

**Microbial Extracellular Enzymes in Marine Sediments:
Methods Development and Potential Activities in the Baltic Sea
Deep Biosphere**

**A Thesis Presented for the
Master of Science
Degree
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ABSTRACT

The deep biosphere is defined as the subsurface ecosystem in which little energy is available to microorganisms and microorganisms can live for thousands of years. Heterotrophic microbes survive in the deep biosphere even though organic matter is limited and highly recalcitrant in nature. Measuring microbial extracellular enzyme activity provides a potential means to evaluate the rate at which microorganisms are performing carbon remineralization in the energy limited sediment beneath the seafloor. Extracellular enzymes breakdown organic compounds so that the nutrients can move inside the cell and be used for energy. This study explored the role extracellular enzymes play in the microbial metabolisms of the deep biosphere by 1) developing reliable methodology for microbial extracellular enzyme assays in aquatic sediments; and 2) measuring the activities of multiple carbon-degrading extracellular enzymes in Baltic Sea sediment. Test assays with fluorophore standards were performed on a BioTek Cytation 3 96-well plate reader and a Promega Glomax multi JR single cuvette fluorimeter and results were compared to determine which yielded the most high resolution and dependable results. The Promega single-cuvette fluorimeter proved to be the more precise method because instrumental drift over time, intra-well variation among replicates, and settling of slurry homogenate over long incubations were observed using the BioTek Cytation 3. Manually measuring fluorescence one cuvette at a time with a Promega Glomax multi JR lead to little instrumental drift, less variation among replicates, and a consistent shading

effect that does not alter final results. Saturation curves created with the Promega Glomax multi JR obeyed Michaelis-Menten kinetics. Next, enzymatic activity measured in the Baltic Sea deep biosphere supported the hypothesis that microbes in the Baltic Sea subsurface utilize extracellular enzymes to acquire organic carbon, nitrogen, and phosphorus. Data suggest the microbial community in the Baltic Sea deep biosphere is seeking nitrogen and phosphorus via amino acids and phosphate more than it is seeking carbon. Enzyme activity is strongly correlated with organic matter content and depth. Residual activity in autoclaved sediments suggest that some enzymes can survive intense heat implying stability and possible hundred-year lifetimes in the deep subsurface biosphere of the Baltic Sea Basin.

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CHAPTER I
INTRODUCTION

The Carbon Cycle

All heterotrophic organisms require organic carbon for energy, growth, and reproduction. Most organic carbon in the ocean is created from CO₂ by primary producers like phytoplankton in the water column (Burdige, 2006). Organisms that cannot produce their own organic carbon via primary production must obtain it via heterotrophy, the degradation of existing organic matter. The two main controls on preservation or degradation of organic carbon in sediments are 1) the chemical composition of the organic matter (the more complex, the less degradable), and 2) the matrix that encloses the organic matter because it offers a level of protection (Burdige, 2006). Simple biochemical components (proteins, sugars, etc.) can be broken down relatively easily, but more complex molecules cannot. The chemically-characterizable fraction of organic matter in the ocean is made up mostly of amino acids (proteins) and carbohydrates (sugars) derived from terrestrial and marine organisms (Burdige, 2006). Other components include lignin from terrestrial plants, and lipids (Burdige, 2006). As these components fall through the water column they get remineralized by microbes (Azam, 1998). Carbon remineralization is the oxidation of organic matter by an electron acceptor which yields CO₂, nutrients, and water (Arndt et al., 2013). Organic carbon remineralization in marine environments is an extremely efficient process: 99.9% of the organic carbon created through primary production is remineralized back to CO₂ by heterotrophic microorganisms in the water column (Hedges, 1992). Particulate organic matter and marine aggregates, sinking

through the water column as marine snow, are hotspots for organic matter degradation and microbial enzymatic activity (Arnosti, 2011). Only 0.1% of global net primary production makes it past the water column and is deposited in marine sediments (Hedges, 1992). Sedimentary rocks contain the largest reservoir of carbon on the Earth's surface (Hedges, 1992). These rocks hold about $60,000 \times 10^{18}$ g of inorganic carbon mostly in the form of carbonate minerals in limestones and $15,000 \times 10^{18}$ g of organic carbon mostly in the form of kerogen in shale (Hedges, 1992). The ocean holds the largest amount of inorganic carbon on Earth, approximately 40×10^{18} g (Hedges, 1992).

Microbial extracellular enzymes potentially act as intermediaries between organic carbon that living organisms use for energy and inorganic carbon that exists in forms such as CO_2 (Arnosti et al., 2014). Only a small amount of organic carbon escapes the work of extracellular enzymes in the water column and ends up sequestered in the deep ocean or subsurface (Hedges, 1992). Organic carbon which escapes microbial activity for a sufficiently long time enters the deep biosphere, defined as the subsurface ecosystem in which little energy is available to microorganisms and microorganisms can live for tens to thousands of years (Jørgensen & Marshall, 2016). If there are microorganisms living in the energy limited sediments beneath the seafloor, their extracellular enzymes could be slowly contributing to the carbon cycle from the deep subsurface.

The bulk reactivity of sedimentary organic matter typically decreases more than tenfold for each tenfold increase in age (Arndt et al., 2013). The most

reactive organic matter gets remineralized first resulting in a growing concentration of recalcitrant components (Middelburg, 1989). These unreactive molecules are collectively known as MUC, molecularly uncharacterized component (Hedges et al., 2000). More than half of the organic matter in marine sediments is considered MUC (Hedges et al., 2000). The basic biochemistry components (amino acids, carbohydrates, and lipids) cannot be detected in MUC via current analytical techniques (Burdige, 2006). The organic C in the deep biosphere is likely highly concentrated with recalcitrant MUC (Arndt et al., 2013; Burdige, 2006; Birgit Dauwe & Middelburg, 1998; Hopkinson & Vallino, 2005), and requires the actions of extracellular enzymes to break it down.

The Deep Biosphere

Similarly to most environments on Earth, microbes play a crucial role in driving biogeochemical cycles in ocean sediments. Microbes survive in the deep biosphere even though the organic matter is limited and highly recalcitrant in nature (Hoehler & Jørgensen, 2013). There are about as many cells (3×10^{29}) in marine sediments as there are in global ocean waters (Kallmeyer et al., 2012), yet we know very little about the role that specific taxa play because only an extremely small fraction of soil or sedimentary microbes have been successfully grown in culture (Handelsman et al., 1998). Coupled with difficulty accessing sediment samples from the deep biosphere, our understanding of the mechanisms behind C recycling and C sequestration in the deep biosphere are particularly lacking. Modern analytical techniques also are incomplete in the

information they provide. 16S ribosomal RNA techniques do not provide insight into cell function, and procedures such as single-cell genomic and metagenomics analyses do not confirm that microbes are performing the functions for which their genes code. The only subsurface archaea that have been successfully cultured and studied in laboratory conditions are a subset genus of methanogens and methanotrophs (Jarrell et al., 2011). Analyzing subsurface sediments off the coast of Peru from Integrated Ocean Drilling Program (IODP) Leg 201, Biddle et al. (2006) found that the isotopic composition of archaeal biomass is very similar to that of total organic carbon in sediments. They concluded that archaea must be receiving a substantial amount of their required carbon from sediments which implies heterotrophy instead of methanogenesis (Biddle et al., 2006).

Contradicting the assumption that bacteria mediate carbon cycling in the deep biosphere, Lloyd et al. (2013) found that the uncultured archaea Bathyarchaeota (Miscellaneous Crenarchaeotal Group, MCG) and marine benthic group D (MBG-D) are not only numerically abundant in the seafloor but produce peptidases (protein-degrading enzymes) and are capable of metabolizing protein. Such evidence suggests that heterotrophy is an important metabolic mode for microorganisms living in the seafloor. Assuming bacteria and archaea in the deep biosphere utilize extracellular enzymes to degrade recalcitrant organic matter from the deep biosphere, this provides a potential means to evaluate the rate at which microorganisms are performing carbon remineralization in the energy limited sediments beneath the seafloor.

Extracellular Enzymes

In order to hydrolyze large, recalcitrant organic compounds, heterotrophic microorganisms in surface environments produce extracellular enzymes. These enzymes breakdown the organic compounds so that the nutrients can move inside the cell and be used for energy (Arnosti, 2011). Extracellular enzymes are created inside the microbial cell, but are then released outside of the cell. They can be tethered to the cell membrane, freely dissolved in water (Arnosti, 2011), or adsorbed to sediment particles (Zimmerman et al., 2011). There is also the likelihood of many free-living enzymes in the environment resulting from cells ruptured by viral lysis (Burns, 1982). The role extracellular enzymes play in the marine carbon cycle is pivotal, for the hydrolysis of organic macromolecules by enzymes is the rate-limiting step in the carbon remineralization process (Arnosti, 2011).

Extracellular enzymes can be grouped into broad classes based on the type of biomolecule they hydrolyze. Peptidases are enzymes that breakdown proteins. Peptidases are further classified based on where they hydrolyze the amino acid chain. Exo-acting peptidases hydrolyze bonds located on the end of a polymer chain, and endo-acting peptidases break a polymer midchain (Obayashi & Suzuki, 2005). Another broad class of enzymes are polysaccharide hydrolases, which break down polysaccharides into simple sugars (McMurry, 2004).

With a few exceptions (Steen & Ziervogel, 2012), the majority of environmental enzymes follow Michaelis-Menten kinetics (German et al., 2011).

The initial hydrolysis rate of the enzyme, V_0 , increases as a function of substrate concentration, $[S]$, until it reaches the maximum hydrolysis rate, V_{max} , at saturated substrate conditions. This relationship can be modeled by the Michaelis-Menten equation:

$$V_0 = \frac{V_{max} [S]}{K_m + [S]}$$

K_m , the Michaelis constant, describes an individual enzyme's affinity for its substrate. While V_{max} is proportional to the number of enzymes present, K_m is a property of individual enzymes. In practice, saturation curves are created via a fluorogenic assay and the Michaelis-Menten equation is fit to the data using non-linear least squares regression. In this sense, K_m is a fitted parameter defined as the substrate concentration that produces half the maximum hydrolysis rate of the enzyme (Figure 1-1). A low K_m value indicates the enzyme has a high affinity for its substrate.

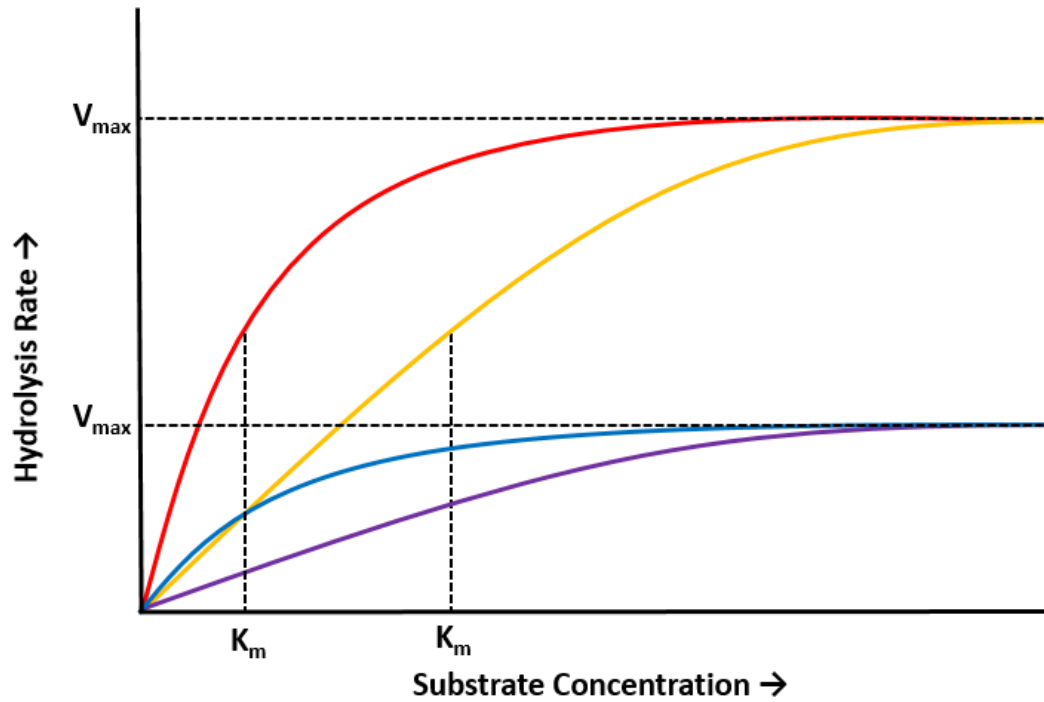


Figure 1-1. Michaelis-Menten Kinetics

Most environmental enzymes obey Michaelis-Menten kinetics, but the parameters can vary widely. Enzymes can exhibit high V_{max} and low K_m (red), high V_{max} and high K_m (yellow), low V_{max} and low K_m (blue), or low V_{max} and high K_m (purple).

