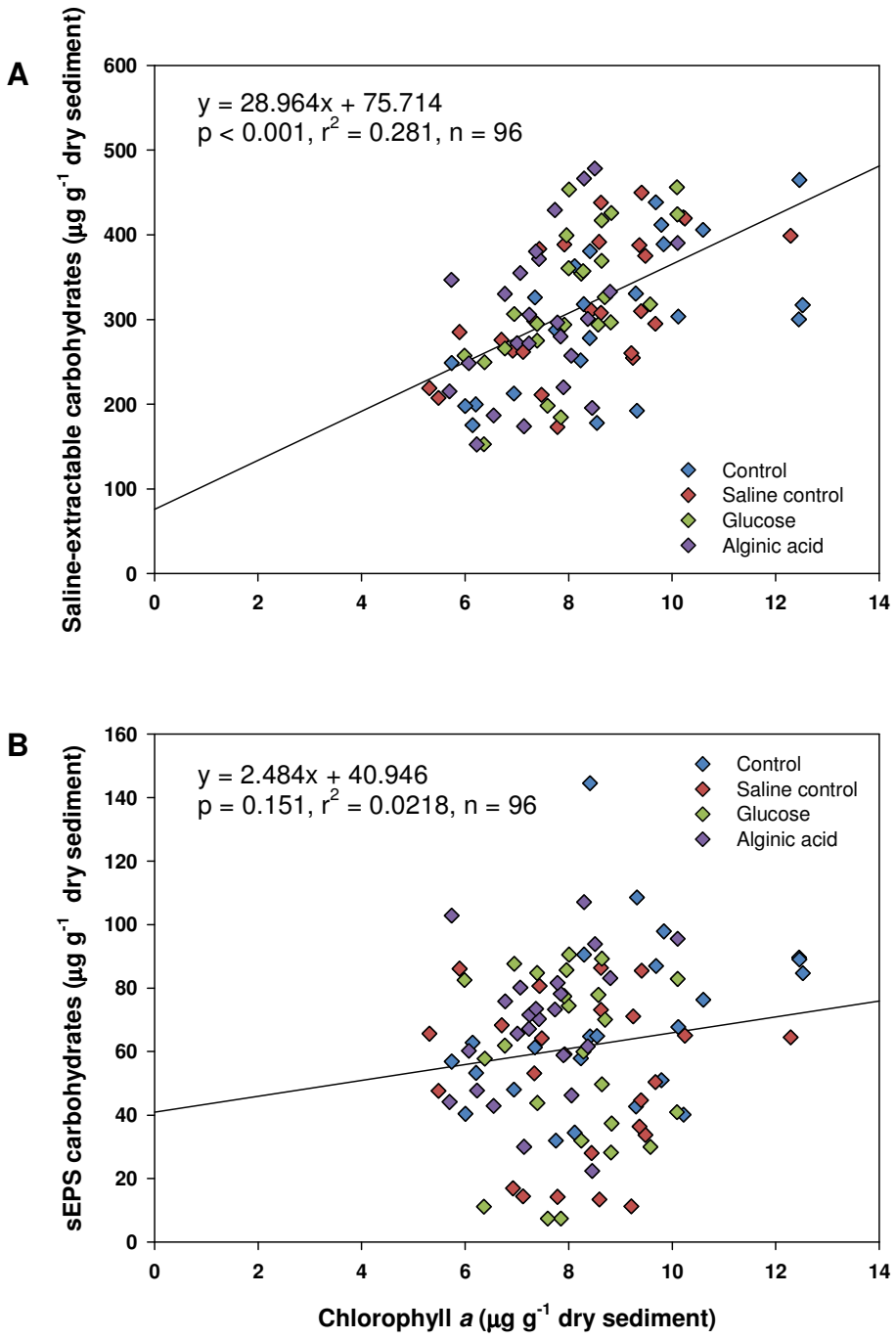


Figure 25: Chlorophyll and carbohydrate relationships with all treatments for Experiment #2.1. Relationships were between chlorophyll *a* and (A) Saline-extractable carbohydrates and (B) sEPS carbohydrates.

Figure 25 displays the linear regressions between chlorophyll *a* concentrations with the saline-extractable carbohydrate concentrations and with the sEPS carbohydrate concentrations for all samples from Experiment #2.1. The regression calculated for the correlation between chlorophyll *a* and sEPS carbohydrates was not significant ($p = 0.151$, $r^2 = 0.0218$, $n = 96$). The regression calculated for the correlation between chlorophyll *a* and the saline-extractable carbohydrates was significant ($p < 0.001$, $r^2 = 0.281$, $n = 96$). Neither regression was very strong. Because the carbohydrate additions could skew this relationship between chlorophyll *a* and the carbohydrate fractions, Figure 26 shows the linear regressions between chlorophyll *a* concentrations with the saline-extractable carbohydrate concentration sand with the sEPS carbohydrate concentrations using the data from only the control treatment and the saline control treatment. The regression calculated for the correlation between chlorophyll *a* and sEPS carbohydrates was not significant ($p = 0.085$, $r^2 = 0.063$, $n = 48$). The regression calculated for the correlation between chlorophyll *a* and the saline-extractable carbohydrates was significant ($p < 0.001$, $r^2 = 0.339$, $n = 48$). Even without the carbohydrate additions, the regressions were not very strong.

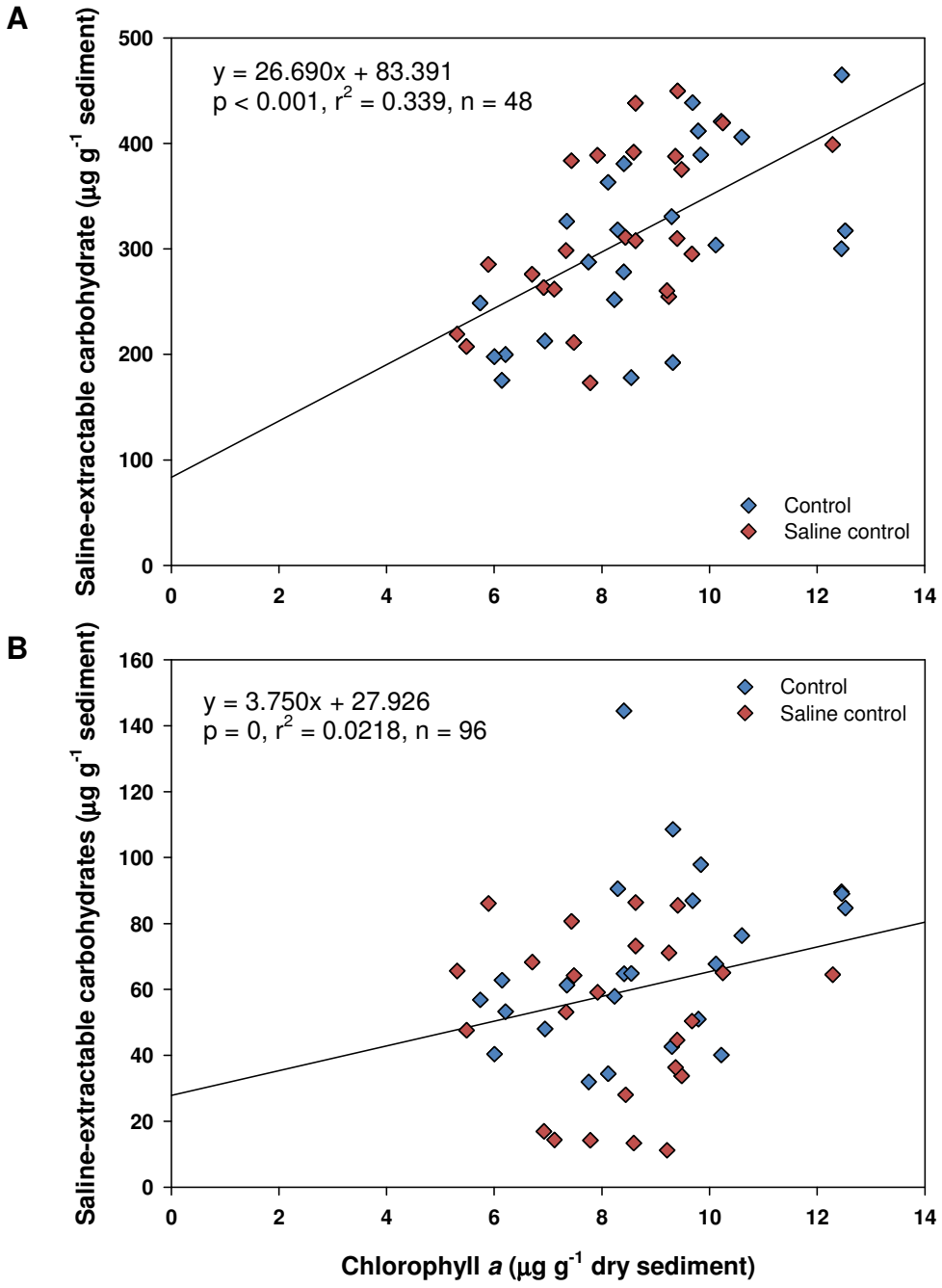


Figure 26: Chlorophyll and carbohydrate relationships with the control treatments for Experiment #2.1. Relationship between chlorophyll *a* and (A) saline-extractable carbohydrates and (B) sEPS carbohydrates with the control treatment and the saline control treatment.

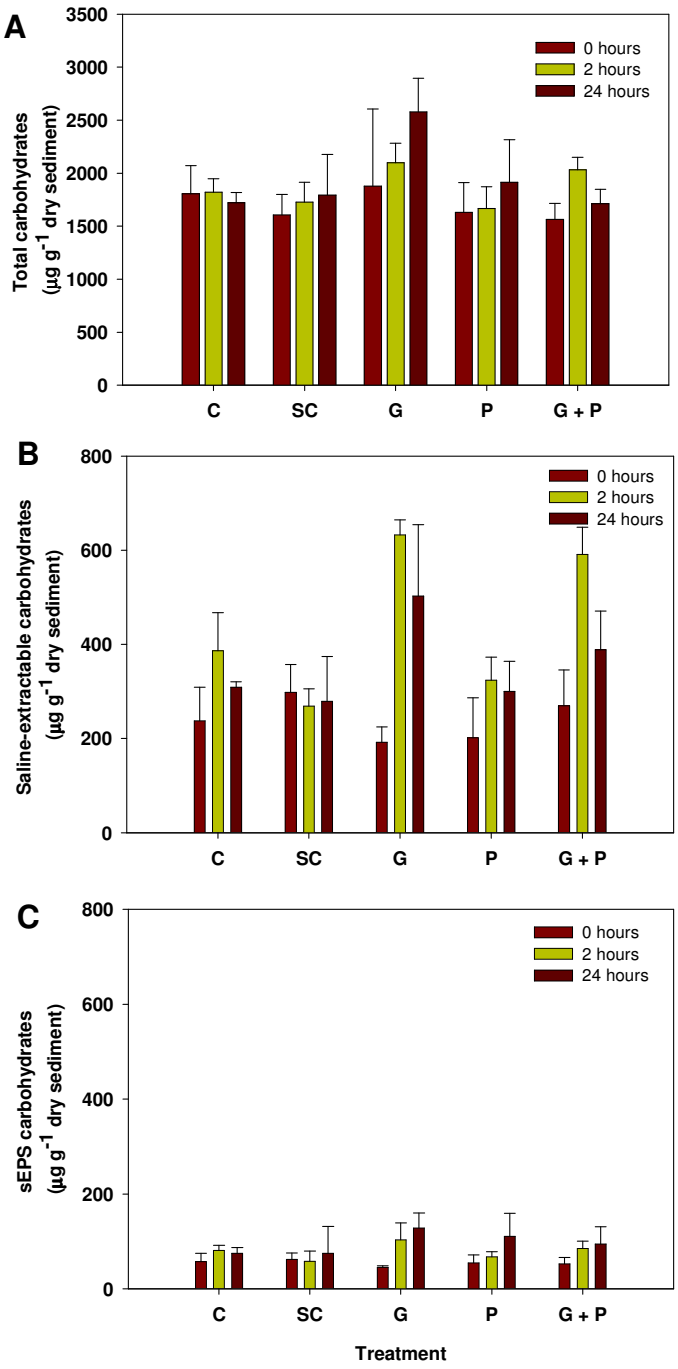


Figure 27: Carbohydrate concentrations for Experiment #2.2. Concentrations were from within the sediment over time for each treatment for Experiment #2.2. (A) Total carbohydrate concentration (B) Saline-extractable carbohydrate concentration (C) sEPS carbohydrate concentration. Treatments were control (C), saline control (SC), glucose addition (G), phosphorus addition (P), and glucose + phosphorus (G + P) addition. Bars represent mean + SD (n = 4).

Figure 27 shows the change in the total carbohydrate concentration, as well as the saline-extractable carbohydrate fraction and the sEPS carbohydrate fraction concentrations within the sediment over time with treatment for Experiment #2.2. There was a significant difference between the treatments for the total carbohydrate concentration ($F_{4, 44} = 4.375$, $p < 0.01$) observed as a significant difference ($p < 0.05$) from the glucose treatment compared with the other four treatments. Also there was a significant difference with time for the total carbohydrate concentrations ($F_{2, 44} = 4.158$, $p < 0.05$). This was observed with the significant increase in the glucose treatment ($p < 0.01$) from the initial concentration of 1879 ± 728 μg g^{-1} sediment to a concentration of 2580 ± 315 μg g^{-1} sediment after 24 hours. There was a significant difference between treatments for the saline-extractable carbohydrate concentrations ($F_{4, 44} = 36.475$, $p < 0.001$). Post-hoc pair wise tests revealed that the glucose treatment and the glucose + phosphorus treatment were significantly higher ($p < 0.01$) over the other three treatments. A significant difference was found with those two treatments with time ($F_{8, 44} = 6.641$, $p < 0.001$) ,which was observed with both the glucose treatment and the glucose + phosphorus treatment increasing dramatically after 2 hours, then decreasing slightly after 24 hours due to the detection of the carbohydrate addition to the sediment. The saline-extractable carbohydrate concentration in the glucose treatment started initially at 191.9 ± 32.9 μg g^{-1} sediment and increased significantly ($p < 0.001$) after 2

hours to a concentration of 632.5 ± 31.8 μg g^{-1} sediment. The saline-extractable carbohydrate concentration then decreased significantly ($p < 0.05$) to a concentration of 502.7 ± 151.8 μg g^{-1} sediment. The saline-extractable carbohydrate concentration in the glucose + phosphorus treatment showed the same pattern significantly increasing ($p < 0.001$) from 269.7 ± 75.9 μg g^{-1} sediment to 590.7 ± 58.1 μg g^{-1} sediment. The saline-extractable carbohydrate concentration then decreased significantly ($p < 0.01$) to 388.4 ± 82.1 μg g^{-1} sediment. Even though both the glucose treatment and the glucose + phosphate treatment saline-extractable carbohydrate means were higher than the other treatments, the glucose treatment had the highest mean concentrations over the whole experiment. There was no significant difference between the treatments for the sEPS carbohydrate concentration. Although the difference between the treatments was not significant, there was a significant difference with time ($F_{2, 44} = 13.244$, $p < 0.001$). Post-hoc pair wise comparisons revealed significant increases ($p < 0.05$) in the sEPS concentrations within the glucose treatment, the phosphate treatment, and the glucose + phosphate treatments. After two hours, the sEPS concentration in the glucose treatment

increased from 45.2 ± 3.3 μg g^{-1} sediment to 102.9 ± 36.0 μg g^{-1} sediment. After 24 hours, the sEPS concentration in the phosphorus treatment increased from 54.6 ± 17.3 μg g^{-1} sediment to 110.9 ± 48.7 μg g^{-1} sediment, and the glucose + phosphate treatment increased over 24 hours from 52.4 ± 13.7 μg g^{-1} sediment to 94.7 ± 36.2 μg g^{-1} sediment.