Enzymes found in sediment are expected to be extremely diverse and are present in very low concentrations in a matrix of organic matter (Steen et al., 2015). While it is possible to isolate a specific enzyme for study, steps taken to purify the enzyme will likely alter its activity (Wang et al., 2012). Therefore, conventional enzyme assays involve adding a substrate to an environmental sample and monitoring the subsequent reaction (Hoppe, 1983). Fluorophore-tagged substrates have become the most commonly used method because it is relatively quick, effective, and allows for multiple enzyme assays (Freeman & Nevison, 1999; Tabatabai, 1982). The fluorophores are quenched until hydrolyzed by an enzyme. The potential hydrolysis rate for an enzyme is then calculated from the increase in fluorescence over time.

Fluorogenic substrates can be hydrolyzed by enzymatic or abiotic processes. Abiotic processes are expected to be first order reactions, the rates of which increase linearly with substrate concentration and are insensitive to prior heat treatment. Enzymatic processes obey Michaelis-Menten kinetics in that they exhibit saturating behavior with increased substrate concentration. Enzymatic processes should, in principle, decrease after heat treatment. In theory, enzymes are denatured by extreme temperature (Tabatabai, 1982) so that enzymatic hydrolysis does not occur and only abiotic processes take place. In practice, however, this is not always the case and possible explanations for that will be addressed here. Heat treated controls are created by autoclaving sediment samples before adding substrates (Bell et al., 2013). When results obtained via a

fluorogenic enzyme assay obey Michaelis-Menten kinetics and decrease with heat treatment, the results are considered representative of enzymatic hydrolysis.

Objectives

Relatively few studies have been conducted on enzyme activities in sediments despite their potential role in the fluxes of carbon in the subseafloor (Arnosti, 2011). This study aims to explore the role extracellular enzymes play in the microbial metabolisms of the deep biosphere. This study had two objectives: 1) develop reliable methodology for microbial extracellular enzyme assays in aquatic sediments; and 2) measure the activities of multiple carbon-degrading extracellular enzymes in Baltic Sea subseafloor sediment.

CHAPTER II

METHODS DEVELOPMENT

Abstract

Enzyme assays in sediments and soils are typically carried out in one of two ways: either using a plate reader, which can read fluorescence in 96 wells automatically, or using a single cuvette fluorescence detector, in which samples must be changed manually. In order to obtain the most accurate and precise possible measurements of extracellular enzyme activities in the Baltic Sea, I assessed whether the plate reader protocol that is standard for soils was optimal for subsurface sediments. In order to compare and contrast results from two different fluorimeters, sediment was collected from the Tennessee River at Sequoyah Park, Knoxville. Despite the convenience of running 96 samples at once on the BioTek fluorimeter, the Promega single-cuvette fluorimeter proved to be the more precise method. Instrumental drift over time, intra-well variation among replicates, and settling of slurry homogenate over long incubations were observed using the BioTek Cytation 3. Manually measuring fluorescence one cuvette at a time using the Promega Glomax multi JR single cuvette reader leads to little instrumental drift, less variation among replicates, and a consistent shading effect that does not alter final results.

Introduction

Investigation of microbial hydrolytic enzymes began in 1918 (Waksman, 1918) and continue today (Arnosti et al., 2014; Steen et al., in prep.). The modern era of environmental enzyme analysis began in 1983 with the use of

fluorogenic substrates (Hoppe, 1983). Adding synthetic substrates labeled with fluorophores such as 7-amino-4-methylcoumarin (AMC) or 4-methylumbelliferone (MUB) to water, sediment, or soil samples became a widely used tool for investigating the role microbial metabolisms play in biogeochemical cycling (German et al., 2011). Even though marine sediments are the largest anoxic environment on Earth (Jørgensen & Marshall, 2016) and contain one of the largest microbial communities on the planet (Kallmeyer et al., 2012), very few studies have looked at the role extracellular enzymes play in biogeochemical cycling in subsurface sediments.

The major goal for this thesis is to determine how enzyme activity changes with depth in deep sediments (4-80 mbsf) in the Baltic Sea. In order to obtain the most accurate and precise possible measurements of extracellular enzyme activities in the Baltic Sea, I decided to test the effectiveness of a high throughput enzyme assay method developed by Bell et al. (2013) and adapted for sediment by Steen et al. (in prep). I revisited the methodology and fluorimeters used for sedimentary enzyme assays to see if a different method would provide higher resolution results. Test assays with fluorophore standards were performed on both a BioTek Cytation 3 96-well plate reader and a Promega Glomax multi JR single cuvette fluorimeter and results were compared to determine which method and fluorimeter yielded the most high resolution and dependable results.

Methods

Preliminary Assay

A push core reaching 61.5 centimeters below the seafloor (cmbsf) was collected from Station H of the White Oak River Estuary in North Carolina in October of 2014 (Kelley, Martens, & Ussler, 1995). The core was transported back to the University of Tennessee where it was sectioned. Enzyme assays were performed on 16 depth intervals using a BioTek Cytation 3 96-well plate reader, and enzyme activity for each section was plotted vs. depth.

Fluorimeter Experiments

In order to compare and contrast results from two different fluorimeters, sediment was collected from the Tennessee River at Sequoyah Park, Knoxville. Samples consisted of the top 10 cm of subaqueous sediment from about 4 meters from the shoreline. The sediment sample was transported back to our lab at the University of Tennessee. 3 g of sediment was added to a laboratory blender and blended for 1 minute with 100 mL of 0.2 M borate buffered saline (BBS) to create a homogenous slurry. The synthetic substrate leucine-AMC was chosen because it is commonly used in enzyme assays and is relatively inexpensive.

Using a deep-well plate, 1960 μL of sediment slurry and 40 μL of 20 mM leucine-AMC were added to well 1. 1960 μL of 0.2 M BBS and 40 μL of 1 mM AMC-standard were added to well 2. 1960 μL of sediment slurry and 40 μL of 1

mM AMC-standard were added to well 3. 2000 μL of sediment slurry was added to well 4. The deep-well plate was centrifuged for 1 min reaching $\sim 3,000 \times g$. 250 μL aliquots of the supernatant were transferred from each well to a 96-well microplate. Fluorescence was measured once every hour for 4 hours in a BioTek Cytation 3 96-well plate reader.

Using single cuvettes, 980 μL of sediment slurry and 20 μL of 20 mM leucine-AMC were added to cuvette 1. 980 μL of 0.2 M BBS and 20 μL of 1 mM AMC-standard were added to cuvette 2. 980 μL of sediment slurry and 20 μL of 1 mM AMC-standard were added to cuvette 3. 1000 μL of sediment slurry was added to cuvette 4. Fluorescence was measured manually once every hour for 4 hours in a Promega Glomax multi JR single-cuvette reader.

Drift Experiments

4 replicate wells of 245 μL of 0.2 M BBS and 5 μL of 100 μM AMC-standard were prepared and measured in the BioTek Cytation3 fluorimeter for 8 hours. A dilution series of sodium fluorescein standard was prepared in 0.1 M phosphate buffered saline (PBS) and 2 blank control wells were measured in the BioTek Cytation 3 fluorimeter for 8 hours. Final well concentrations of sodium fluorescein ranged from 21 μM to 117 μM .

Saturation Curves

Sediment was collected from the same location in the Tennessee River at Sequoyah Park, Knoxville. A sediment slurry was prepared as described above.

Cuvettes with concentrations of 0 – 800 μM of MUB-N-acetyl- β -D-glucosaminide, MUB- PO_4 , and ornithine-AMC were created with the single-cuvette method and measured over 24 hours using the Promega Glomax multi JR single cuvette reader.

Results and Discussion

Preliminary Assay

The sensitivity of the 96-well plate method did not detect changes in enzyme activity below 20 cmbfs (Figure 2-1). Results were not precise enough for further analyses.

Fluorimeter Experiments

The BioTek Cytation 3 96-well plate reader produced unreliable results. First, the rate at which fluorescence increased over a 3 hour period in the well containing sediment slurry and Leu-AMC was noticeably high (Figure 2-2). The fluorescence in that sample reached the same amount of fluorescence as the well containing BBS and AMC-standard. One possible reason is that enough sediment settled out of the homogenate slurry over 3 hours that it reached equal fluorescence as the particle-free buffer solution. We tentatively conclude that a shading effect was affecting fluorescence readings in this high-throughput method as sediment settled out of the slurry throughout the assay. Second, significant periodic drift was observed in the well containing BBS and AMC-standard (Figure 2-2). The drift in the wells containing AMC-standard follow the

same temporal trends (i.e., fluorescence in well 2 increased at the same time as in well 3) implying that this is a result of instrumental drift instead of complications with our buffer or standard solutions. Lastly, results from the well containing only sediment slurry showed that natural fluorescence in the organic matter itself did not significantly impact assay results.

The Promega Glomax multi JR single cuvette reader did not display these artifacts. First, the rate at which fluorescence increased over a 3 hour period in the cuvette containing sediment slurry and Leu-AMC was reasonable in that it did not increase higher than that in the cuvette containing sediment slurry and AMC-standard (Figure 2-3). The shading effect observed in the 96-well plate was controlled by manually inverting each cuvette before each fluorescence measurement in order to keep the slurry homogenous throughout the assay. No periodic drift was observed in the cuvettes containing AMC-standard (Figure 2-3). Fluorescence in the cuvette containing BBS and AMC-standard is noticeably higher than the others because it does not contain sediment particles. In this method, the shading effect still exists but is kept constant throughout the assay so that final results are not affected. Lastly, the cuvette containing only sediment slurry showed that the natural fluorescence of organic matter in the sediment does not significantly impact assay results.

Drift Experiments

Fluorescence increased in all 4 replicate wells of AMC-standard over the 8 hour incubation period (Figure 2-4). Significant intra-well variation also existed

among the replicates, despite the fact that the same amount of standard was added to each well. To ensure this was not a problem with our AMC-standard, the fluorescence of a different standard, sodium fluorescein, was measured over 8 hours. Wells containing a dilution series of sodium fluorescein, increased over an 8 hour incubation period (Figure 2-5). These tests confirmed that the BioTek Cytation 3 96-well plate reader was yielding unreliable results that we attributed to instrumental drift.

Saturation Curves

Saturation curves for MUB-N-acetyl- β -D-glucosaminide (Figure 2-6), MUB-PO₄ (Figure 2-7), and ornithine-AMC (Figure 2-8) were created with the Promega Glomax multi JR fluorimeter to ensure it was reliable at measuring enzymatic activity. The close fit of the results to the Michaelis-Menten formula by all 3 substrates indicated that it was indeed an effective and reliable method.

Conclusions

Despite the convenience of running 96 samples at once on the BioTek fluorimeter, the Promega single-cuvette fluorimeter proved to be the more precise method. Major factors included in this decision were instrumental drift over time, intra-well variation among replicates, and settling of slurry homogenate over long incubations in the BioTek Cytation 3. While the amount of samples that can be assayed at once drastically decreases when using the Promega Glomax multi JR single cuvette reader, manually measuring fluorescence one cuvette at

a time leads to little instrumental drift, less variation among replicates, and a consistent shading effect that does not alter final results. Saturation curves created with the Promega Glomax multi JR obeyed Michaelis-Menten kinetics. Based on this comparison, it appears that enzyme assays measured using the single-cuvette method are more accurate and precise.

Appendix

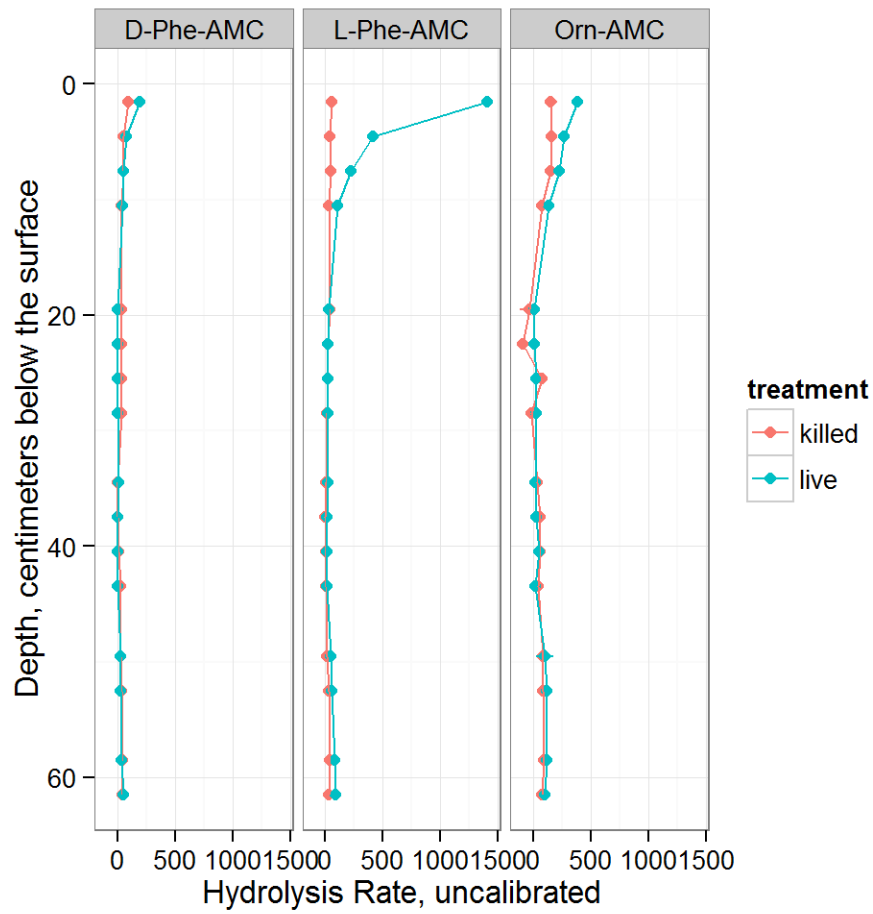


Figure 2-1. BioTek Cytation 3 Preliminary Assay

Uncalibrated hydrolysis rates versus depth for 3 different peptidases in the White Oak River Estuary, N.C.

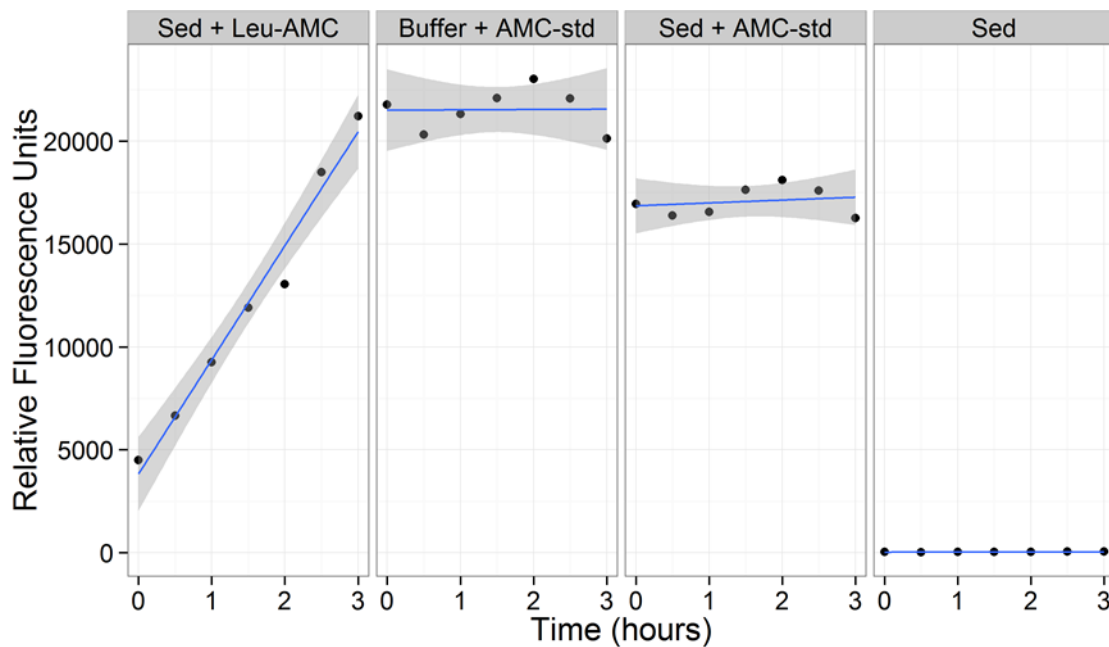


Figure 2-2. BioTek 96-well Plate Reader Test Assay

Significant periodic drift was observed in standards, along with a relatively high increase in fluorescence over time of leucine-AMC. Natural fluorescence of organic matter does not significantly impact assay results.

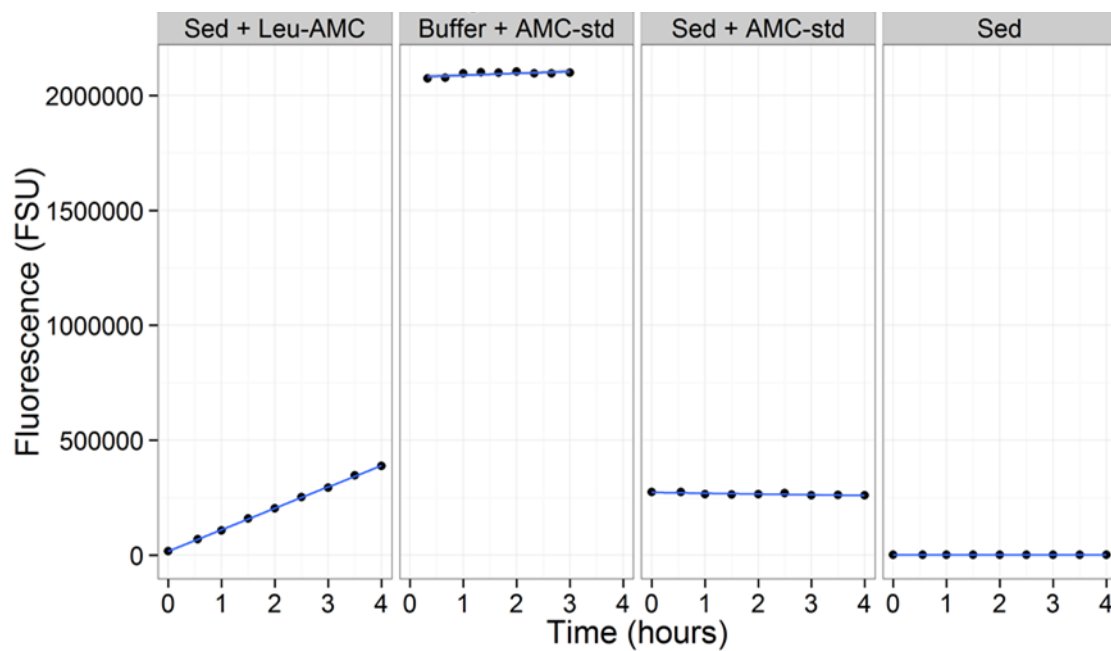


Figure 2-3. Promega Glomax multi JR Single Cuvette Reader Test Assay

Very little periodic drift was observed in standards, along with an accurate increase in fluorescence over time of leucine-AMC. Natural fluorescence of organic matter does not significantly impact assay results.

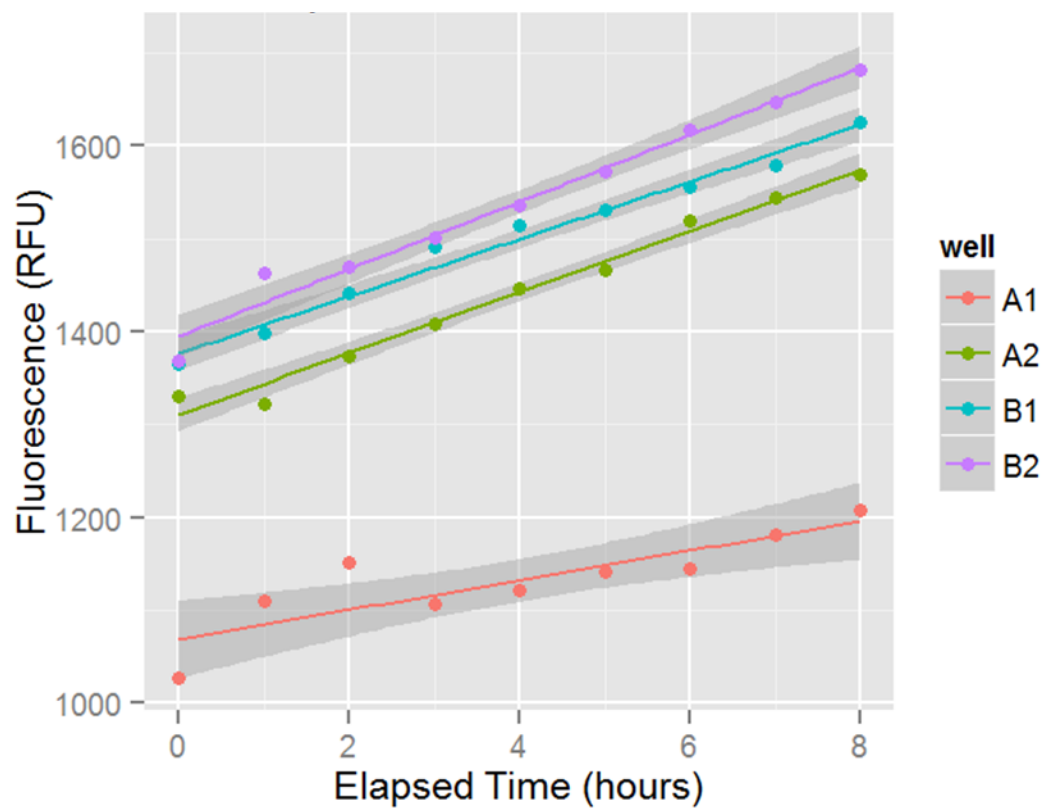


Figure 2-4. BioTek 96-well Plate Reader Standards Test.

Four replicates of AMC-standard + borate buffered saline showing standards increasing over 8 hours and intra-well variation among replicates.