For Experiment #2.2, the hot-water extracted carbohydrate concentrations and the hot-bicarbonate extracted carbohydrate concentrations were calculated, and the changes with these carbohydrate fractions for each treatment with time can be seen in Figure 28. The hot-water extracted carbohydrate concentration showed no significant difference between treatments or within the treatments with time. After pooling all the hot-water extracted carbohydrate data, the mean concentration was 660.5 ± 86.2 μg g^{-1} sediment. The hot-bicarbonate extracted carbohydrate concentrations also showed no significant difference between the treatments or within the treatments with time. After pooling all the hot-bicarbonate extracted carbohydrate data, the mean concentration was 221.4 ± 42.4 μg g^{-1} sediment.

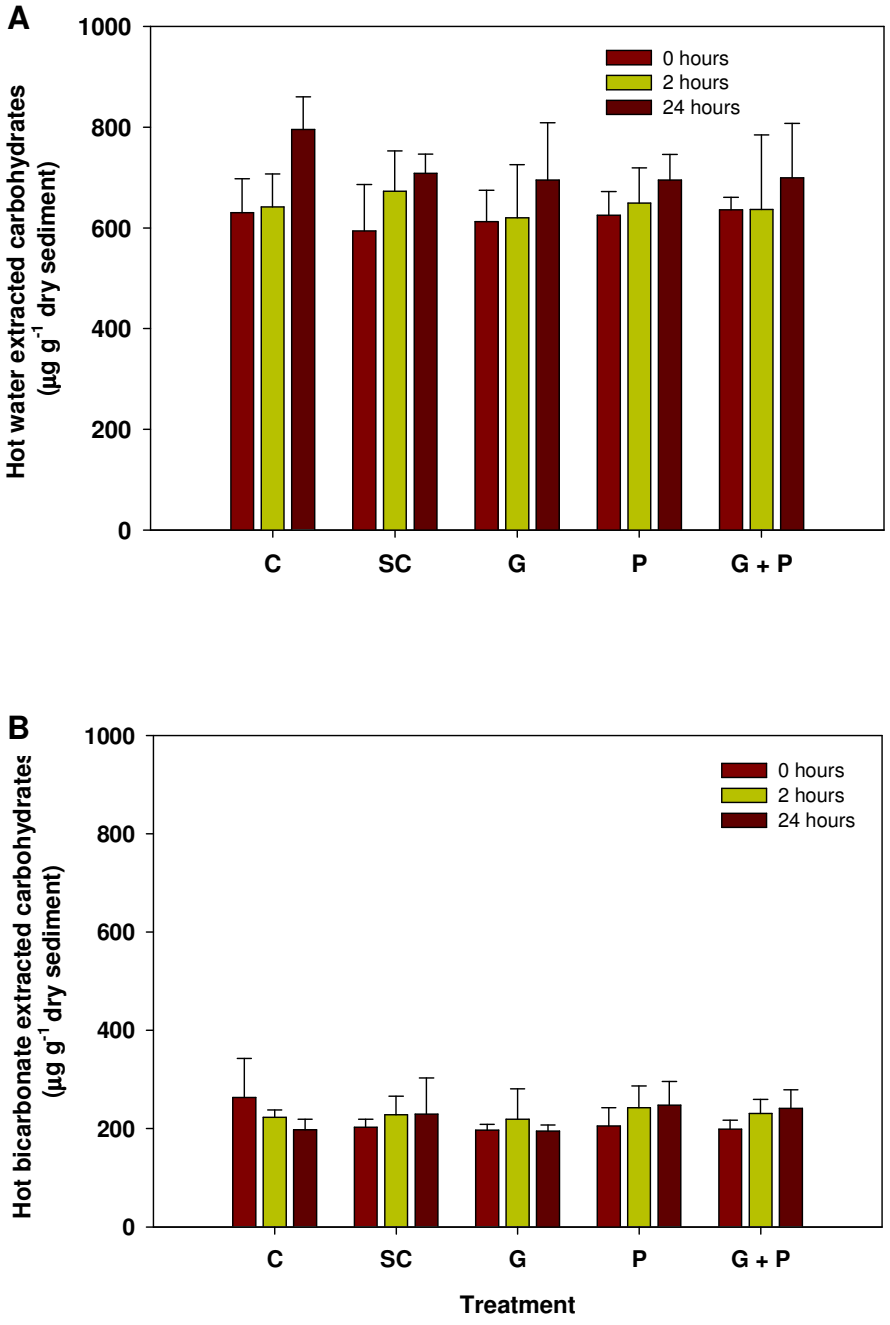


Figure 28: Hot water and hot bicarbonate carbohydrate concentrations for Experiment #2.2. Concentrations were from within the sediment over time for each treatment for Experiment #2.2. (A) hot-water extracted carbohydrate concentration (B) hot-bicarbonate extracted carbohydrate concentration. Treatments were control (C), saline control (SC), glucose addition (G), phosphorus addition (P), and glucose + phosphorus addition (G + P). Bars represent mean + SD (n = 4).

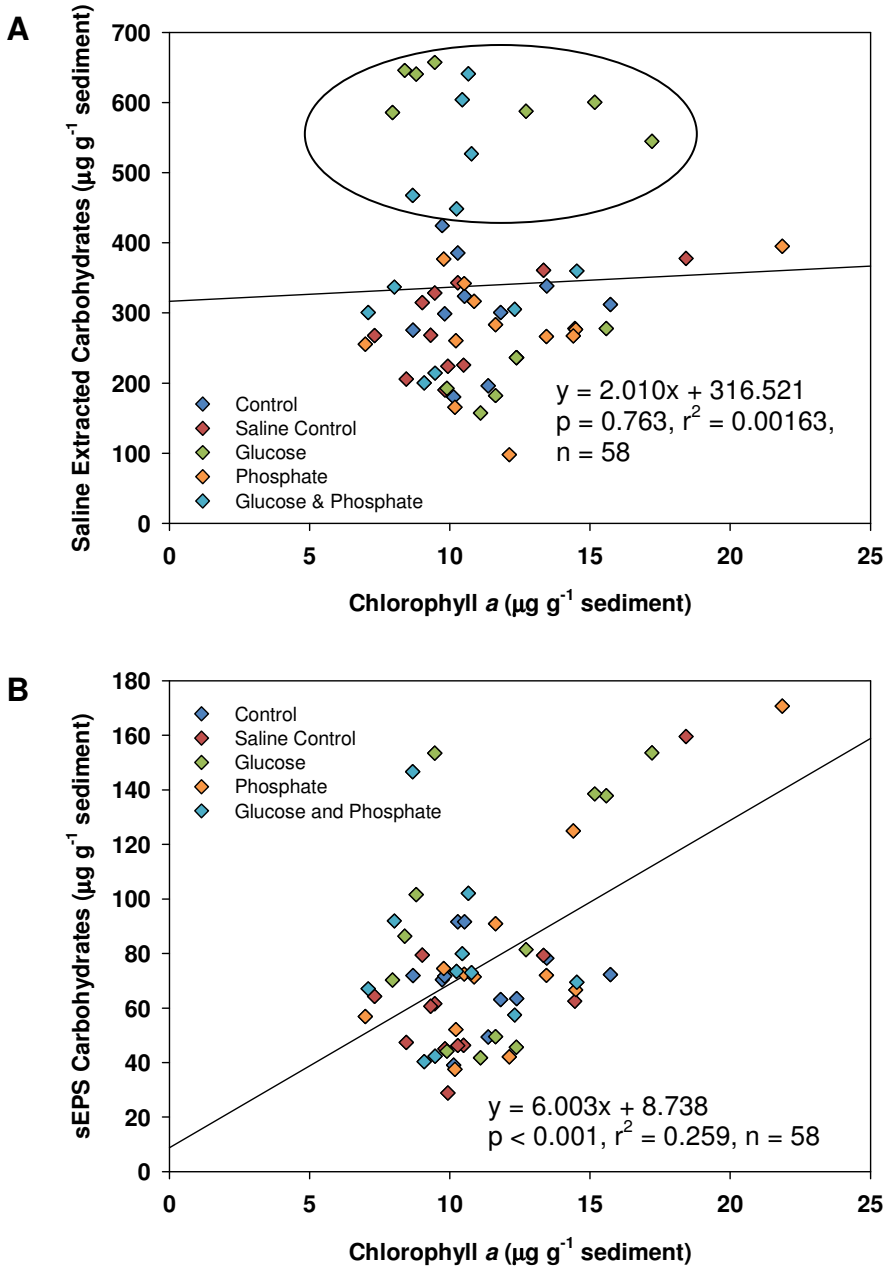


Figure 29: Chlorophyll and carbohydrate relationships with all treatments for Experiment #2.2. Relationship between chlorophyll *a* with (A) Saline-extractable carbohydrates (the circle surrounds samples from glucose and glucose + phosphate treatments) and (B) sEPS carbohydrates.

Figure 29 displays the linear regressions between chlorophyll *a* concentrations with the saline-extractable carbohydrate concentrations and with the sEPS carbohydrate concentrations for all samples from Experiment #2.2. The regression calculated for the correlation between chlorophyll *a* and the saline-extracted carbohydrates was not significant ($p = 0.763$, $r^2 = 0.00163$, $n = 58$). The circle seen in Figure 29A surrounds the data points representing the concentrations from the glucose treatment and the glucose + phosphate treatment. The regression was most likely skewed due to the additional carbohydrates added to the sediment. The regression calculated for the correlation between chlorophyll *a* and the sEPS carbohydrates was significant ($p < 0.001$, $r^2 = 0.259$, $n = 58$). Neither regression is very strong. Because the carbohydrate additions skewed the relationship between chlorophyll *a* and the carbohydrate fractions, Figure 30 shows the regressions of chlorophyll *a* with the saline-extractable carbohydrate concentration and the sEPS carbohydrate concentrations using only the control treatment and the saline control treatment. The regression calculated for the relationship between chlorophyll *a* and the saline-extractable carbohydrates was not significant ($p = 0.154$, $r^2 = 0.0943$, $n = 23$). The regression calculated for the relationship between chlorophyll *a* and the sEPS carbohydrates was significant ($p < 0.01$, $r^2 = 0.344$, $n = 23$). Even without the treatments of carbohydrate additions, the regressions are not very strong.

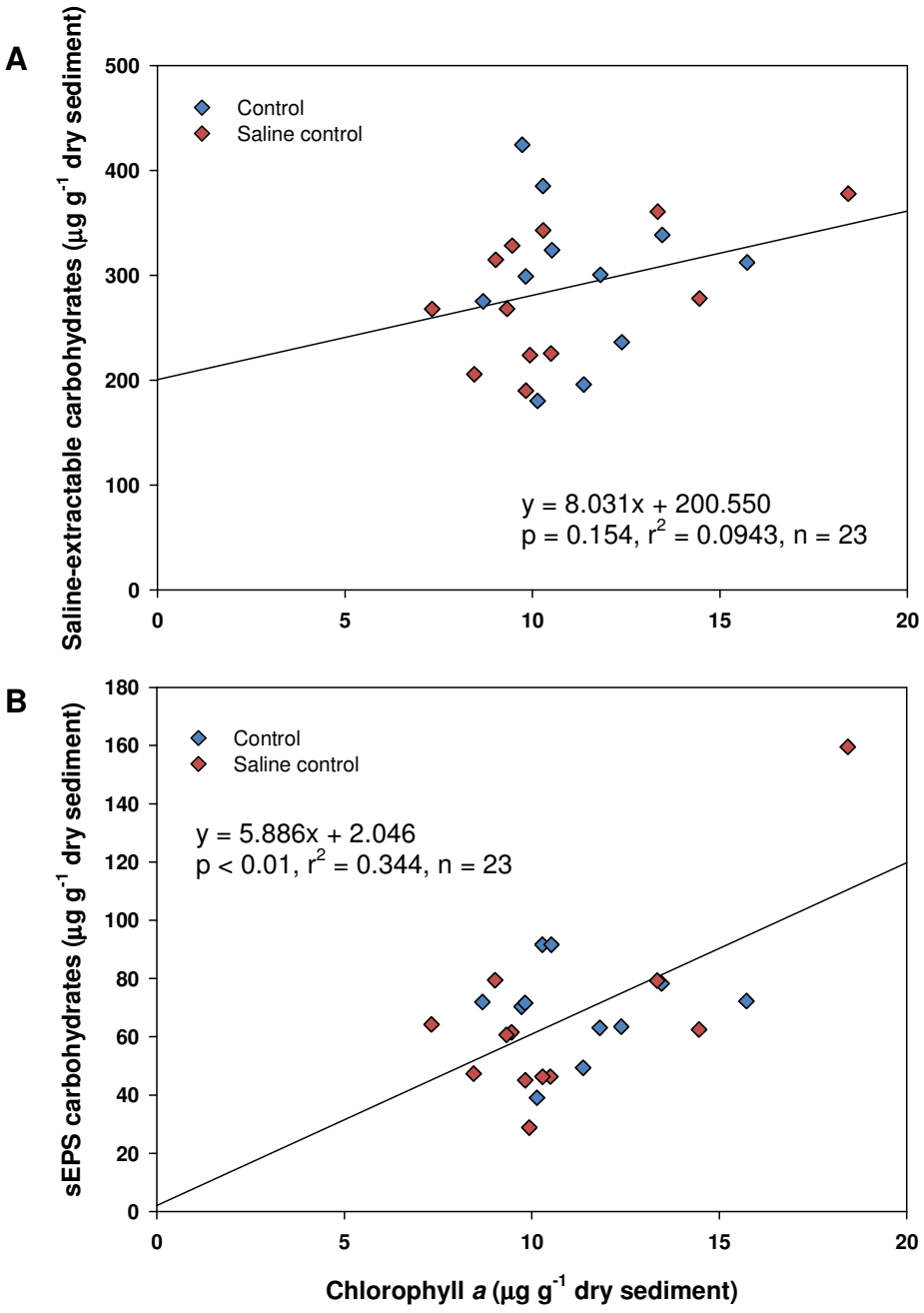


Figure 30: Chlorophyll and carbohydrate relationships with the control treatments for Experiment #2.2. Relationship between chlorophyll *a* with (A) Saline-extractable carbohydrates and (B) sEPS carbohydrates.