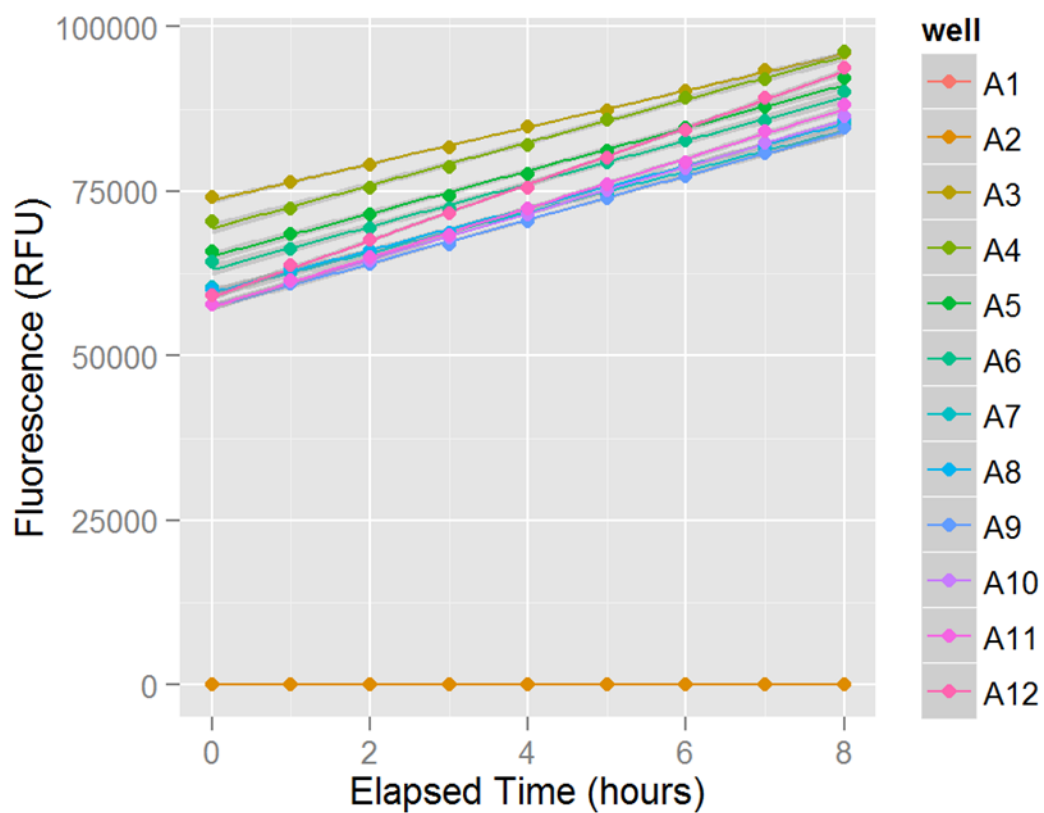


Figure 2-5. BioTek 96-well Plate Reader Standards Test.

Dilution Series of sodium fluorescein + phosphorus buffered saline (wells A3-A12). Wells A1 and A2 are blank controls. Sodium fluorescein standard also increased over 8 hours.

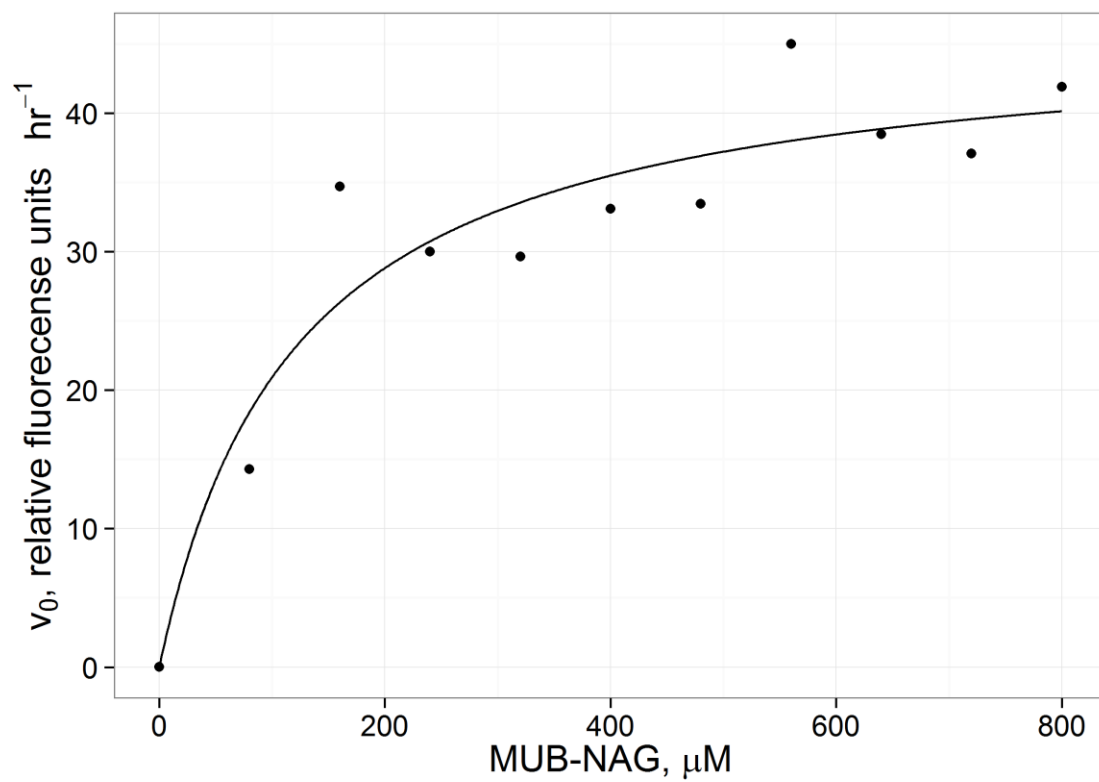


Figure 2-6. Promega Glomax Jr. Saturation Curve

TN River surface sediment assayed with series of MUB-N-acetyl- β -D-glucosaminide concentrations.

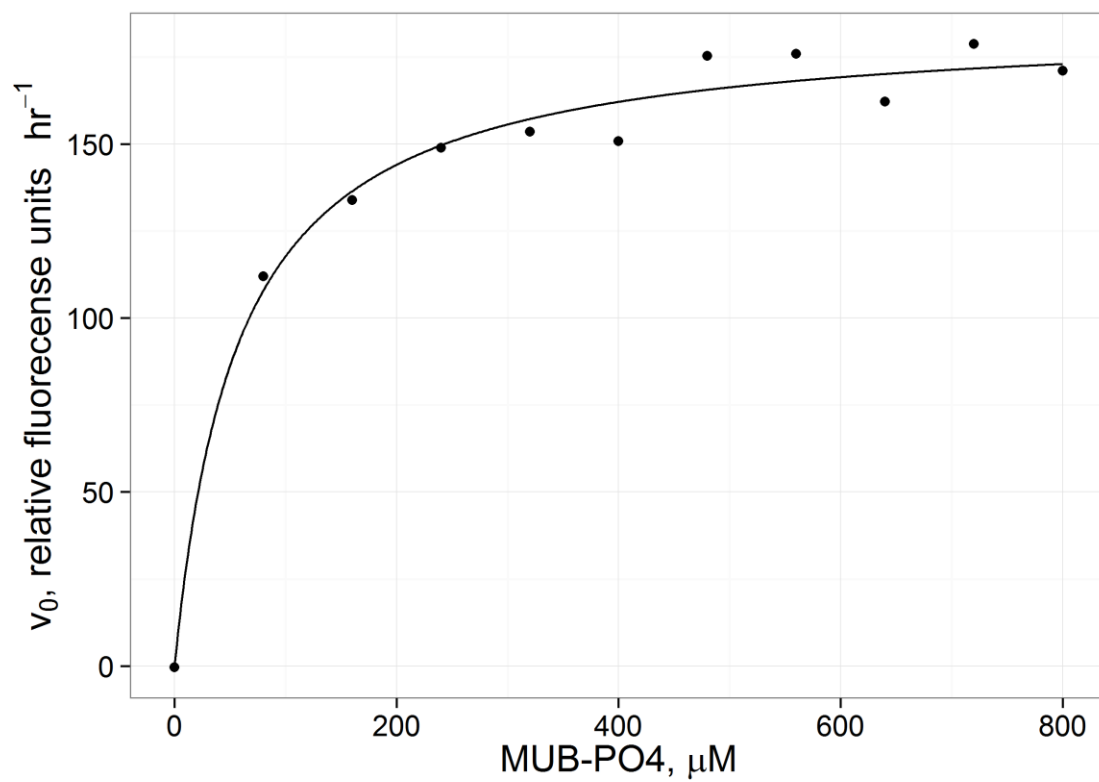


Figure 2-7. Promega Glomax Jr. Saturation Curve

TN River surface sediment assayed with series of MUB-PO₄ concentrations.

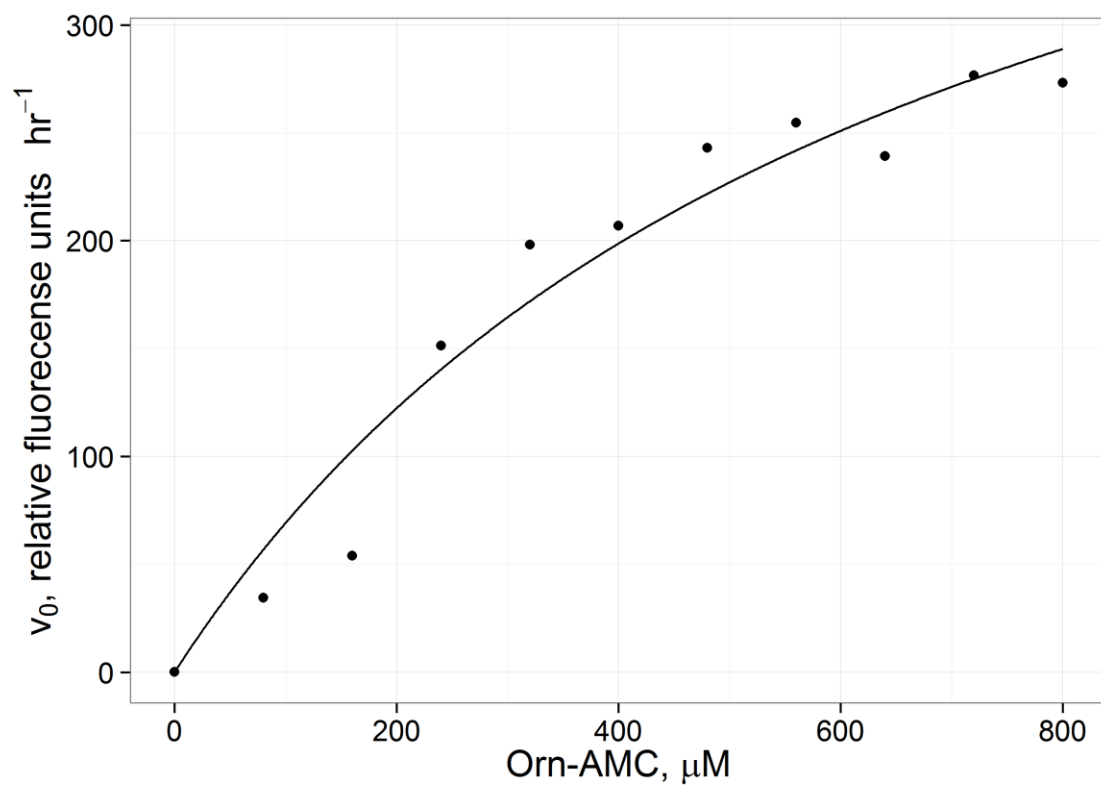


Figure 2-8. Promega Glomax Jr. Saturation Curve

TN River sediment assayed with series of Orn-AMC concentrations.

CHAPTER III

POTENTIAL ACTIVITIES OF EXTRACELLULAR ENZYMES IN THE

BALTIC SEA DEEP BIOSPHERE

Abstract

Heterotrophic microorganisms in sediments produce extracellular enzymes to hydrolyze organic macromolecules so they can be transported inside the cell and used for energy and growth. Using core samples from the Baltic Sea Basin obtained by International Ocean Discovery Program (IODP) Expedition 347, the potential activities of multiple carbon degrading enzymes were measured using small substrate proxies containing the fluorescent compounds 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (AMC). The potential maximum hydrolysis rates ($V_{\max}$) were measured at depths down to 80 meters below seafloor (mbsf) for the following enzymes: alkaline phosphatase, β -D-xylosidase, β -D-cellobiohydrolase, N-acetyl- β -D- glucosaminidase, β -glucosidase, α -glucosidase, leucyl aminopeptidase, arginyl aminopeptidase, prolyl aminopeptidase, gingipain and clostripain. Bulk enzymatic activity decreased with depth and was related to organic carbon content. Activities of extracellular peptidases were detectable at depths less than 55 mbsf. Gingipain and clostripain showed the highest potential activities. Activity of alkaline phosphatase was detectable throughout the core, albeit against a relatively high activity in autoclaved sediments. β -glucosidase activities were detected above 30 mbsf, however activities of other polysaccharide hydrolases (β -D-xylosidase, β -D-cellobiohydrolase, N-acetyl- β -D- glucosaminidase and α -glucosidase) were generally indistinguishable from zero at all depths. These data suggest the microbial community is looking to acquire N and P via amino acids and

phosphate. This is consistent with the behavior of a nutrient starved community, despite high nutrient concentrations in sediment porewater. Residual activity in autoclaved sediments suggest that some enzymes can survive intense heat implying stability and possible hundred-year lifetimes in the deep subsurface biosphere of the Baltic Sea Basin.

Introduction

Situated along the western and southern borders of Sweden, the Baltic Sea Basin (BSB) is in the direct path of the Scandinavian ice sheet and has a long glacial-interglacial history (Andrén et al., 2012). The BSB has high potential for organic matter preservation because it is a primarily depositional environment with high sedimentation rates and low oxygen content (Hedges & Keil, 1995) leading to short oxygen exposure times (Hartnett et al., 1998). However, organic matter oxidation in deep sediments is ultimately driven by the activities of anaerobic microbes. Studying the extracellular enzymes that these microbes produce can shine light on pathways of organic matter preservation or oxidation in deep sediments.

There are ten enzymes that are commonly targeted in environmental samples. Five of them, β -D-xylosidase, β -D-cellobiohydrolase, N-acetyl- β -D-glucosaminidase, β -glucosidase, and α -glucosidase, are polysaccharide hydrolases which are helping microbes obtain organic carbon (Arnosti & Steen, 2013). Others, leucyl aminopeptidase and phosphatase provide information specific to N and P cycling (German et al., 2011; Saiya-Cork, Sinsabaugh, & Zak,

2002). In fact, leucyl aminopeptidase is such a common choice for enzyme assays that very few studies have looked any other peptidases (Arnosti & Steen, 2013; Obayashi & Suzuki, 2005; Steen & Arnosti, 2013).

Past studies of microbial extracellular enzymes report that surface sediments exhibit the highest amount of enzyme activity, and that activity decreases rapidly with depth (Arnosti, 2011; King, 1986). It has also been found that sedimentary extracellular enzymes are capable of hydrolyzing substrates that are often not accessible to enzymes in the water column, which implies that the sediment microbial community is different from surface microbial communities with respect to the ability to breakdown recalcitrant organics (Arnosti, 2000; Arnosti, 2008). Extracellular peptidases in sediments of the White Oak River estuary, North Carolina showed no trend in $V_{\max}$ relative to cell count and a strong increase in $V_{\max}$ relative to modeled carbon oxidation rates (Steen et al. in prep).

By measuring extracellular enzymatic activity in the Baltic Sea Basin, I shed light on whether extracellular enzymes are a viable way for microbes to acquire organic nutrients in the subsurface, and if extracellular enzymes play a role in the transformation of sequestered organic carbon to carbon dioxide in the subsurface. I hypothesized that microbes in the Baltic Sea subsurface utilize extracellular enzymes to acquire organic carbon, nitrogen, and phosphorus and that enzyme substrate affinity increases with depth (lower K_m) as a result of enzymatic adaptation to lower substrate concentrations in the subsurface.

Methods

Description of Samples

On September 19, 2013, the *Greatship Manisha* collected sediment cores from Hole M0059C at Lille Bælt in the Baltic Sea as part of Integrated Ocean Drilling Program (IODP) Expedition 347 (Andrén et al., 2015a). Sediments from hole M0059C were collected beneath 37.1 meters of water using a piston corer system (Andrén et al., 2015b). The core was estimated to represent 140,000 years of sediment deposition (Expedition 347 Scientists, 2014). Sampling of this hole was completed on September 20 and reached a final depth of 83 meters below the seafloor (mbsf) (Andrén et al., 2015b). Cores were sectioned within 1-2 hours of recovery on deck (Andrén et al., 2015a). After being sub-sampled for shipboard analyses, 5 cm core sections were labeled, capped and stored at 4 °C (Andrén et al., 2015a). Upon arrival at The University of Tennessee Microbiology Department, samples were stored at -80 °C until time of analysis.

Clay minerals were the dominant lithology of Hole M0059C (Andrén et al., 2015b). Visual inspections of recovered sediment by shipboard scientists separate the uppermost 83 mbsf of the site M0059 core into three main units. The mineralogy of unit 1a (0 – 47.1 mbsf) consists of biosiliceous clay deposits from a Holocene marine setting (Andrén et al., 2015b). These sediments are black to dark-brown suggesting high organic content. Unit 1b (47.1 – 51.68 mbsf) is a transition zone which consists of clay with some silt (Andrén et al., 2015b). The presence of freshwater diatoms in unit 1b suggests the sediment was

deposited in a brackish or freshwater setting (Andrén et al., 2015b). Unit 2 (51.68 – 51.73 mbsf) represents an erosional unconformity likely created by a lowstand in sea level (Andrén et al., 2015b). Lastly, Unit 3 (51.73 – 83 mbsf) consists of clayey silt and silty clay. Sand content increases with depth and is grey to light green in color, interpreted to represent glacial deposits (Andrén et al., 2015b).

Based on shipboard geochemical analysis, porewater nutrient levels are highest in the top 50 meters of the core (Figure 3-1). Total carbon content (TC) is high throughout the profile, with organic carbon as the dominant form (Andrén et al., 2015b). Total organic carbon (TOC) content in Unit I is between 6-8 wt%, and then decreases to about 4-6 wt% (Figure 3-2). A distinct geochemical boundary is noticeable at the unconformity at Unit II. At this depth, the TOC content decreases to less than 1 wt% and remains low for the remainder of the core (Andrén et al., 2015b).

Enzyme Assays

Substrate Selection

Substrates were selected based on the known composition of organic matter in marine sediments. Three broad classes of enzymes were targeted in this study: peptidases which degrade proteins to obtain N and C, polysaccharide hydrolases which degrade carbohydrates to obtain C, and phosphatase which releases phosphate.

Peptidases were chosen because amino acids originating from dead terrestrial and marine organisms make up a significant component of characterized organic matter in sediments (Burdige, 2006). Amino acids make up proteins and are also a major component in peptidoglycan which make up bacterial cell walls (Burdige, 2006). Despite small differences in the usage of proteins among organisms, the bulk amino acid composition of most organisms is the same (Burdige, 2006).

The synthetic substrate leucine-AMC was chosen because the neutral amino acid, leucine, was expected to be present in our sediment based on work by Dauwe and colleagues (Dauwe et al., 1999; Dauwe & Middelburg, 1998). Leucine tends to be preferentially remineralized (Burdige, 2006), and leucine-AMC is the most common substrate used for enzyme assays possibly because the synthetic substrate leucine-AMC is relatively inexpensive. L-arginine-AMC was chosen because the amino acid arginine has a higher N content than the other protein substrates. This basic amino acid was used to test for signs of organic nitrogen limitation in the sediments. Leucine and arginine are expected to be selectively degraded in the water column (Dauwe & Middelburg, 1998). Because it is a basic amino acid, arginine is expected to be preferentially remineralized before the neutral amino acids. H-proline-AMC was chosen in order to assay another neutral amino acid, proline. Ornithine-AMC was chosen because ornithine is a product of deamination of arginine, which seems to be part

of early protein diagenesis (Wakeham & Lee, 1993). Unlike leucine and arginine, ornithine content is expected to be enriched in aged sediments.

Lloyd et al. (2013) found that archaea in sediments of Aarhus Bay, Denmark, 600 cmbsf, produce extracellular clostripain and gingipain to degrade proteins. Z-phenylalanine-arginine-AMC and Z-phenylalanine-valine-arginine-AMC were chosen to target the peptidases gingipain and clostripain, respectively. Gingipain R. (EC 3.4.22.37) was isolated from the bacteria *Porphyromonas gingivalis*, and is specific for the arginyl peptide bond (Chen, Potempa, Polanowski, Wikstrom, & Travis, 1992). Clostripain (EC 3.4.4.20) was isolated from the culture of *Clostridium histolyticum* (Kocholaty and Krejci, 1948). Clostripain is selective to arginine and lysine peptide bonds or arginine alone (Cole et al., 1971).

Polysaccharide hydrolases were chosen because carbohydrates make up another significant component of characterized organic matter in sediments (Burdige, 2006). Carbohydrates, or polysaccharides, are used by organisms for structural purposes and carbon and energy storage. The synthetic substrate MUB- β -D-xylopyranoside was selected because the neutral sugar, xylose, is a major structural component in wood and some red algae (Turvey & Williams, 1970). Burdige et al. (2000) estimate that neutral sugars make up the majority of carbohydrates in particulate organic matter. MUB- β -D-cellobioside was chosen because the disaccharide, cellobiose, was hypothesized to be a major component of sedimentary polysaccharides. The polysaccharide chitin makes up

the structures of many marine organisms like arthropods, for example (Burdige, 2006). MUB-N-acetyl- β -D-glucosaminide was selected since chitin is a polymer of N-acetyl-glucosamine and is another component of bacterial peptidoglycan (Burdige, 2006). MUB- β -D-glucopyranoside and MUB- α -D-glucopyranoside were chosen to represent other glucose containing polysaccharides of various structures.

In order to provide substrates that cover the nutrients - C, N and P - the last synthetic substrate selected was MUB-phosphate. This substrate represented phospholipids that release phosphate when hydrolyzed by alkaline phosphatase.

Table 3-1 summarizes the selected enzymes, their properties, their substrates (German et al., 2011), and their assigned enzyme commission (EC) number. Enzymes are assigned EC numbers by the International Union of Biochemistry and Molecular Biology and are organized based on the type of biological reactions they catalyze.

Potential Maximum Hydrolysis Rates

For the $V_{\max}$ analysis, each sample (depth) was assayed separately using the Promega Glomax multi JR single-cuvette method described in Chapter 2. On the day of analysis, a sample was removed from -80°C freezer. A sterilized electric hand drill was used to drill out a 1 cm diameter sediment core from the frozen sediment sample. For use as a sterile control, 3 g of defrosted sediment was placed in a serum vial, covered in foil, and autoclaved for 60 min on a liquid cycle at 121°C. In theory, 121°C should kill all microbes and denature the

proteins that make up enzymes. 3 g of defrosted sediment was used as untreated sediment. An autoclaved sediment slurry and an un-autoclaved sediment slurry were prepared separately by blending 3 g of sediment with 100 mL of borate buffered saline (BBS) in a laboratory blender for 1 minute. Immediately after the slurries were prepared, supplies were placed inside a glove box. The glove box was purged with nitrogen gas to create an anoxic atmosphere. Cuvettes were filled with slurry and substrate to a final volume of 1.0 mL. Calibration curves were created using AMC and MUB standards and live sediment slurry. Cuvettes were capped, inverted to prevent settling, and incubated inside the anoxic glove box. Fluorescence was measured 3 to 5 times over a 24-hour period using the Promega Glomax multi JR single cuvette reader inside the anoxic glove box.