Enzyme Activities

Figure 31 shows the mean activity for β-glucosidase, β-xylosidase, and β-galactosidase measured from sediment from each treatment over the course of Experiment #2.1. The β-glucosidase activity is very variable between and within treatments, but there was a significant difference with time with all four treatments ($F_{5, 264} = 46.501$, $p < 0.001$). Post-hoc pair wise comparisons revealed a significant increase ($p < 0.05$) over the first 24 hours of the experiment, then decreasing over the course of the rest of the experiment. For β-xylosidase, there was a significant difference between treatments ($F_{3, 264} = 10.667$, $p < 0.001$) and with time ($F_{5, 264} = 79.009$, $p < 0.001$). Activities for β-xylosidase was significantly lower ($p < 0.001$) in the glucose treatment and the alginic acid treatment compared to both the control treatment and the saline control treatment. The same trend, of an initial increase over the first 24 hours, followed by a decrease over the rest of the experiment seen with the activity for β-glucosidase, was also seen for β-xylosidase. After 24 hours, there was a significant decrease ($p < 0.001$) over the rest of the experiment in β-xylosidase mean enzyme activity for all the treatments. There was no significance difference in mean enzyme activity for β-galactosidase between treatments or over time for Experiment #2.1. The enzyme activities for all three enzymes, β-glucosidase, β-xylosidase, and β-galactosidase, are all

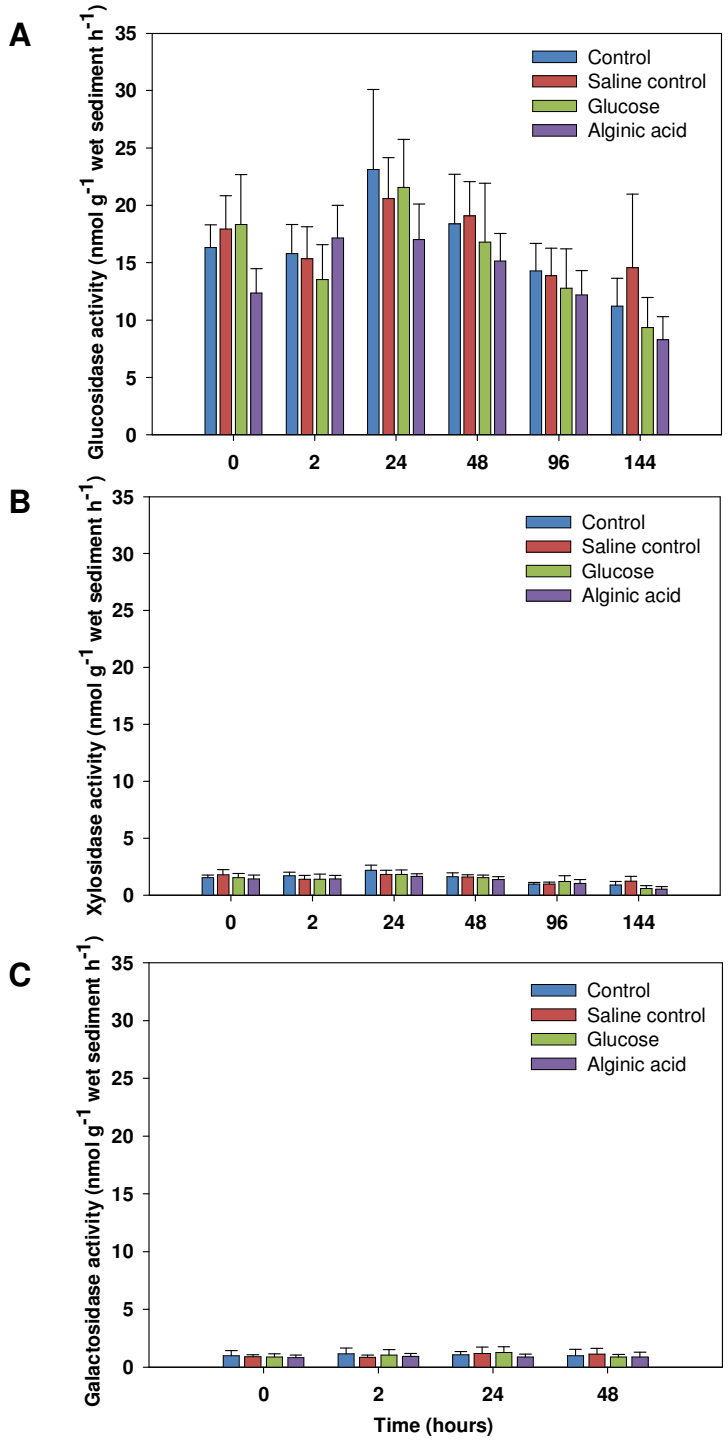


Figure 31: Enzyme activities for Experiment #2.1. Activities measured with a substrate concentration of 500 µM over time with treatment. (A) β-glucosidase activity, (B) β-xylosidase activity, (C) β-galactosidase activity. Bars show means + SD (n = 4).

Table 1: Mean enzyme activities for Experiment #2.1. Average β-glucosidase activity, β-xylosidase activity, and β-galactosidase activity reported with time and treatment for Experiment #2.1. Values in table are means ± SD (n = 4).

	Control			Saline Control			Glucose			Alginic Acid		
	β-glu	**β-xyl**	**β-galac**	**β-glu**	**β-xyl**	**β-galac**	**β-glu**	**β-xyl**	**β-galac**	**β-glu**	**β-xyl**	**β-galac**
0 hours	16.3 ± 2.0	1.5 ± 0.3	1.0 ± 0.4	17.9 ± 2.9	1.8 ± 0.5	0.9 ± 0.2	18.3 ± 4.4	1.5 ± 0.4	0.9 ± 0.3	12.3 ± 2.1	1.4 ± 0.3	0.8 ± 0.2
2 hours	15.8 ± 2.5	1.7 ± 0.3	1.2 ± 0.5	15.3 ± 2.8	1.4 ± 0.3	0.9 ± 0.2	13.5 ± 3.0	1.4 ± 0.4	1.1 ± 0.5	17.2 ± 2.9	1.4 ± 0.3	0.9 ± 0.3
24 hours	23.1 ± 7.0	2.2 ± 0.5	1.1 ± 0.3	20.6 ± 3.5	2.0 ± 0.4	1.2 ± 0.5	21.6 ± 4.2	1.8 ± 0.4	1.3 ± 0.5	17.0 ± 3.1	1.7 ± 0.2	0.9 ± 0.3
48 hours	18.4 ± 4.3	1.6 ± 0.4	1.0 ± 0.6	19.1 ± 3.0	1.6 ± 0.2	1.1 ± 0.5	16.8 ± 5.1	1.6 ± 0.2	0.9 ± 0.3	15.2 ± 2.4	1.4 ± 0.3	0.9 ± 0.4
96 hours	14.3 ± 2.4	1.0 ± 0.2	ND	13.9 ± 2.4	1.0 ± 0.2	ND	15.0 ± 8.4	1.2 ± 0.5	ND	12.2 ± 2.1	1.0 ± 0.3	ND
144 hours	11.2 ± 2.4	0.9 ± 0.3	ND	14.6 ± 6.4	1.2 ± 0.4	ND	9.3 ± 2.0	0.6 ± 0.2	ND	8.3 ± 2.0	0.5 ± 0.2	ND

Table 2: Mean enzyme activities for Experiment #2.2. Average β-glucosidase activity, β-xylosidase activity, and β-galactosidase activity reported with time and treatment for Experiment #2.2. Values in table are means ± SD (n = 4).

	Control			Saline Control			Glucose			Phosphate			Glucose and Phosphate		
	β-glu	**β-xyl**	**β-gala**	**β-glu**	**β-xyl**	**β-gala**	**β-glu**	**β-xyl**	**β-gala**	**β-glu**	**β-xyl**	**β-gala**	**β-glu**	**β-xyl**	**β-gala**
0 hours	15.3 ± 3.4	5.8 ± 1.2	8.4 ± 2.0	15.6 ± 3.1	9.5 ± 4.1	8.2 ± 2.2	13.7 ± 4.8	6.5 ± 0.4	8.7 ± 2.6	15.6 ± 3.1	6.4 ± 2.4	7.9 ± 1.5	13.7 ± 1.7	5.9 ± 1.1	7.2 ± 1.3
2 hours	17.7 ± 2.3	6.2 ± 1.3	9.1 ± 2.0	14.9 ± 1.7	5.8 ± 0.2	7.7 ± 1.5	13.4 ± 1.7	5.9 ± 1.3	8.7 ± 1.0	16.9 ± 2.8	6.3 ± 0.4	7.5 ± 2.4	14.0 ± 2.7	7.8 ± 2.4	6.9 ± 1.9
24 hours	12.6 ± 2.8	5.1 ± 1.9	6.7 ± 1.1	11.4 ± 1.8	5.9 ± 2.1	7.2 ± 1.1	18.1 ± 2.0	7.7 ± 4.8	8.5 ± 1.4	15.5 ± 1.1	5.3 ± 0.8	7.9 ± 0.5	15.5 ± 3.5	5.5 ± 0.9	8.0 ± 1.2

significantly different ($H = 570.907$, $df = 2$, $p < 0.001$) from each other, with β-glucosidase activity higher than β-xylosidase activity, which is higher than β-galactosidase activity (Table 1).

Figure 32 shows the mean enzyme activity for β-glucosidase, β-xylosidase, and β-galactosidase measured from sediment from each treatment over the course of Experiment #2.2. There was a significant interaction of treatment with time for the β-glucosidase activities ($F_{8, 165} = 8.076$, $p < 0.001$). After 24 hours, the β-glucosidase activity was significantly higher ($p < 0.05$) in the glucose treatment and the phosphate treatment compared to the β-glucosidase activity in the control treatment and the saline control treatment. There was no significant difference in mean activity for β-xylosidase and β-galactosidase either with treatment or time. The enzyme activities for all three enzymes, β-glucosidase, β-xylosidase, and β-galactosidase, are all significantly different ($F_{2, 297} = 400.140$, $p < 0.001$) from each other, with β-glucosidase activity higher than β-galactosidase activity, which was higher than β-xylosidase activity (Table 2).

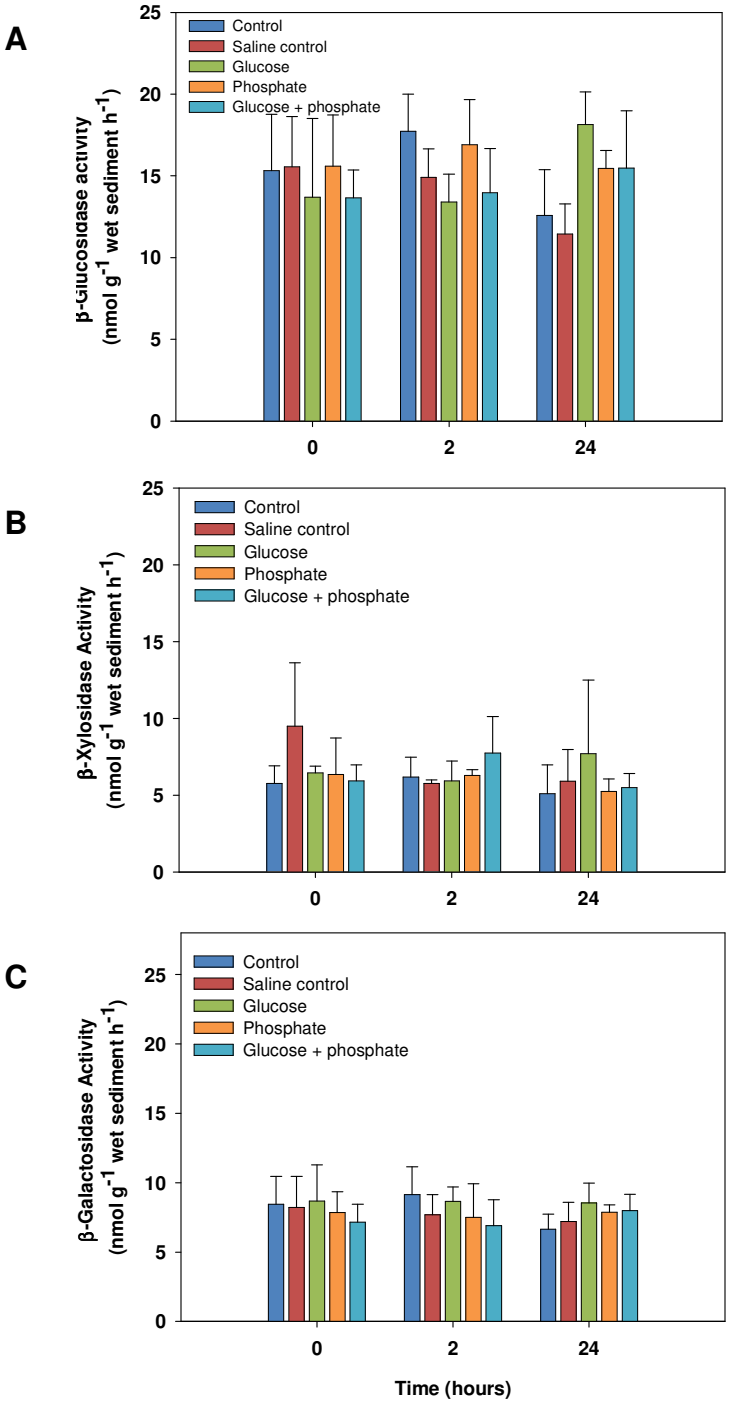


Figure 32: Enzyme activities for Experiment #2.2. Activities measured with a substrate concentration of 900 µM over time with treatment for Experiment #2.2. (A) β-glucosidase activity, (B) β-xylosidase activity, (C) β-galactosidase activity. Bars show means + SD (n = 4).

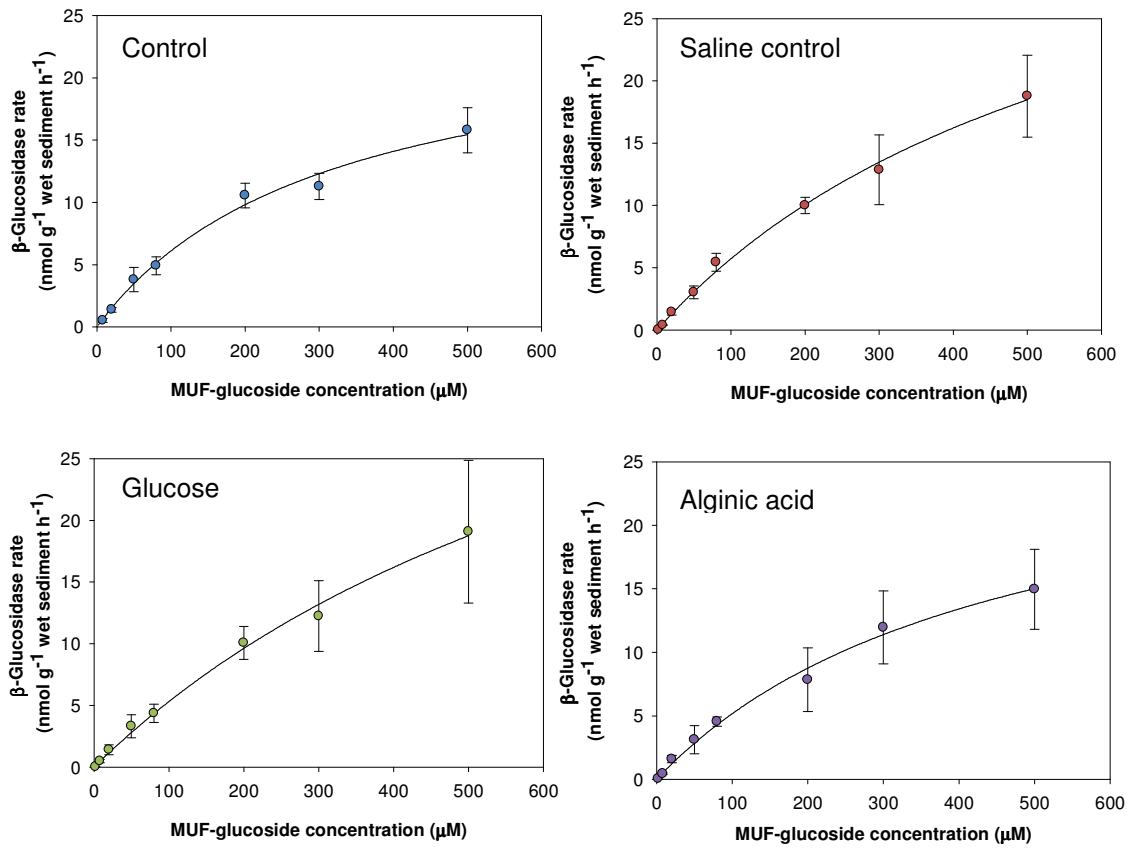


Figure 33: β-glucosidase kinetics curves for Experiment #2.1. Curves were measured for each treatment after 48 hours. Points show means ± SD (n = 4).

Enzyme Kinetics

Figure 33 shows the β-glucosidase kinetic curves for each treatment 48 hours into Experiment #2.1. The curves are very similar, but each treatment had high variability between activity rates, seen with the error bars. Table 3 shows the mean K_m and V_{max} values for the treatments after fitting a Michaelis-Menten relationship to the data from each treatment.

Table 3: V_{max} and K_m values for the β-glucosidase kinetics curves for Experiment #2.1.

		Control	**Saline Control**	**Glucose**	**Alginic Acid**
48 hours	**V_{max}**	26.02 ± 5.99	44.53 ± 16.01	35.24 ± 10.37	29.04 ± 11.16
	K_m	334.42 ± 115.77	675.87 ± 256.60	578.29 ± 140.68	377.38 ± 175.42

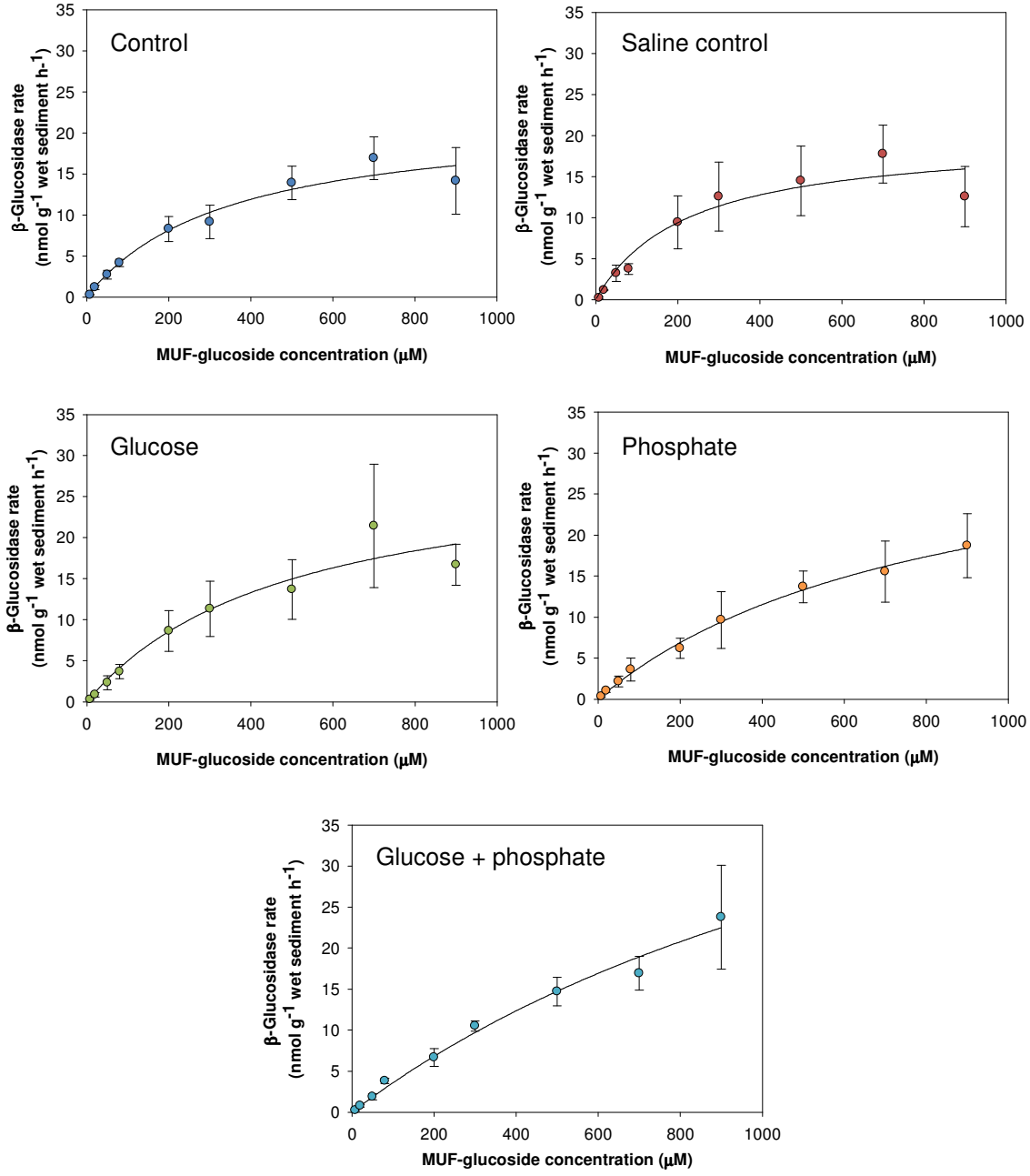


Figure 34: β-glucosidase kinetics curves at time 0 for Experiment #2.2. Points show means ± SD (n = 4).

Figures 34-36 show the β-glucosidase kinetic curves for each treatment at each sample time for Experiment #2.2. One set of figures corresponds with one sampling time. Again although the curves look very similar, there is high variability of enzyme activity within the treatments. Many of the curves hint at a possible substrate inhibition at the concentration of 900 μM. Table 4 shows the mean K_m and V_{max} values for the treatments after fitting a Michaelis-Menten relationship to the date from each treatment.

Table 4: V_{max} and K_m values for the β-glucosidase kinetics curves for Experiment #2.2.

		Control	Saline Control	Glucose	Phosphate	Glucose and Phosphate
0 hours	**V_{max}**	22.57 ± 3.88	21.04 ± 5.57	31.15 ± 9.63	36.60 ± 11.58	41.31 ± 6.37
	K_m	362.95 ± 92.72	265.35 ± 102.37	609.70 ± 233.83	872.58 ± 314.42	938.60 ± 107.06
2 hours	**V_{max}**	40.14 ± 9.61	33.35 ± 8.08	36.32 ± 7.87	38.06 ± 16.18	29.66 ± 5.07
	K_m	649.93 ± 422.89	477.85 ± 231.21	738.33 ± 346.38	548.83 ± 269.03	504.03 ± 98.60
24 hours	**V_{max}**	28.47 ± 6.93	31.93 ± 4.07	34.32 ± 16.24	28.61 ± 5.96	22.43 ± 5.13
	K_m	311.80 ± 43.10	489.78 ± 155.25	450.95 ± 250.13	395.93 ± 170.90	378.73 ± 204.27

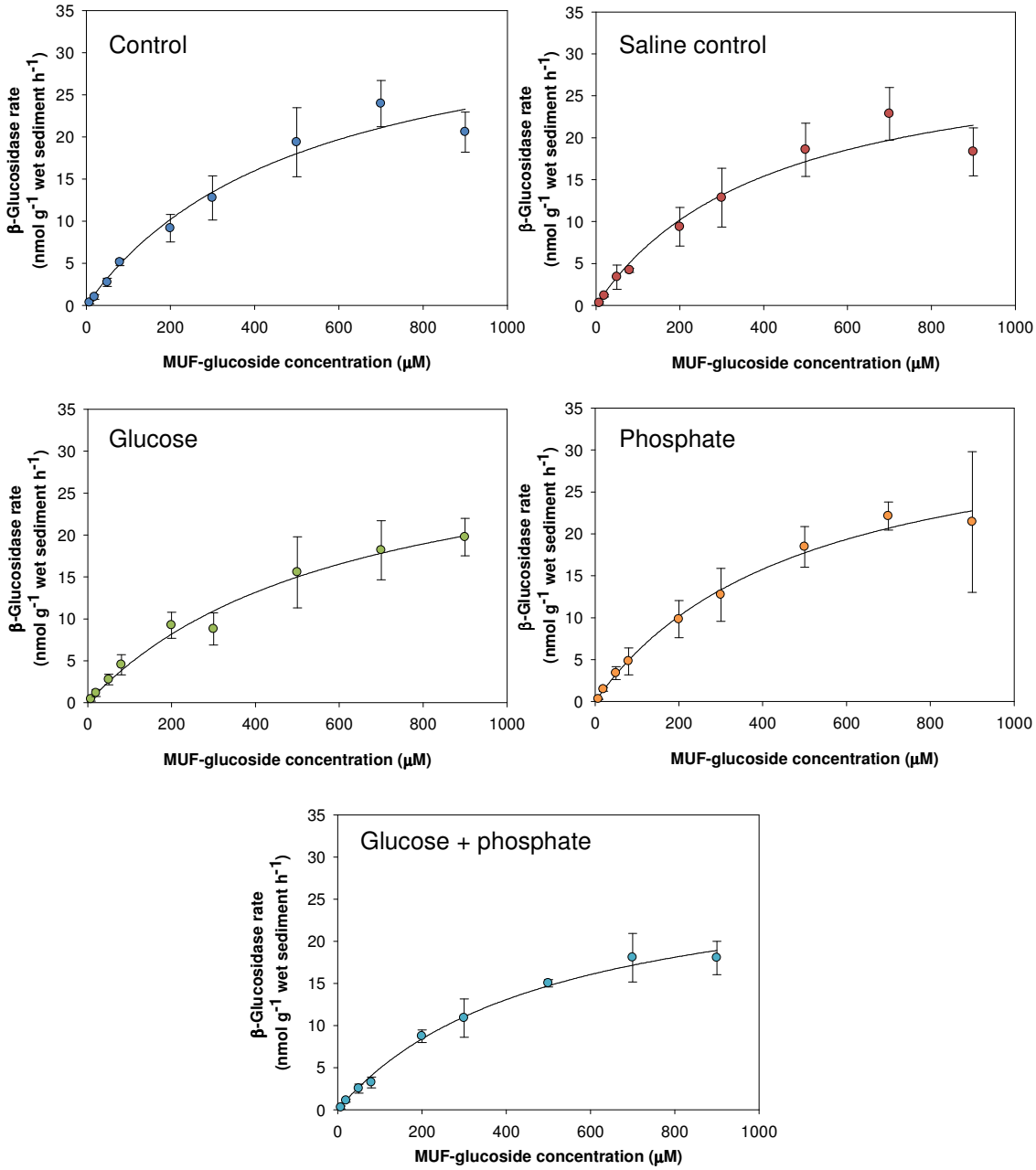


Figure 35: β-glucosidase kinetics curves after 2 hours for Experiment #2.2. Points show means ± SD (n = 4).

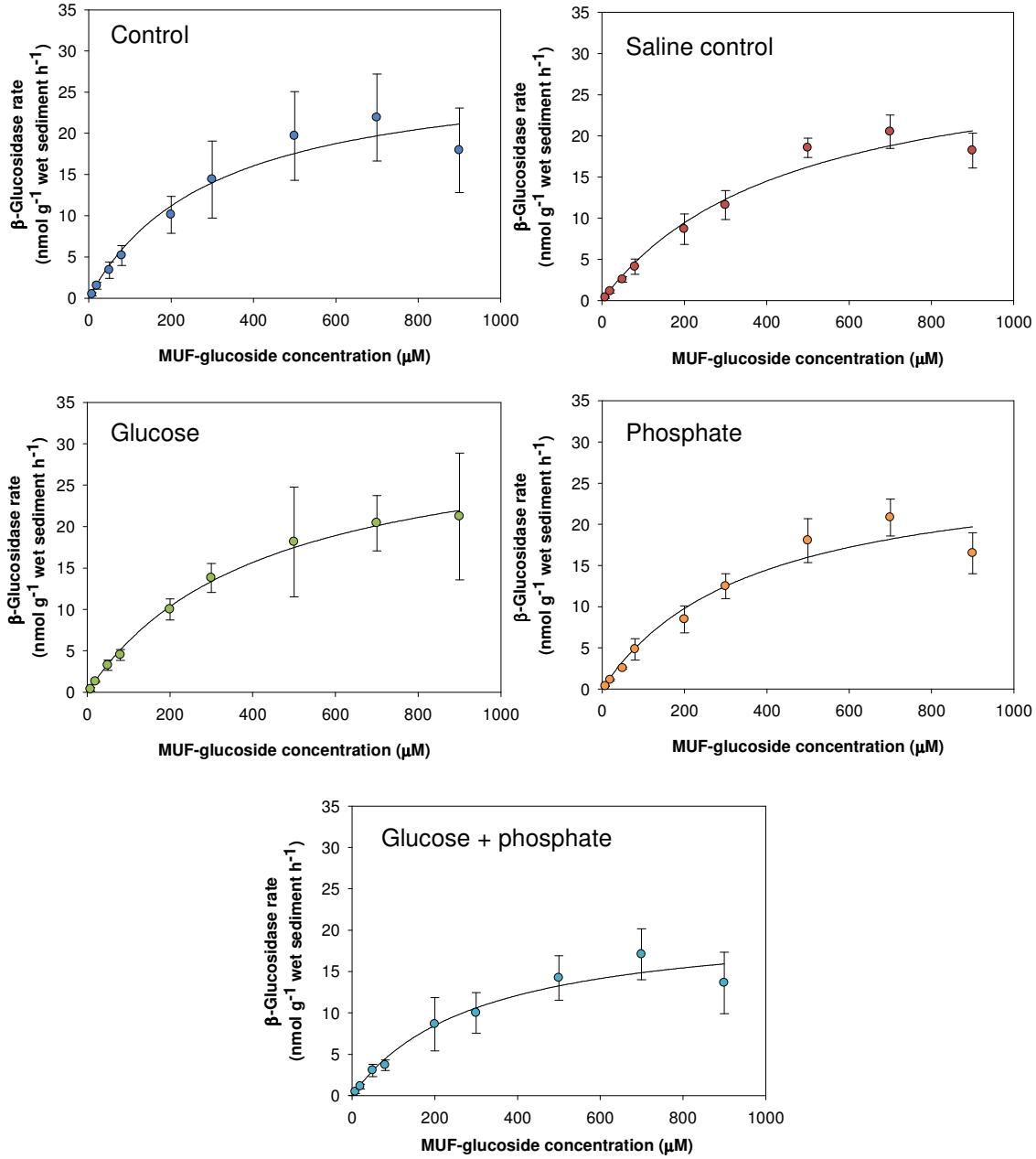


Figure 36: β-glucosidase kinetics curves after 24 hours for Experiment #2.2. Points show means ± SD (n = 4).