0.02 M BBS was chosen to dilute our samples and maintain a pH of 8. Other common buffers (phosphate buffer, carbonate buffer) were avoided so as to not interfere with the substrates being analyzed. The sediment to buffer ratio and shading effect were kept the same for each cuvette so that they could be accurately compared to one another and calibrated. A standard curve was used to calibrate all assays, as opposed to a single concentration standard, because quenching can vary with standard concentration (German et al., 2011). Each assay included a series of controls such as cuvettes containing only BBS and substrate to account for background noise and other reactions by assay reagents (German et al., 2011).

Cell-Specific Enzyme Activities

Cell counts were obtained from BSB site M0059 by Buongiorno et al. (submitted) via SYBR-gold technique. Counts were used to normalize enzyme activity results to cell abundance. Because enzyme activity data and cell count data were measured at different depths, a LOESS spline was drawn through the data. Because of the presence of an erosional unconformity, one line was fit to cell count data above the unconformity and one was fit to the cell count data below the unconformity. Enzyme activities were then divided by cell count estimates and plotted on a per cell basis.

Changes in Enzyme Kinetics with Depth

In order to analyze how enzyme kinetics changed down-core, saturation curves were created using the substrate Phe-Val-Arg-AMC with sediments from 4.5, 11.1, 17.6 and 43.15 meters below the seafloor. Clostripain was chosen because it exhibited the highest amount of activity in the $V_{\max}$ analysis. Un-autoclaved and autoclaved sediment slurries were prepared the same as previously except a set of cuvettes for each depth contained a 0 – 800 μM dilution series of Phe-Val-Arg-AMC. Results were calibrated with AMC-standard.

Results and Discussion

Patterns of Enzyme Activity vs. Depth

For many substrates, at depths above 50 m, substrate hydrolysis was significantly faster in un-autoclaved samples than in autoclaved samples. Furthermore, kinetics of Phe-Val-Arg-AMC hydrolysis fit the Michaelis-Menten

relationship. Both of these facts are consistent with hydrolysis of substrates by extracellular enzymes present in sediments, rather than by abiotic factors.

Most variability in enzyme activity reflects differences in microbial biomass C or organic matter content (Sinsabaugh et al., 2008; German et al., 2011). Here, bulk enzymatic activity decreased with depth (Figure 3-3). Observed enzymatic activities followed organic carbon content (Figure 3-2), with a slight increase in both at about 10 meters below the surface. With the exception of alkaline phosphatase, all enzymatic activity dropped to zero when the organic matter content dropped to below 1 wt%.

Data here provided a maximum rate the enzymes could hydrolyze the given substrate when concentrations of the substrate were saturating (i.e., $[S] \gg K_m$). In situ rates are likely much lower because in situ substrate concentrations are much lower (German et al., 2011). Potential maximum hydrolysis rates still provide useful information on how microbes make a living in the subsurface. Unlike many genomics techniques, enzyme assays provide information on the functional characteristics of the microbial community.

Patterns of Enzyme Activity by Enzyme Class

Peptidases

Enzymatic activity was detected in all 6 peptidases that were assayed (Figure 3-3). Gingipain and clostripain showed the highest potential activities. These two enzymes are endo-acting peptidases meaning they break bonds within the molecule as opposed to exo-acting peptidases that break terminal

molecular bonds. Their higher $V_{\max}$ may indicate that the microbial community accesses degraded organic matter by endo-hydrolysis. Peak potential clostripain activity reached 0.04 μmol substrate per gram sediment per hour.

Peptidase activity was highest in the top 30 meters of the core. Arginine-aminopeptidase, leucine-aminopeptidase, and ornithine-aminopeptidase exhibited similar potential activities. Ornithine aminopeptidase exhibited high activity in autoclaved sediments, and was the only peptidase to show activity below the unconformity suggesting that it could be a highly stable enzyme that has adapted to recalcitrant organic matter. While ornithine is known to be a common non-protein amino acid in particulate marine organic matter (Keil, Tsamakis, & Hedges, 2000) and Erlanger (1950) claims to have isolated it, there is no EC number for ornithyl aminopeptidase. The data here suggest that not only could ornithine-aminopeptidase exist in the deep subsurface, but it could be an extremely stable and specialized subsurface enzyme. Proline-aminopeptidase exhibited a relatively small amount of activity throughout the core.

There is evidence that microbes inhabiting sediment consisting mostly of recalcitrant organic matter may be much more nitrogen-limited than surface microbial communities (Dauwe & Middelburg, 1998). Peptidase data obtained here suggest the microbial community in the deep biosphere actively produces peptidases to obtain nitrogen via amino acids. However, microbes did not seem to favor arginine-AMC over other amino acid substrates that do not contain as much nitrogen. The fact that almost all peptidase activity dropped out below the

unconformity at 51.68 mbsf implies that microbes below this depth do not utilize extracellular enzymes (enough for us to detect) and perhaps have a different pathway for obtaining nitrogen.

Polysaccharide Hydrolases

β -D-glycosidase and N-acetyl- β -D-glucosaminidase were the only polysaccharide hydrolases we assayed that exhibited detectable enzymatic activity (Figure 3-3). α -D-glucosidase activity was observed in a single sample, although this may be due to contamination rather than the presence of enzymes in the sample. β -D-glucosidase exhibited the highest polysaccharide hydrolase activity at about 0.015 μ mol substrate per gram sediment per hour. This is similar in magnitude yet lower than potential peptidase activities. α -D-glucosidase activity was detected only at 24 meter below the seafloor. β -D-cellobiohydrolase and β -D-xylosidase activities were indistinguishable from zero throughout the core (Figure 3-3). Below the unconformity at 51.68 mbsf, all polysaccharide activities were below the detection limit of the Promega fluorimeter suggesting that polysaccharide hydrolases may not be active there. Small amounts of hydrolytic activity was observed in autoclaved sediment in the polysaccharide hydrolase assays as well which mimicked the measured enzymatic activity.

The paucity of polysaccharide hydrolase activity implies the microbial community spends energy to acquire nitrogen and phosphorus before spending energy to acquire carbon. This has been supported by studies reporting that bacterial genes coding for amino acid uptake were found to be more common

than genes coding for the uptake of carbohydrates (Sinsabaugh et al., 2010). It is also possible that other polysaccharide hydrolases are present and active in the Baltic Sea subsurface that simply could not hydrolyze the synthetic substrates provided.

Another possible reason that polysaccharide activities were scarce is that freezing a sample can have an effect on residual activity (Gianfreda & Ruggiero, 2006). DeForest (2009) found that freezing samples before analysis does not affect β -glucosidase activities but does impact N-acetyl- β -D- glucosaminidase activities. Those results were from soils which have very different physico-chemical properties than marine sediments, but it is possible that sedimentary β -glucosidase is more robust than other enzymes. Because all samples used in this study were frozen, it is not possible to test this hypothesis within the dataset, but future studies on the effect of freezing in marine sediments could suggest whether polysaccharide hydrolases were inactivated by sample storage.

Alkaline Phosphatase

Alkaline phosphatase exhibited the highest activity out of all the enzymes assayed. At 4.5 meters below the seafloor, potential alkaline phosphatase activity reached about 0.075 μmol substrate per gram sediment per hour (Figure 3-3). Alkaline phosphatase was the only enzyme other than ornithine aminopeptidase that showed detectable activity below the unconformity at 51.68 mbsf. Autoclaved sediments showed relatively high activities which closely mimicked

the enzymatic activity suggesting alkaline phosphatase could be a highly stabilized enzyme in sediment.

There is evidence for the preferential remineralization of phosphorous in the water column (Clark et al., 1998) which results in an environment highly depleted in phosphorus. While labile dissolved organic carbon has a Redfield ratio of 199:20:1, recalcitrant dissolved organic matter can have a Redfield ratio of 3,511:202:1 (Hopkinson & Vallino, 2005). Perhaps the microbial community produces plentiful alkaline phosphatase enzymes in order to extract as much phosphorus from their environment as possible. Conversely, it is possible that subsurface sedimentary communities express alkaline phosphatase in order to more easily access the carbon rich tails of phospholipids as has been suggested in the Indian Ocean water column (Hoppe & Ullrich, 1999).

Cell-Specific Enzyme Activities vs. Depth

Cell counts decreased with depth with much uncertainty in count numbers near the unconformity (Figure 3-4). Cell counts from holes M0059C and M0059E are slightly higher than the global average number of bacteria and archaea in sediment (Buongiorno et al., submitted). Normalizing our enzyme activity data to cell counts by Buongiorno et al. (submitted) allowed for quantification of enzyme activity per cell. $V_{\max}$ per cell decreased faster than bulk $V_{\max}$ suggesting that enzymes become less important to heterotrophic metabolism with depth (Figure 3-5). Cell-specific peptidase activities measured here ranged from 0 to 150 amol substrate per cell per hour. These data are on a similar order of magnitude to

that of Vetter and colleagues who reported cell-specific extracellular peptidase activity in Arctic waters to be between 5 and 90 amol substrate per cell per hour (Vetter & Deming, 1994).

Patterns of Enzyme Affinity (K_m) vs Depth

Enzymatic activity was detected down to 43.25 meters below the seafloor using the substrate Phe-Val-Arg-AMC. Clostripain V_{max} decreased rapidly down-core (Figure 3-6). Clostripain K_m slightly increased with depth (Figure 3-7). However, this increase was found to be not statistically significant. The hypothesis that an enzyme's affinity for its substrate would increase with depth was not supported by the results obtained here. These data suggest either that clostripain is not adapted to lower substrate concentrations at depths, or that all adaptation has occurred at depth shallower than 4.5 mbsf.

Enzyme Stability

Autoclaved sediment was heated on a 60 minute liquid cycle that reached a peak temperature of 121°C. This temperature is theoretically sufficient to denature extracellular enzymes so that any possible non-enzymatic hydrolysis can be quantified and corrected for. This heating technique works well when enzyme activities are measured in water (Steen, unpublished data). However, residual activity was consistently observed in autoclaved sediment.

The residual activity measured in autoclaved sediment in this study was not attributed to abiotic factors for the following reasons. First, the Michaelis-

Menten equation was successfully fit to the autoclaved sediment data of substrates arginine-AMC and ornithine-AMC (Figure 3-8) using a non-linear least squares model in R (R Core Team, 2015). Second, the killed control activity often closely mimicked the real enzymatic activity for each enzyme but at a lower rate. Samples with high activity in autoclaved samples tended to show higher activity in un-autoclaved sediment as well. Third, the activity in the autoclaved sediment dropped to zero at the unconformity just like the real enzymatic activity does for most enzymes assayed in this study. Likewise, Figures 3-9 and 3-10 show that different enzymes seem to survive the autoclave at different rates. Ornithyl aminopeptidase exhibited higher activities in autoclaved sediment than in untreated sediment. The slope of the relationship of fluorescence production rates in autoclaved versus un-autoclaved samples suggests that approximately 25% of arginyl aminopeptidases were not denatured during the autoclave cycle (Figure 3-10).

Microbial cells in the deep biosphere are known to have extremely long lifetimes and very slow metabolic rates (Jørgensen & Marshall, 2016; Lomstein et al., 2012). Jørgensen and colleagues performed calculations on data collected from Peru shelf sediment (Ocean Drilling Program site 1227, D'Hondt et al., 2003) and estimated that cell doubling times rapidly increased from 100 years at 1 mbsf and 2,500 years at 40 mbsf (Jørgensen & Marshall, 2016). Such slow cell turnover rates would suggest extracellular enzymes have long lifetimes as well.