No correlations were found between K_m or V_{max} and the saline-extractable carbohydrate concentrations or between K_m or V_{max} and the sEPS carbohydrate concentrations.

Bacteria Abundance

Figure 37 shows the change seen in bacterial abundance in Experiment #2.2 in the control treatment, saline control treatment, glucose treatment, and the phosphate treatment over 24 hours. There was a significant difference in treatment ($F_{3, 24} = 7.746$, $p < 0.001$). The bacteria abundance in the glucose treatment was significantly higher ($p < 0.001$) after 24 hours increasing from $4.32x10^9 \pm 7.32x10^8$ cm^{-3} sediment to $6.04x10^9 \pm 5.18x10^8$ cm^{-3} sediment.

Phosphate Concentrations

Figure 38 shows the change in pore water phosphate concentrations over time with treatment for Experiment #2.2. There was no significant difference between treatments or with time for phosphate concentrations.

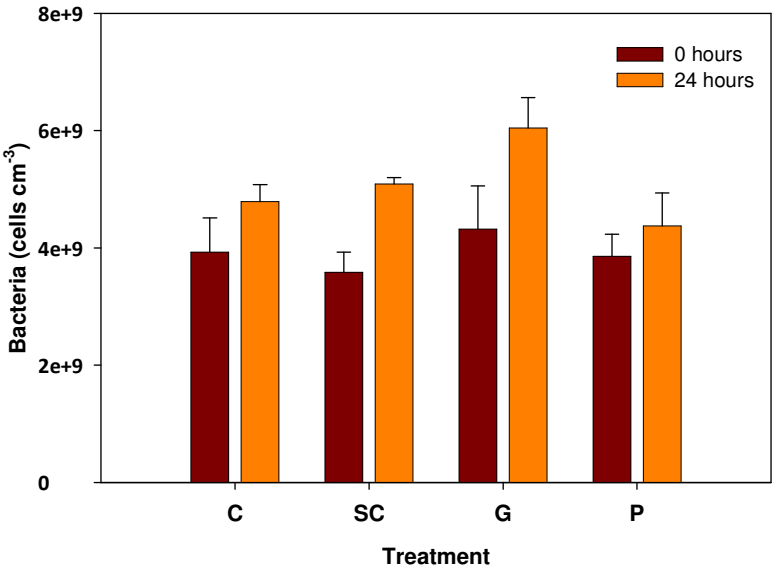


Figure 37: Bacteria abundances for Experiment #2.2. Abundances measured within the sediment over time with treatment. Treatments were control (C), saline control (SC), glucose addition (G), and phosphate addition (P). Bars represent means + SD (n = 8).

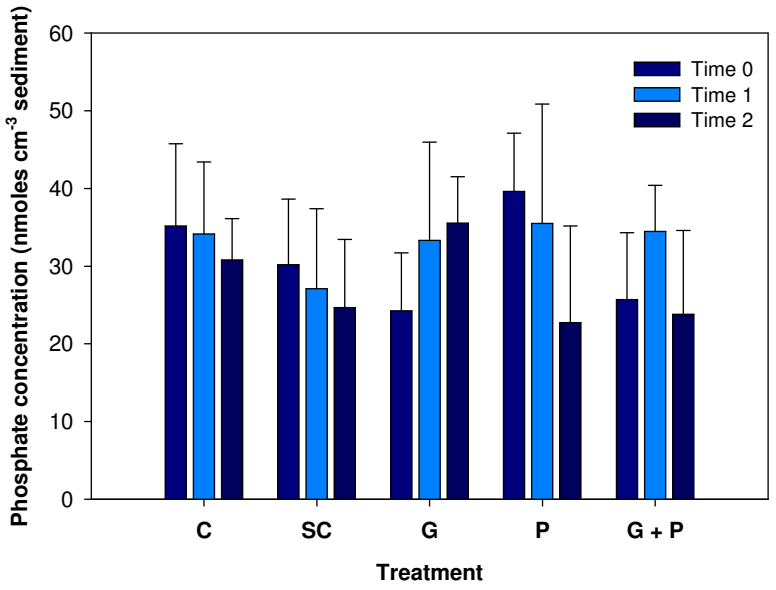


Figure 38: Sediment phosphate concentrations for Experiment #2.2. Concentrations measured within the sediment over time with treatments. Treatments were control (C), saline control (SC), glucose addition (G), phosphate addition (P), glucose + phosphate addition (G + P). Bars represent means + SD (n = 4).

Discussion

While the first experiment looked at the changes in BMA biomass and carbohydrate production by the BMA with nutrient additions, this experiment looked at the possible hydrolysis of these carbohydrates by exoenzymes (β-glucosidase, β-xylosidase, and β-galactosidase) into monosaccharides necessary for the heterotrophic bacteria living in the biofilms with the BMA. Research on exoenzyme activity has occurred in coastal sediments in the past, but rarely has there been work on glycosidases in BMA biofilms.

Chlorophyll a *and Carbohydrate Responses to Phosphate and Carbohydrate Additions*

As in the first experiment, there was no change in the BMA biomass with treatment after the chlorophyll and carbohydrate analyses. Once again, the results showed high variability. BMA within the sediment tend to grow in patches depending on environmental factors, such as the nutrients available to them, grazing pressures, salinity, and temperature changes. There may have been patches of BMA growth within the plot enclosures, which when sampled, would lead to the variability seen in the chlorophyll *a* and carbohydrate concentrations. Interestingly, the glucose addition was not seen in the saline-extractable carbohydrate concentrations in Experiment #2.1, but it was clearly seen in the saline-extractable carbohydrate concentrations in Experiment #2.2. The glucose addition was strong enough to show an increase in the total carbohydrate concentrations and the sEPS carbohydrate concentrations for Experiment #2.2 as well. It is unclear why an addition of glucose would lead to an increase in sEPS

unless some of the low-molecular weight carbohydrates were being incorporated into the sEPS or the glucose was contaminating the sEPS analysis. The phosphate treatment displayed an increase in sEPS hinting at a possible growth response in the BMA with this treatment, but this was not seen in the chlorophyll *a* concentration. An increase in sEPS in the alginic acid treatment occurred because the alginic acid added to the sediments would also be extracted with the sEPS, which would increase that concentration in the analysis. The phosphate addition, instead of promoting BMA growth, may have caused them to produce more sEPS. Other studies have noticed an increased production of sEPS when BMA cells become nutrient limited. This provides evidence that the BMA are not phosphorus limited as previously suspected (Underwood & Smith 1998, Underwood et al. 2004).

In Experiment #2.2, the hot water extracted (HW) and hot bicarbonate (HB) extracted carbohydrate pools were measured as well. The HW polymers tend to be glucose dominated, and thus have been used in the past as a measurement of stored photosynthetic carbon created by the BMA (Hanlon et al. 2006). The HW polymers tend to be intracellular carbohydrates that tend to be storage for carbon fixed through photosynthesis (Bellinger et al. 2005). With no changes seen in the HW carbohydrate concentration, along with little change seen in the chlorophyll *a* concentration, it seems that there was not an increase in BMA biomass with these treatments. An increase in BMA biomass would lead to an increase in photosynthetic activity within the sediment. The HB polymers are similar in monosaccharide composition as sEPS, but are considered to be tightly bound capsular polymers used as structure for the BMA and for

the biofilm (Hofmann et al. 2009). Previous work has shown little correlation between HB polymers and BMA biomass indicating a longer turn-over time for the HB carbohydrates than the other carbohydrate fractions produced by BMA. This turn over time is long enough to decouple the HB concentration with the BMA even though they are the source of the HB carbohydrates. The HB carbohydrates linger long enough within the biofilm that they tend to act as a sediment stabilizer (Bellinger et al. 2009). Thus, it would be unlikely to see a change in the HB carbohydrate concentration, unless there was a noticeable decrease in the saline-extractable carbohydrates and the sEPS carbohydrates, as exoenzymes released by the bacteria attack the carbohydrate polymers preferentially with hydrolysis of HB carbohydrates occurring after sEPS.

Enzymatic Response to Carbohydrate Additions

In the past, β-glucosidase has been identified as the hydrolytic enzyme used as an indicator of the overall enzymatic activity within the sediment, and most researchers attempt to add saturating concentrations of the MUF-substrate for this enzyme in order to measure the maximum rate of enzyme activity (V_{max}). While this has been attempted in the past, each researcher has a slightly different idea on what the saturating concentration should be. Haynes et al. (2007) added a 1 mM concentration of the substrate to the samples, and Meyer-Reil (1986) decided that a concentration of 5 mM was still not saturating. In this study, the saturating concentration was increased from 500 μM to 900 μM from Experiment #2.1 to Experiment #2.2. Preliminary kinetics graphs using sediment near the sampling site indicated that the saturating concentration

was 500 μM. After reviewing the kinetics curves for Experiment #2.1 (Fig. 31), the curves were still clearly increasing at that concentration. Preliminary kinetics tests were run again using sediment from the sample site increasing the saturating concentration to 900 μM. The curves were visibly flattening as it reached 500 μM in these preliminary tests, so that was the concentration chosen for Experiment #2.1. Clearly there was a change in the enzyme kinetics between the preliminary tests and Experiment #2.1 which indicated the change in β-glucosidase kinetics between two slightly different intertidal sediment patches. This change could have been spatially, but it might also be temporally, as much of the work to develop this method of measuring enzyme kinetics was completed over the summer months prior to sampling. Enzyme activities for all three enzymes did increase from Experiment #2.1 to Experiment #2.2 with the change in the concentration, thus 900 μM was to closer to the saturating substrate concentration for glycosidase in this salt marsh. Michaelis-Menten enzyme kinetics curves were calculated using several different concentrations of the MUF-substrates over the range of 0 to 900 μM. Among many of the kinetics curves calculated for Experiment #2.2, there seemed to be an indication of MUF-substrate inhibition for β-glucosidase activity, which actually suggests that 900 μM is too high as an estimate of the β-glucosidase saturating concentration for this salt marsh.

This study is the first study to incorporate enzyme kinetics to further characterize changes in the carbohydrate pools within sediment. Hydrolysis rates of glucose, xylose, and galactose are related to the substrate concentration and the maximum activity of the exoenzyme. The kinetics of an exoenzyme is described by the half-saturation constant

(K_m) and the maximum velocity (V_{max}) determined using the Michaelis-Menten equation (Nagata 2008). These parameters describe the enzyme hydrolysis rate for each exoenzyme and variations in the hydrolysis rate tend to occur with spatial variability, shifts in community compositions of the bacteria, and changes in the expression of the enzyme by the same bacteria species (Nagata 2008). This experiment showed little difference in the enzyme hydrolysis rate for each exoenzyme, thus the treatments added to the sediment had little effect upon the factors that alter the rate.

This was also one of the first experiments to measure the enzyme activities of β-xylosidase and β-galactosidase in intertidal sediments. One other study by King (1986) measured rates of these enzymes, as well as β-glucosidase rates, in a temperate intertidal sediment, but the fluorescent-tagged substrate concentration added to the samples was only 1 μM. Typically, the substrate concentration is much higher in order to measure accurate enzyme activities. King also measured enzyme rates over the top 2 cm of sediment, which incorporates a larger carbohydrate pool containing extracellular carbohydrates that would have been buried in the sediment. This study focused instead on the portion of sediment containing the extracellular carbohydrates produced by the BMA because these carbohydrates are ecologically significant within the surface biofilms.

These carbohydrate pools within the sediment may have high glucose content, but xylose and galactose are also common monosaccharides found in these carbohydrate polymers (Bellinger et al. 2009, Hofmann et al. 2009, Hanlon et al. 2006). Exoenzyme activities can be used as an indicator of polysaccharide composition within the sediment.

The production of enzymes will only occur if there is suitable substrate available, therefore the activity of glucosidase, galactosidase, and xylosidase indicates the presence of glucose, galactose, and xylose in the sediment carbohydrates. Glycosidases can also be used as a tool to investigate the linkages between monosaccharides in polymers. For example, the enzymes used in this work show that there were β-linkages between the monosaccharides within the sediment. Enzyme activity of α-glycosidases would indicate α-linkages between the monosaccharides.

In both experiments, β-glucosidase activities were significantly higher that the activities of β-xylosidase and β-galactosidase, supporting the second hypothesis. This indicates either higher glucose content within the sediment than xylose or galactose or the preferential selection of glucose over xylose or galactose by the bacteria releasing these enzymes. The β-xylosidase and β-galactosidase activities were significantly different from each other in both experiments, but the higher activities of these two enzymes switched between the two experiments. This could have been an artifact from changing the saturating concentration between Experiment #1 and Experiment #2, or there have been different contents of xylose and galactose within the sediment. Haynes et al (2007) saw a shift in the bacteria species composition with additions of sEPS to the sediment, which many have also occurred in the experiment. A shift in the bacteria community could also lead to a shift in the amounts of β-xylosidase and β-galactosidase released by the bacteria. The β-glucosidase activities measured for this experiment are slightly lower than activities measured by van Duyl et al. (1999) in the Ems-Dollard estuary, but they are slightly higher than activities measured in the Colne Estuary by

Haynes et al. (2007). Both of these estuaries are located at higher latitudes than Galveston Bay and have different environmental conditions which can affect the hydrolysis rate of an exoenzyme making it somewhat difficult to compare activities between different intertidal mudflats.

Past research has shown variable responses in β-glucosidase with an increase in low molecular weight carbohydrates within the sediment. Romani et al. (2004) found a suppression in β-glucosidase activity with an addition of low molecular weight carbohydrates, but van Duyl (1999) noticed that the β-glucosidase activity remained constant throughout tidal cycles and with changes in the low molecular weight carbohydrates. Haynes et al. (2007) found an increase in β-glucosidase activity with increased concentrations of structural polysaccharides. For this study, two different responses in β-glucosidase arose for both experiments. For Experiment #2.2, β-glucosidase activities were significantly higher with the glucose additions over the time period. There was also a significant increase in the bacteria abundance for this treatment, which would explain the increase in β-glucosidase. Glucose may be such a necessary carbon source for the bacteria that instead of suppressing the release of β-glucosidase, this enzyme is continually released in order to provide a steady supply of glucose. The glucose addition may have lead to the bloom in bacteria abundance in that treatment. Assuming β-glucosidase is continually released, this bloom would allow for more of the enzyme to be available with the increase in bacteria. On the other hand, a bacteria bloom may have depleted the labile carbon sources, leading to more glycosidases to be released in order to hydrolyze more refractory carbohydrates. For

Experiment #2.1, all three enzymes, β-glucosidase, β-xylosidase, and β-galactosidase, increased in activity over the first 24 hours of the experiment and then significantly decreased over the rest of the experiment. This hints at another environmental cue, instead of the carbohydrate additions, that could be affecting the change in enzyme activities.

This experiment displayed changes in the enzyme activity with glucose addition but not with alginic acid additions, which partially supports the third hypothesis. Although sEPS can contain uronic acids similar to the uronic acid polymers in alginic acid (Hanlon et al. 2006, Bellinger et al. 2009), none of the exoenzymes measured would hydrolyze alginic acid as it does not contain glucose, xylose or galactose. Alginic acid is composed of uronic acid polymers of β-1,4-linked D-mannuronic acid and α-1,4-linked L-glucouronic acid (FAO 1997), which is targeted by different enzymes to hydrolyze these polymers. sEPS consists of multiple monosaccharide components, such as glucose, xylose, galactose, fucose, and rhamnose, as well as uronic acids (Hanlon et al 2006, Bellinger et al. 2009, Hofmann et. al 2009). Aldohexoses, such as glucose and galactose, tend to be rapidly utilized by bacteria. Uronic acids in the sEPS have the potential to increase biostabilization within the biofilm through cation links (Panagiotopoulos & Sempere 2005, Bellinger et al 2009).

Enzymatic Response to Phosphate Additions

Sundareshwar et al. (2003) hypothesized phosphorus limitation instead of nitrogen limitation within coastal sediments, indicated by high levels of phosphatase

activity, and this phosphorus limitation was seen within the heterotrophic microbial community, while at the same time the BMA were nitrogen limited. The heterotrophic microbial community may be phosphorus limited, which could increase the loss of nitrogen within the sediment because nitrogen fixation can be limited by the availability of appropriate carbon fractions. With an increase in the heterotrophic microbial community with phosphorus additions, they compete for the available carbon sources (Sundareshwar et al. 2003). The BMA biomass did not respond with the phosphate additions in Experiment #2.2. There was an increase in the sEPS concentration and β-glucosidase activity with the phosphate additions. Even though there was an increase in β-glucosidase activity, there was no change in the bacteria abundance that might be expected based on the bacteria and β-glucosidase response seen with the glucose additions. It is unclear if this result really supports the fourth hypothesis stating that the limiting nutrient will result in an increase in enzyme activity. Although there was an increase in β-glucosidase activity, the data did not support the hypothesis that phosphorus was the limiting nutrient in this salt marsh.

Methodological Improvements

In order to continue with this research, it would be necessary to conduct the enzyme activity analysis sooner after sample collection. The amount of enzyme within the sediment may have changed with the commute between College Station and Galveston, thus the instrumentation for the analysis would need to be relocated to a lab in Galveston. Preservation strategies were employed in an attempt to prevent production

of new exoenzymes or the degradation of the enzymes already present within the sediment, but the samples would have to sit for a day or two due to transportation issues before the enzyme activities could be measured. Moving the operation to Galveston would also allow for the Harrison's artificial seawater solution to be closer to the *in situ* salinity, which would decrease any effects the different salinity of Harrison's might have on the enzyme samples.

In the future, this experiment could be extended to consider seasonal effects on the enzyme activity within these sediments, as well as identifying the monosaccharide components that make up the sEPS, hot-water extracted carbohydrates, and hot-bicarbonate carbohydrates. Hanlon et al. (2006) noticed a seasonal change in the monosaccaride composition for those polysaccharides, and this change could affect the activities of the exoenzymes acting upon the polymers.

Other studies have tried to identify the different bacteria in the sediment population in an attempt to determine the bacteria that release different exoenzymes (Haynes et al 2007, Misic & Harriague 2007, Hanlon et al. 2006). Shifts in the bacteria species composition may correlate with some of the processes of carbon and nutrient cycling affected by the exoenzymes.

Also, to gain a better understanding the effect of nutrient and organic additions for Galveston Bay, it would be useful to identify other sampling areas around the bay. The sampling area used for this study is close to the entrance to the Gulf of Mexico, so a site closer to the head of the bay, where the Trinity River and the San Jacinto River enter the bay, would provide an interesting contrast. One might expect higher concentrations

of nutrients from non-point source runoff to affect the sediment in this area more than the sample site used for this study.

CHAPTER IV

CONCLUSIONS

Summary

During this study, the limiting nutrient could not be identified for this salt marsh, and it was difficult to clearly identify enzyme activity changes within the sediment. The measurements for all the analyses were highly variable masking possible effects from the treatments added to the sediment.

The nutrient additions did not reveal very much response in the carbohydrate production from the BMA, which was one of the objectives for this study. It is possible that there is some other environmental cue that is acting as the limiting factor for growth of the BMA and for increased carbohydrate production.

This was the first study to demonstrate β-xylosidase and β-galactosidase activities in an intertidal BMA biofilm. The β-glucosidase activities and the bacteria abundance both increased in the glucose treatment, but not for any other treatments. Bacteria groups tend to utilize the carbon available to them selectively, and this utilization can sometimes be difficult to measure. Microbial degradation of carbohydrate particles requires colonization of the particles by the bacteria, which can deliver varying measurements of enzyme activities (Boetius & Lochte 1994). Clearly, glucose was more preferred than xylose and galactose due to the higher activities, and this also indicates a higher concentration of glucose components within the salt marsh over xylose and galactose.

This was the first time that exoenzyme kinetics have been used to describe the enzyme response of glycosides with nutrient and organic additions to the sediment.

This study demonstrated the dynamic and adaptive ecosystem mudflats can be. With the nutrient and carbohydrate additions, overall no major changes were seen in the BMA biomass, extracellular carbohydrate concentrations, and enzyme activities. The system was able to adapt to the additions quickly. Enzyme activities for all three enzymes were measured throughout the experiment with little changes see in the extracellular carbohydrate concentrations. Thus, the carbohydrates were quickly cycled with new production replacing the carbohydrates being hydrolyzed. The rapid turnover of sediment carbohydrate has also been documented in other studies over a timescale of hours (Arnosti & Homer 1999, Steen et al. 2008, Hofmann et al. 2009).

What Does This Mean for Galveston Bay?

There are not very many studies that relate enzyme activity with a degree of organic pollution, but reports of really high enzyme activities can be found in salt marshes located near industries and cities (Costa et al 2007). The Galveston Bay Estuary Program has identified non-point source pollution as a real issue that needs to be decreased and monitored (Lester & Gonzales 2002), and much of the oil refinery industry in Houston is located near the shore of the bay. Based on this study though, enzyme activities may not be the best way to monitor carbohydrate loss within the sediment through bacterial utilization with changes in nutrient concentrations. The microbial community within the surface of the sediment may be so used to

environmental changes that the community is very adept at responding and adapting to these changes without too much effort. Further studies located in different areas around the bay would provide a better picture of the microbial community and their responses to nutrient and organic additions.

REFERENCES

Abdullahi AS, Underwood GJC, Gretz MR (2006) Extracellular matrix assembly in diatoms (Bacillariophyceae) v. environmental effects on polysaccharide synthesis in the model diatom, *Phaeodactylum tricornutum*. Journal of Phycology 42:363-378

Admiraal W, Peletier H, Brouwer T (1984) The seasonal succession patterns of diatom species on an intertidal mudflat: an experimental analysis. Oikos 42:30-40

Aminot A, Kerovel R. (1982) Dosage automatique de l'uree dans l'ea de mer: une methode tres sensible a la diacelylmonoxime. Canadian Journal of Fisheries and Aquatic Sciences 39:174-183

Armitage AR, Frankovich TA, Fourqurean JW (2006) Variable responses within epiphytic and benthic microalgal communities to nutrient enrichment. Hydrobiologia 596:423-435

Armstrong FA, Stearns CR, Strickland JD (1967) The measurement of upwelling and subsequent biological processes by means of the Technicon Autoanalyzer and associated equipment. Deep-Sea Research 14(3): 381-389.

Arnosti C, Durkin S, Jeffrey WH (2005) Patterns of extracellular enzyme activities among pelagic marine microbial communities: implications for cycling of dissolved organic carbon. Aquatic Microbial Ecology 38:135-145

Arnosti C, Holmer M (1999) Carbohydrate dynamics and contributions to the carbon budget of an organic-rich coastal sediment. Geochemica et Cosmochimica Acta 63(3/4):393-403

Bellinger BJ, Abdullahi AS, Gretz MR, Underwood GJC (2005) Biofilm polymers: relationship between carbohydrate biopolymers from estuarine mudflats and unialgal cultures of benthic diatoms. Aquatic Microbial Ecology 38:169-180

Bellinger BJ, Underwood GJC, Ziegler SE, Gretz MR (2009) Significance of diatom-derived polymers in carbon flow dynamics within estuarine biofilms determined through isotopic enrichment. Aquatic Microbial Ecology 55:169-187

Berges JA, Franklin DJ, Harrison PJ (2001) Evolution of an artificial seawater medium: Improvements in enriched seawater, artificial water over the past two decades. Journal of Phycology 37:1138-1145

Bernhardt H, Wilhelms A (1967). The continuous determination of low level iron, soluble phosphate and total phosphate with the AutoAnalyzer. Technicon Symposium.

Boetius A, Lochte K (1996) Effect of organic enrichments on hydrolytic potentials and growth of bacteria on deep-sea sediments. Marine Ecology Progress Series 140:239-250

Costa AL, Carolino M, Cacador I (2007) Microbial activity profiles in Tagus estuary salt marsh sediments. Hydrobiologia 587:169-175

de Brouwer JFC, Stahl LJ (2001) Short-term dynamics in microphytobenthos distribution and associalted extracellular carbohydrates in surface sediments of an intertidal mudflat. Marine Ecology Progress Series 218:33-44

Decho AW (2000) Microbial biofilms in intertidal systems: an overview. Continental Shelf Research 20:1257-1273

Defew EC, Tolhurst TJ, Paterson DM (2002) Site-specific features influence sediment stability of intertidal flats. Hydrology and Earth Systems Science 6:971-982

Dong LF, Thornton DCO, Nedwell DB, Underwood GJC (2000) Denitrification in sediments of the River Colne estuary, England. Marine Ecology Progress Series 203:109-122

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356

FAO (1997) Compendium of food additive specifications. Addendum 5. Food and Agriculture Organization of the United Nations. http://www.fao.org/docrep/W6355E/w6355e00.htm

Graneli E, Sündback K (1985) The response of planktonic and microbenthic algal assemblages to nutrient enrichment in shallow coastal waters, southwest Sweden. Journal of Experimental Marine Biology and Ecology 85:253-268

Haese RR, Murray EJ, Smith CS, Smith J (2007) Diatoms control nutrient cycles in a temperate, wave-dominated estuary. Limnology and Oceanography 52:2686-2700

Hanlon ARM, Bellinger B, Haynes K, Xiao G, Hofmann TA, Gretz MR, Ball AS, Osborn AM, Underwood GJC (2006) Dynamics of extracellular polymeric substances (EPS) production and loss in an estuarine, diatom-dominated, microalgal biofilm over tidal emersion-immersion period. Limnology and Oceanography 51:79-93

Harrison PJ, Waters RE, Taylor FJR (1980) A broad spectrum artificial seawater medium for coastal and open ocean phytoplankton. Journal of Phycology 16:28-35

Harwood JE, Kerovel R (1970) A colorimetric method for ammonia in natural waters. Water Research 4:805-811

Haynes K, Hofmann TA, Smith CJ, Ball AS, Underwood GJC, Osborn AM (2007) Diatom-derived carbohydrates as factors affecting bacterial community composition in estuarine sediments. Applied and Environmental Microbiology 73:6112-6124

Hillebrand H, Worm B, Lotze HK (2000) Marine microbenthic community structure regulated by nitrogen loasing and grazing pressure. Marine Ecology Progress Series 204:27-38

Hofmann T, Hanlon ARM, Taylor JD, Ball AS, Osborn AM, Underwood GJC (2009) Dynamics and compositional changes in extracellular carbohydrates in estuarine sediments during degradation. Marine Ecology Progress Series 379:45-58

Hopkins JT (1963) A study of the diatoms of the Ouse estuary, Sussex. I. The movement of the mudflat diatoms in response to some chemical and physical changes. Journal of the Marine Biological Association of the United Kingdom 43:653-663

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

King GM (1986) Characterization of β-glucosidase activity in intertidal marine sediments. Applied and Environmental Microbiology 51(2):373-380

Lester J, Gonzales L (2002) The State of the Bay: A Characterization of the Galveston Bay Ecosystem, The Galveston Bay Estuary Program, Houston, TX

Lorenzen, CJ (1967) Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography 12:343-346

Madsen KN, Nilsson P, Sündback K (1993) The influence of benthic microalgae on the stability of a subtidal sediment. Journal of Experimental Marine Biology and Ecology 170:159-177

Marx MC, Wood M, Jarvis SC (2001) A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biology and Biochemistry 33:1633-1640

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

Middelburg JJ, Barranguet C, Boschker HTS, Herman PMJ, Moens T, Heip CHR (2000) The fate of intertidal microphytobenthos carbon: an in situ ^{13}C-labeling study. Limnology and Oceanography 45:1224-1234

Misic C, Harriague AC (2007) Enzymatic activity and organic substrates on a sandy beach of the Ligurian Sea (NW Mediterranean) influenced by anthropogenic pressure. Aquatic Microbial Ecology 47:239-251

Mulholland MR, Lee C, Gilbert PM (2003) Extracellular enzyme activity and uptake of carbon and nitrogen along an estuarine salinity and nutrient gradient. Marine Ecology Progress Series 258:3-17

Murphy J, Riley JP (1962) A modified single solution method for determination of phosphate in natural waters. Analytica Chimica Acta 26:31-36