These results are consistent with long-lived enzymes which are stabilized by sorption to sediments. In soils, it has been observed that enzymes which are adsorbed to mineral surfaces are stabilized and protected from degradation (Allison, 2006). It seems likely that such a mechanism would function in sediments as well. Residual enzyme activity in autoclaved sediments implies that sedimentary extracellular enzymes are extremely stable, and therefore likely have very long active lifetimes. If an enzyme is capable of surviving a 60 minute autoclave cycle reaching 121° C, it might be capable of surviving in the 4° C subsurface for hundreds or even thousands of years.

Conclusions

Processes in the deep biosphere occur on timescales that cannot be accessed in laboratory conditions (Hoehler & Jørgensen, 2013). Unlike in surface environments where most biochemical reactions occur in hours to days, the subsurface processes of biomass turnover occurs over centuries or even millennia (Hoehler & Jørgensen, 2013). Even so, enzymatic activity was measured in the Baltic Sea deep biosphere supporting the hypothesis that microbes in the Baltic Sea subsurface utilize extracellular enzymes to acquire organic carbon, nitrogen, and phosphorus. These data suggest the microbial community in the deep biosphere is seeking nitrogen and phosphorus via amino acids and phosphate more than it is seeking carbon. Enzyme activity is strongly correlated with organic matter content and depth. Substrate affinity of clostripain showed a statistically insignificant decrease with organic matter content and

depth. Lastly, these data show that some enzymes can survive intense heat implying stability and possible thousand-year lifetimes in the deep subsurface biosphere of the Baltic Sea Basin.

Appendix

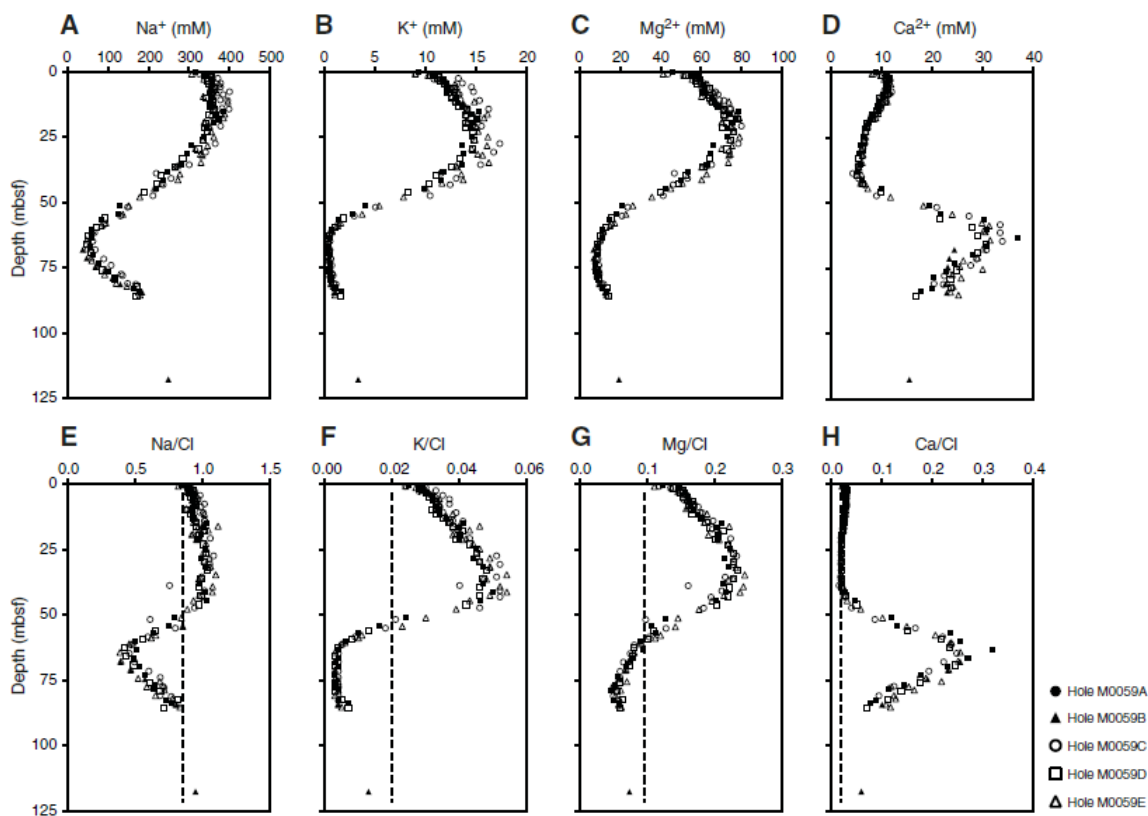


Figure 3-1. Concentration of nutrients in site M0059 sediment pore water.

Figure reproduced from Andren et al. (2015b). Hole M0059C was used in this study.

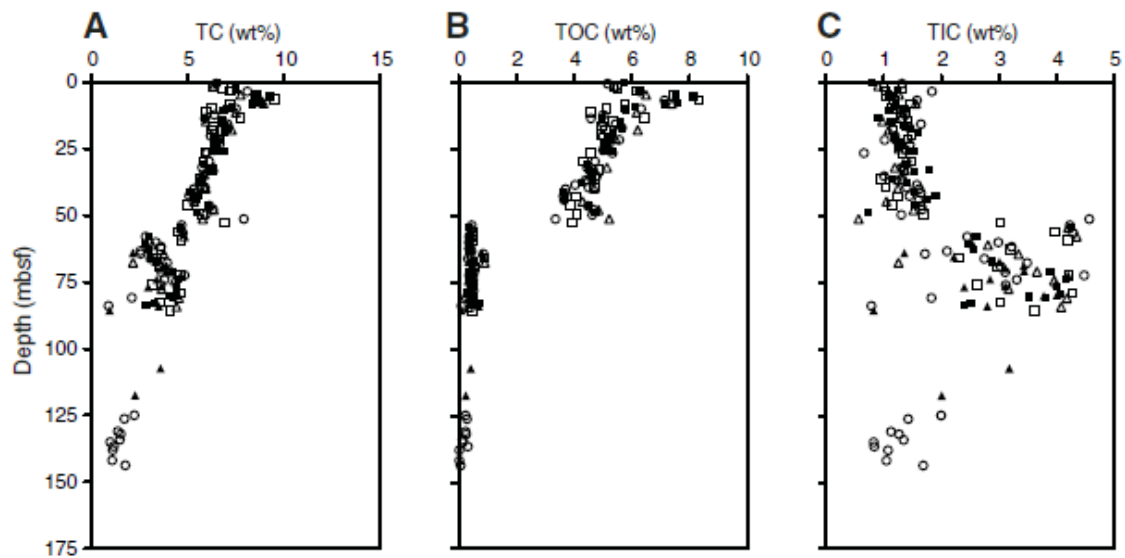


Figure 3-2. Concentration of total carbon (A), total organic carbon (B), and total inorganic carbon (C) at site M0059 in the Baltic Sea Basin.

Figure reproduced from Andren et al. (2015b). Symbols represent different drill holes from the same location. Hole M0059C was used in this study (open circles).

Table 3-1. Enzymes, Enzyme Classes, Substrates and EC Classification

The enzymes assayed in BSB sediment samples along with their class, substrate, and enzyme commission (EC) number.

Enzyme	Class	Substrate	EC
Leucyl aminopeptidase	Exopeptidase	Leucine-AMC	3.4.11.1
Arginyl aminopeptidase	Exopeptidase	L-arginine-AMC	3.4.11.6
Prolyl aminopeptidase	Exopeptidase	H-proline-AMC	3.4.11.5
Ornithyl aminopeptidase	Exopeptidase	Ornithine-AMC	--
Gingipain R.	Endopeptidase	Z-phenylalanine-arginine-AMC	3.4.22.37
Clostripain	Endopeptidase	Z-phenylalanine-valine-arginine-AMC	3.4.22.8
β -d-xylosidase	Polysaccharide hydrolase	MUB- β -D-xylopyranoside	3.2.1.37
β -d-cellobiohydrolase	Polysaccharide hydrolase	MUB- β -D-cellobioside	3.2.1.91
N-acetyl- β -d-glucosaminidase	Polysaccharide hydrolase	MUB-N-acetyl- β -D-glucosaminide	3.2.1.52
β -glucosidase	Polysaccharide hydrolase	MUB- β -D-glucopyranoside	3.2.1.21
α -glucosidase	Polysaccharide hydrolase	MUB- α -D-glucopyranoside	3.2.1.20
Alkaline phosphatase	Phosphatase	MUB-PO ₄	3.1.3.1

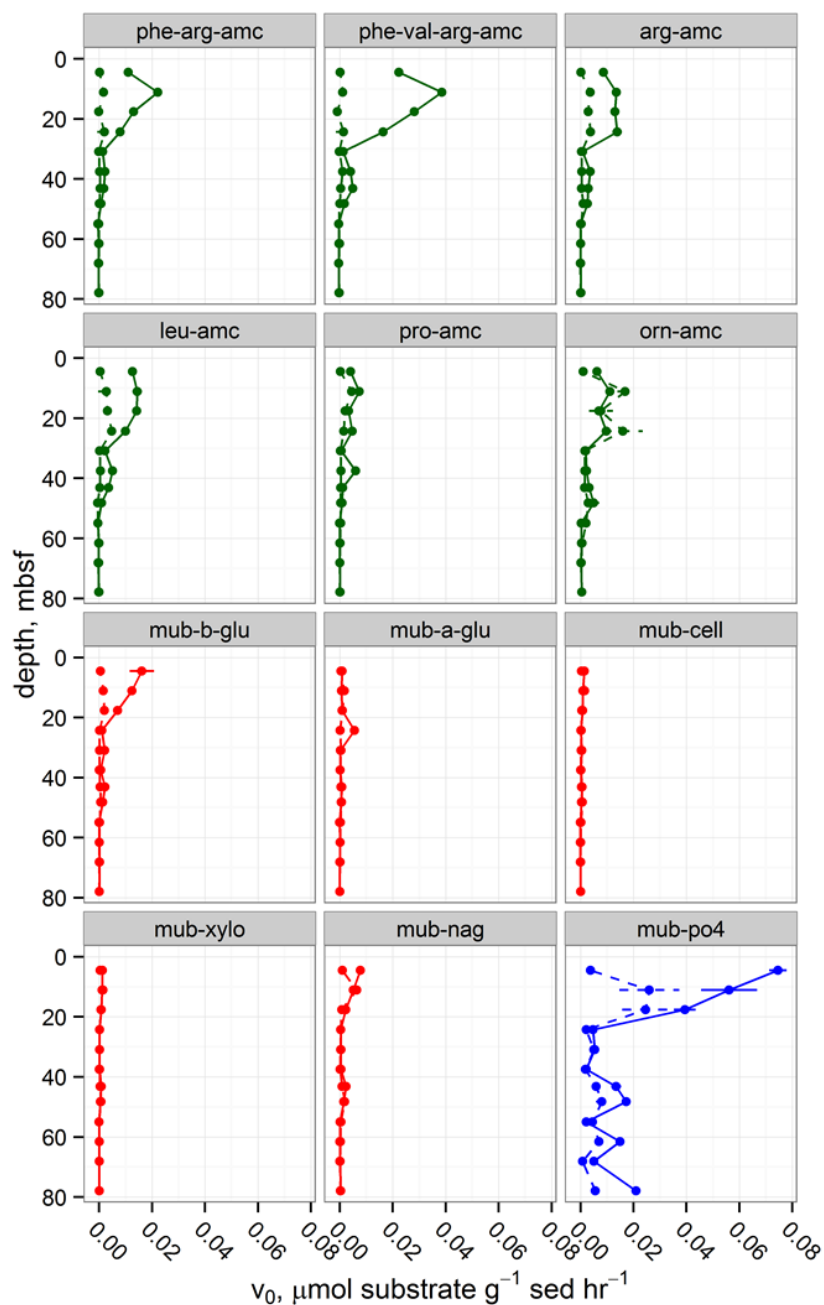


Figure 3-3. Potential Maximum Hydrolysis Rates in BSB

Peptidases (green), polysaccharide hydrolases (red), and alkaline phosphatase (blue) in the BSB; showing activity from untreated sediment (solid) and activity from autoclaved sediment (dashed). Substrate used to target each enzyme is in grey.