Nagata T (2008) Organic matter -- bacteria interactions in seawater. In: Kirchman DL (ed) Microbial Ecology of the Oceans. John Wiley and Sons. Inc., Hoboken, NJ, p 207-241

Panagiotopoulos C, Sempere R (2005) Analytical methods for the determination of sugars in marine samples: A historical perspective and future directions. Limnology and Oceanography 3:419-454

Paterson DM (1986) The migratory behavior of diatom assemblages in a laboratory tidal microecosystem examined by low temperature scanning electron microscopy. Diatom Research 1:227-239

Penton CR, Newman S (2007) Enzyme activity responses to nutrient loading in subtropical wetlands. Biogeochemistry 84:83-98

Perkins RG, Underwood GJC, Brotas V, Snow GC, Jesus B, Ribiero L (2001) Responses of microphytobenthos to light: primary production and carbohydrate allocation over an emersion period. Marine Ecology Progress Series 223:101-112

Pinckney J, Paerl HW, Fitzpatrick M (1995) Impacts of seasonality and nutrients on microbial mat community structure and function. Marine Ecology Progress Series 123:207-216

Pinckney JL, Lee AR (2008) Spatiotemporal patterns of subtidal benthic microalgal biomass and community composition in Galveston Bay, Texas, USA. Estuaries and Coasts 31:444-454

Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography 25:943-948

Romani AM, Giorgi A, Acuna V, Sabater S (2004) The influence of substratum type and nutrient supply on biofilm organic matter utilization in streams. Limnology and Oceanography 49:1713-1721

Sala MM, Karner M, Arin L, Marrase C (2001) Measurement of ectoenzyme activities as an indication of inorganic nutrient imbalance in microbial communities. Aquatic Microbial Ecology 23:301-311

Santschi PH (1995) Seasonality in nutrient concentrations in Galveston Bay. Marine Environmental Research 40:337-362

Sinsabaugh RL, Lauber CL, Weintraub MN, Ahmed B, Allison SD, Crenshaw C, Contosta AR, Cusack D, Frey S, Gallo ME, Gartner TB, Hobbie SE, Holland K, Keeler BL, Powers JS, Stursova M, Takacs-Vesbach C, Waldrop MP, Wallenstein MD, Zak DR, Zeglin LH (2008) Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11:1252-1264

Stal LJ (2003) Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. Geomicrobiology Journal 20:463-478

Stahl LJ, Vangemerden H, Krumbein WE (1984) The simultaneous assay of chlorophyll and bacteriochlorophyll in natural microbial communities. Journal of Microbial Methods 2:295-306

Steen AD, Hamdan LJ, Arnosti C (2008) Dynamics of dissolved carbohydrates in the Chesapeake Bay: insights from enzyme activities, concentrations, and microbial metabolism. Limnology and Oceanography 53(3):936-947

Stutes AL, Cebrian J, Corcoran AA (2006) Effects of nutrient enrichment and shading on sediment primary production and metabolism in eutrophic estuaries. Marine Ecology Progress Series 312:29-43

Sundareshwar PV, Morris JT, Koepfler EK, Fornwalt B (2003) Phosphorus limitation of coastal ecosystem processes. Science 299:563-565

Sündback K, Graneli W (1988) Influence of microphytobenthos on the nutrient flux between sediment and water: a laboratory study. Marine Ecology Progress Series 43:63-69

Sündback K, Snoeijs P (1991) Effects of nutrient enrichment on microalgal community composition on a coastal shallow-water sediment system: an experimental study. Botanica Marina 34:341-358

Sutherland IW (1999) Biofilm exopolysaccharides In: Wingender J, Neu TR, Flemming H-C (ed) Microbial Extracellular Polymeric Substances. Characterization, Structure, and Function. Springer, Berlin, p73-92

Thornton DCO, Dong LF, Underwood GJC, Nedwell DB (2002) Factors affecting microphytobenthic biomass, species composition and production in the Colne Estuary (UK). Aquatic Microbial Ecology 27:285-300

Thornton DCO, Dong LF, Underwood GJC, Nedwell DB (2007) Sediment-water inorganic nutrient exchange and nitrogen budgets in the Colne Estuary, UK. Marine Ecology Progress Series 337:63-77

Thornton DCO, Visser LA (2009) Measurement of acid polysaccharides (APS) associated with microphytobenthos in salt marsh sediments. Aquatic Microbial Ecology 54:185-198

Tso SF, Taghon GL (1997) Enumeration of protozoa and bacteria in muddy sediment. Microbial Ecology 33:144-148

Underwood GJC, Boulcott M, Raines CA, Waldron K (2004) Environmental effects on exopolymer production by marine benthic diatoms: dynamics, changes in composition, and pathways of production. Journal of Phycology 40:293-304

Underwood GJC, Paterson DM (2003) The importance of extracellular carbohydrate production by epipelic diatoms. Advances in Botanical Research 40:183-240

Underwood GJC, Paterson DM, Parkes RJ (1995) The measurement of microbial carbohydrate exopolymers from intertidal sediments. Limnology and Oceanography 40:1243-1253

Underwood GJC, Phillips J, Saunders K (1998) Distribution of estuarine benthic diatom species along salinity and nutrient gradients. European Journal of Phycology 33:173-183

van Duyl FCv, Winder Bd, Kop AJ, Wollenzien U (1999) Tidal coupling between carbohydrate concentrations and bacterial activities in diatom-inhabited intertidal mudflats. Marine Ecology Progress Series 191:19-32

van Duyl FCv, Winder Bd, Kop AJ, Wollenzien U (2000) Consequences of diatom mat erosion for carbohydrate concentrations and heterotrophic bacterial activities in intertidal sediments of the Ems-Dollard estuary. Continental Shelf Research 20:1335-1349

Zimmerman AR, Benner R (1994) Denitrification, nutrient regeneration and carbon mineralization in sediments of Galveston Bay, Texas, USA. Marine Ecology Progress Series 114:275-288

APPENDIX A

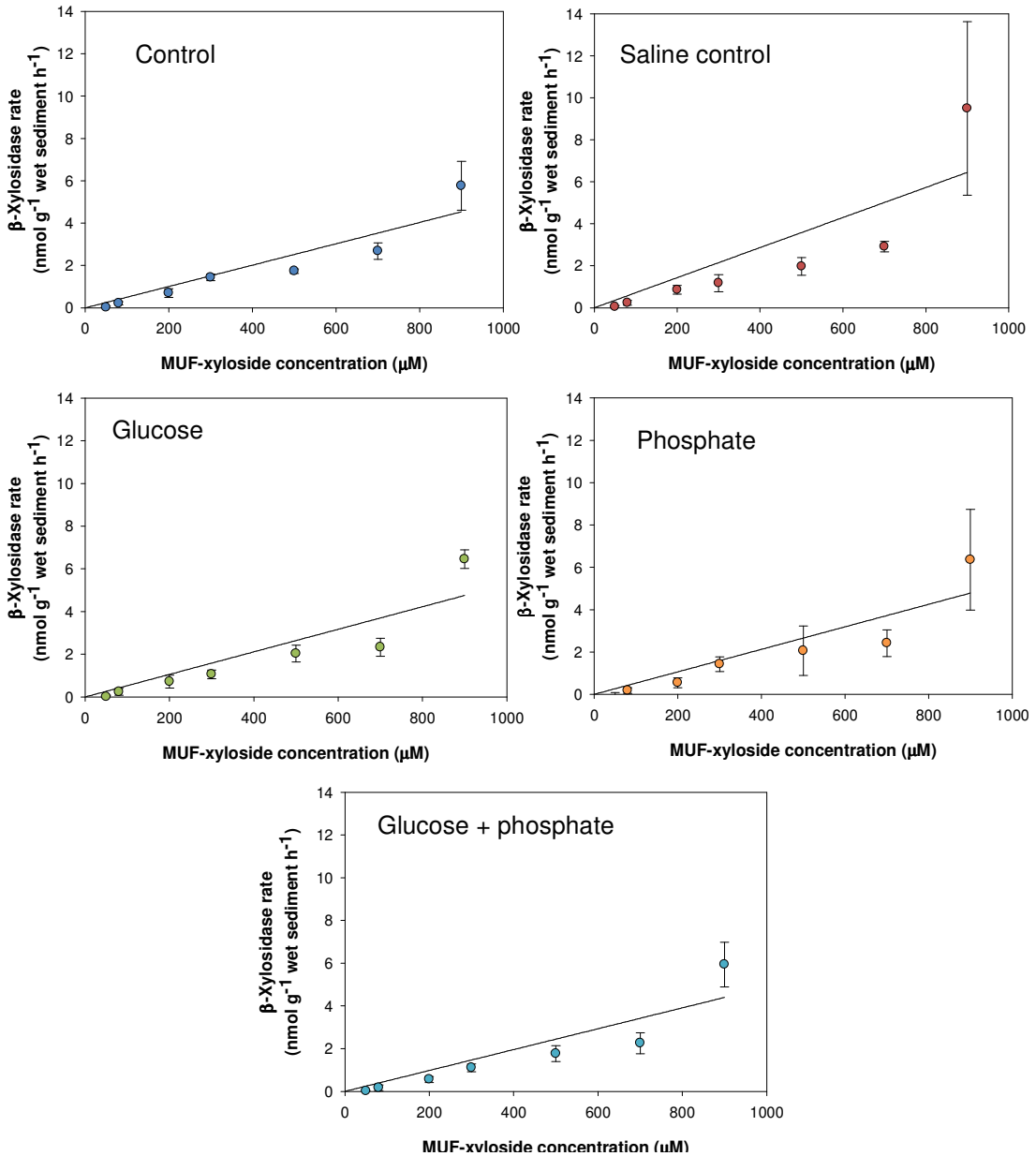


Figure A1: β-Xylosidase kinetics curves at time 0 for Experiment #2.2. Points show means ± SD (n=4).

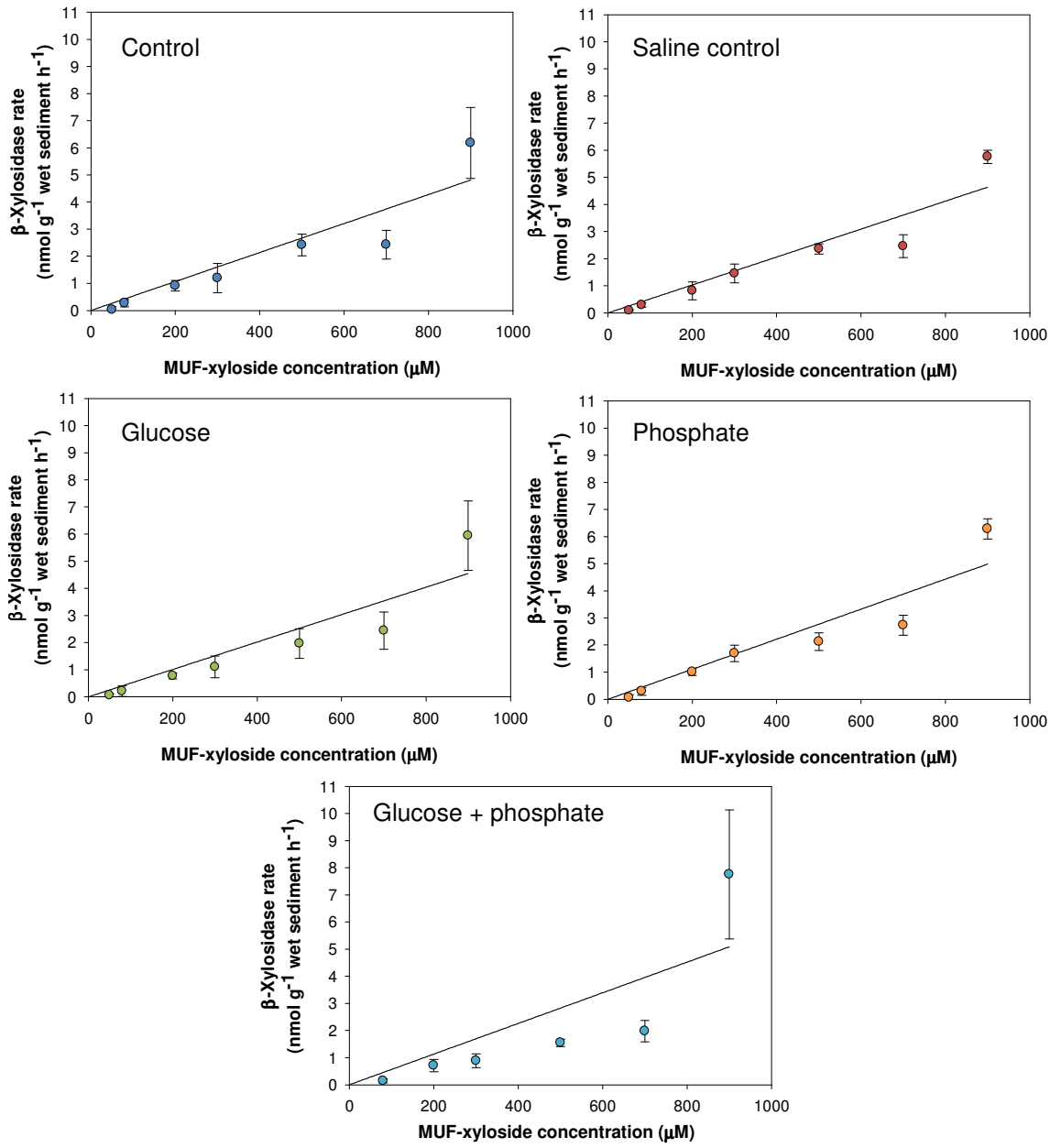


Figure A2: β-Xylosidase kinetics curves after 2 hours for Experiment #2.2. Points show means ± SD (n=4).

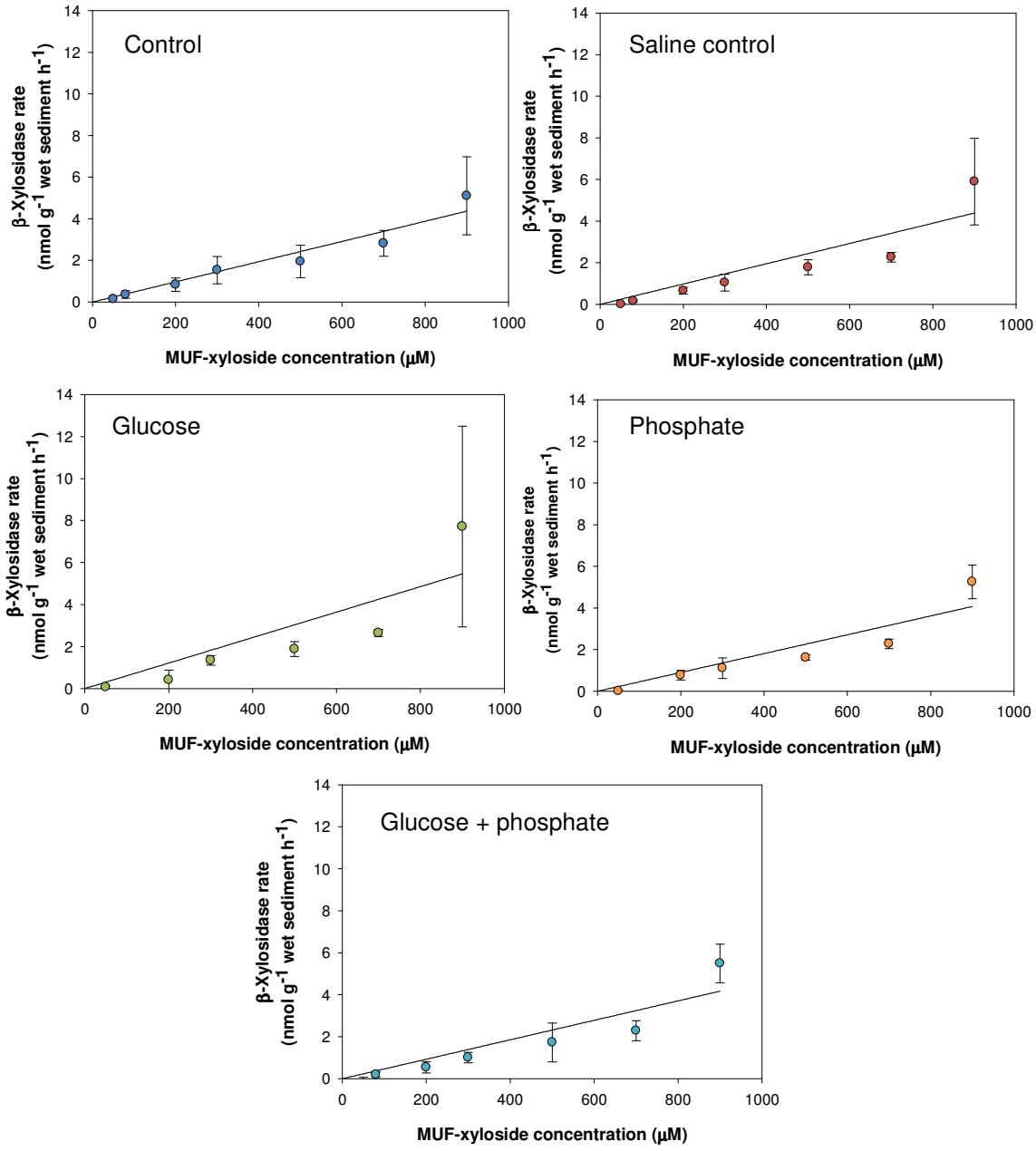


Figure A3: β-Xylosidase kinetics curves after 24 hours for Experiment #2.2. Points show means ± SD (n=4).

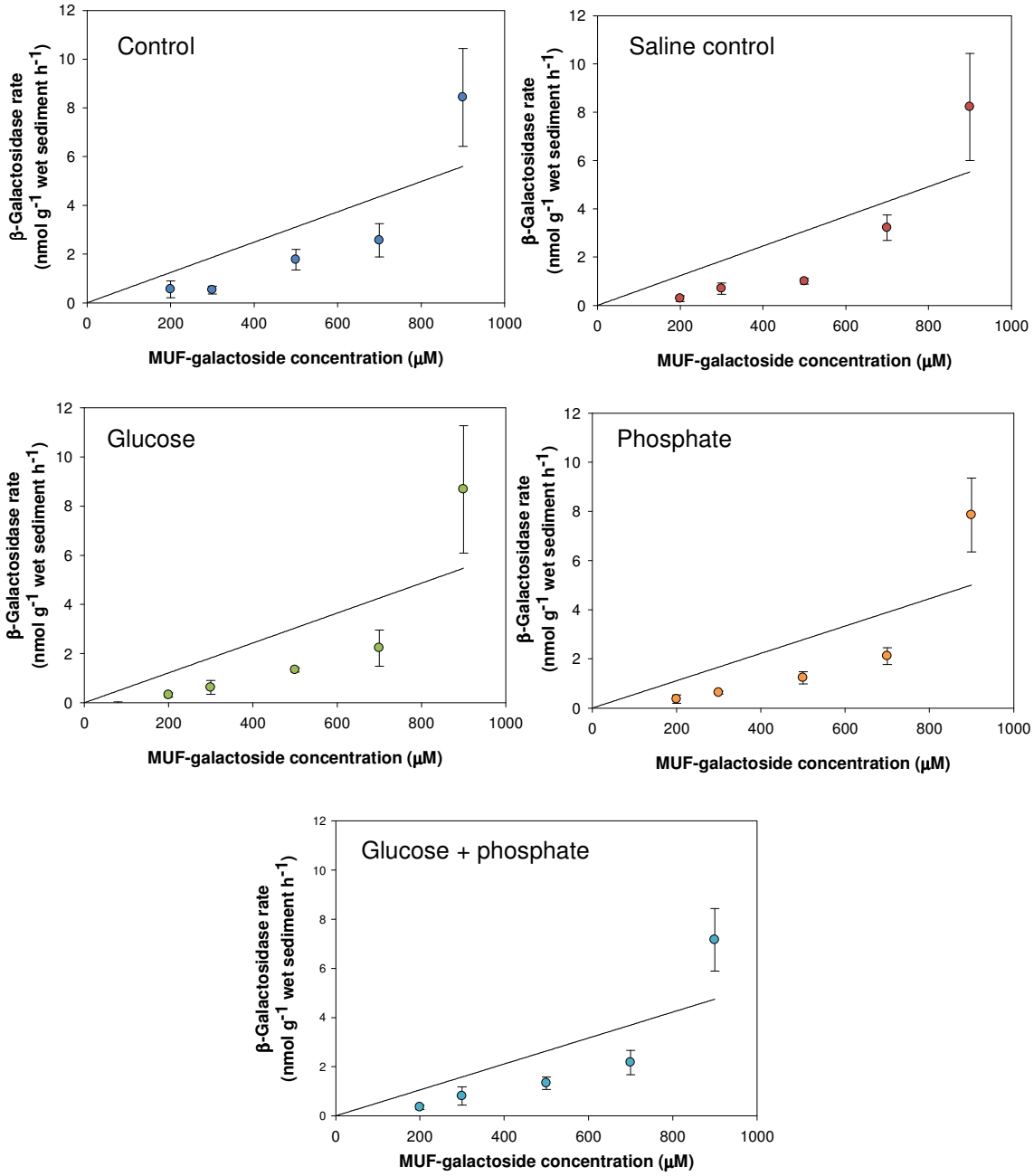


Figure A4: β-Galactosidase kinetics curves at time 0 for Experiment #2.2. Points show means ± SD (n=4).

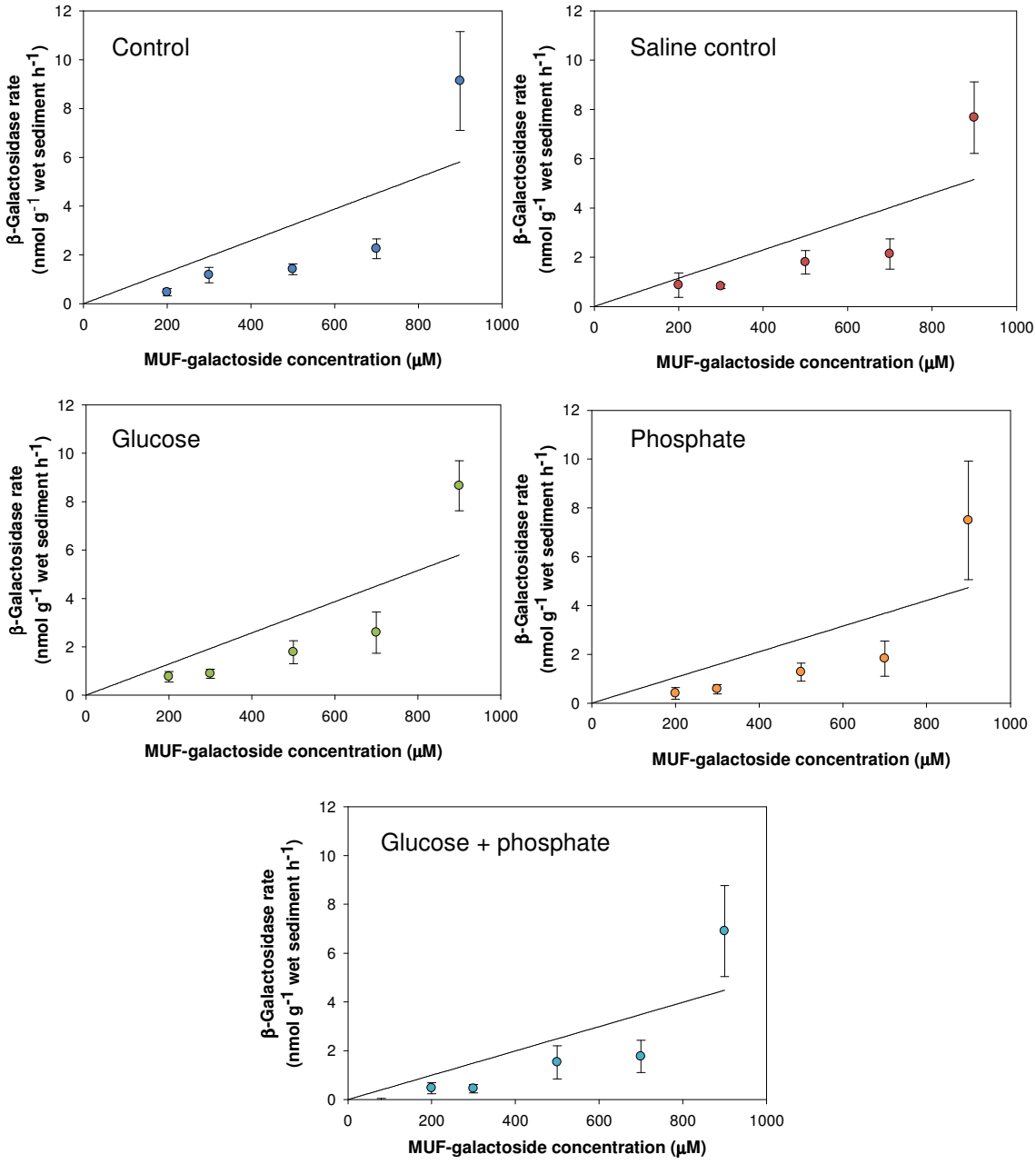


Figure A5: β-Galactosidase kinetics curves after 2 hours for Experiment #2.2. Points show means ± SD (n=4).

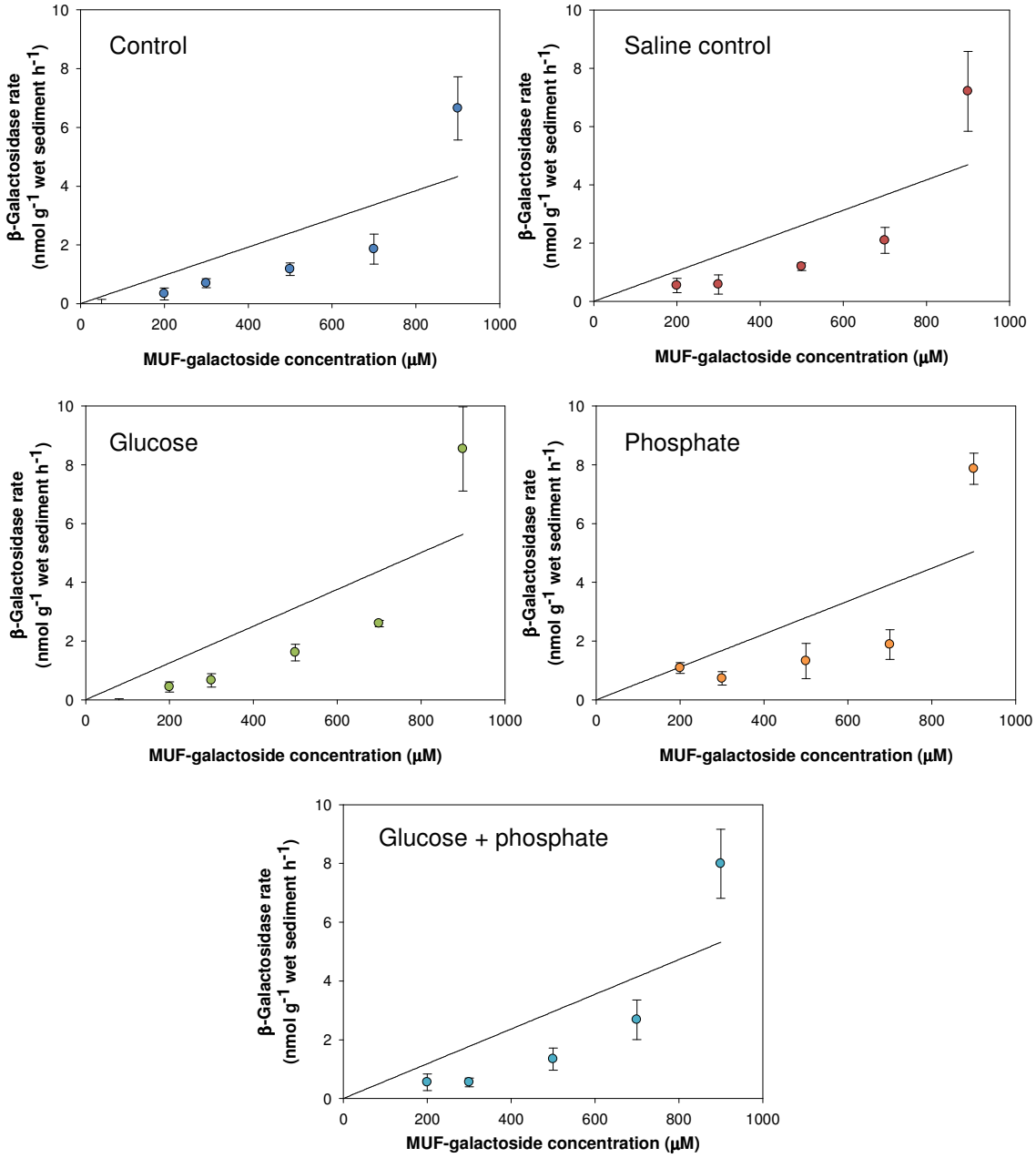


Figure A6: β-Galactosidase kinetics curves after 24 hours for Experiment #2.2. Points show means ± SD (n=4).

VITA

Name:	Carolyn Elaine Wilson
Address:	Texas A&M University, Department of Oceanography, 3146 TAMU, College Station, TX 77843
Email Address:	carolynewilson@gmail.com
Education:	B.A., Biology, Austin College, 2003 M.S., Oceanography, Texas A&M University, 2009
Past Experience:	United States Peace Corps Volunteer in Yap, Micronesia, 2003-2005