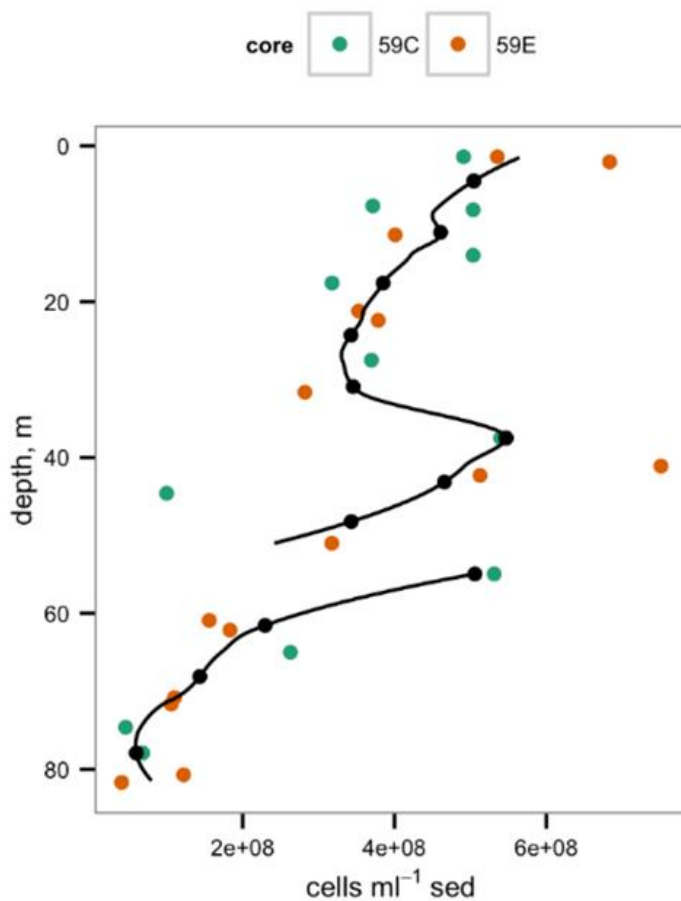


Figure 3-4. BSB Cell Counts

Cell counts obtained via SYBR-gold method for hole M0059C (green) and hole M0059E (orange) in the Baltic Sea Basin (Buongiorno et al., submitted to FEMS Microbiology-Ecology).

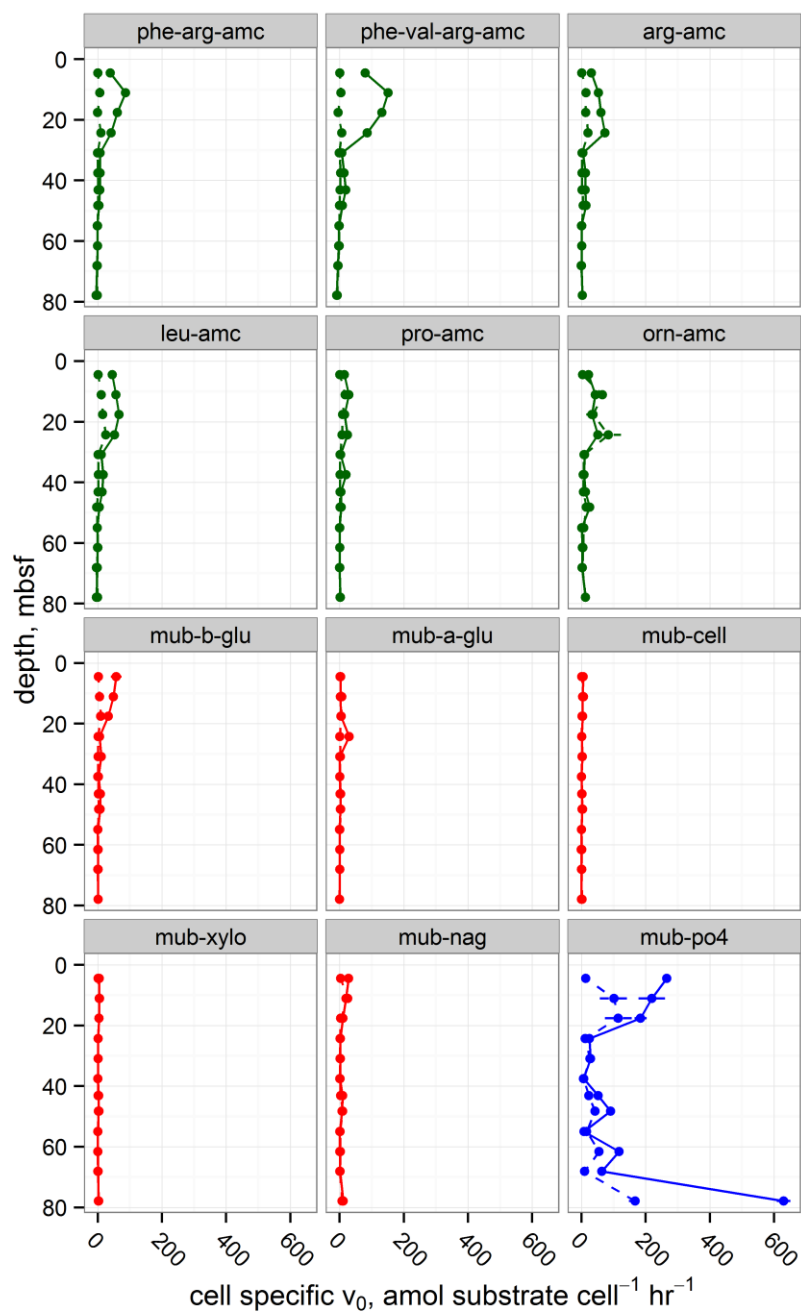


Figure 3-5. Cell Specific Potential Maximum Hydrolysis Rates in BSB

Peptidases (green), polysaccharide hydrolases (red), and alkaline phosphatase (blue) activity from untreated sediment (solid) and activity from autoclaved sediment (dashed). Substrate used to target each enzyme is in grey.

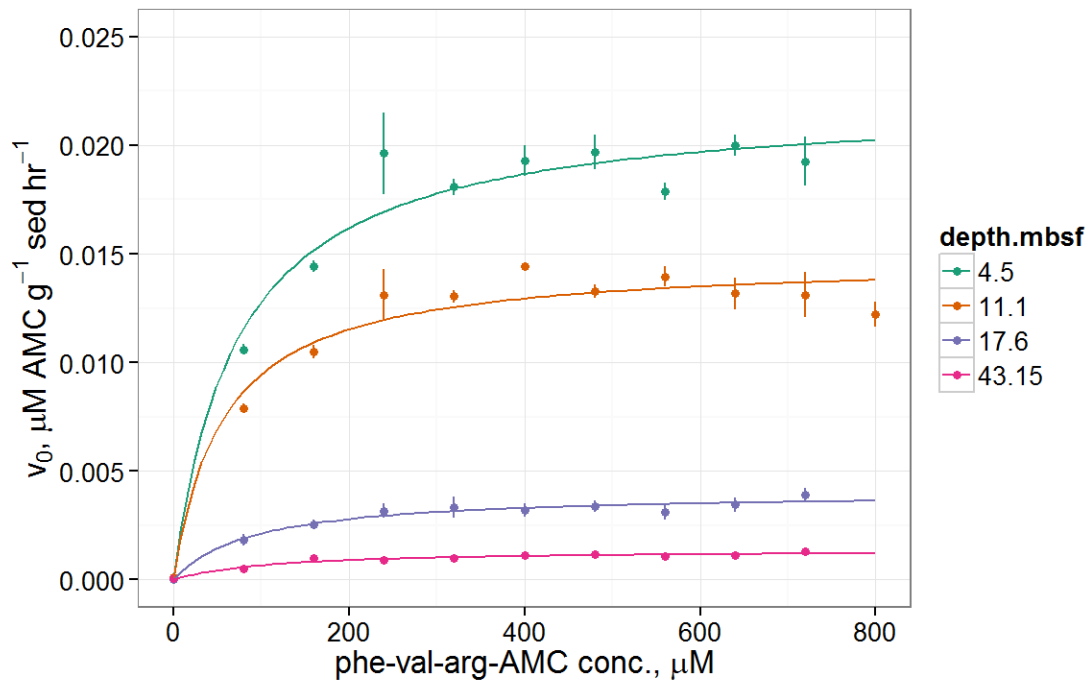


Figure 3-6. Clostripain Saturation Curves

Saturation curves created with the substrate phenylalanine-valine-arginine-AMC which targets the enzyme clostripain. Clostripain $V_{\max}$ decreases with depth in the Baltic Sea Basin.

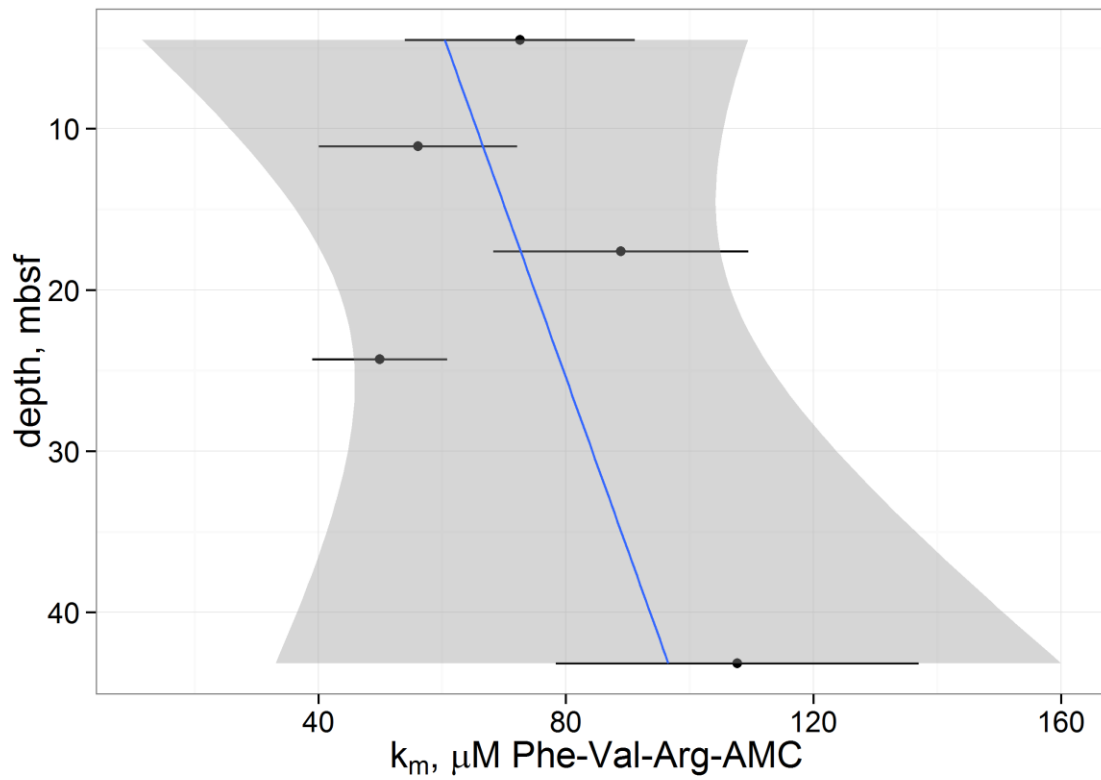


Figure 3-7. Changes in Clostripain K_m with Depth

Clostripain K_m values are calculated from the Michaelis-Menten equation fitted to the data. Values are plotted here with error. The blue line is the overall trend in the data. The shaded area is the 95% confidence interval with no statistically significant trend with depth.

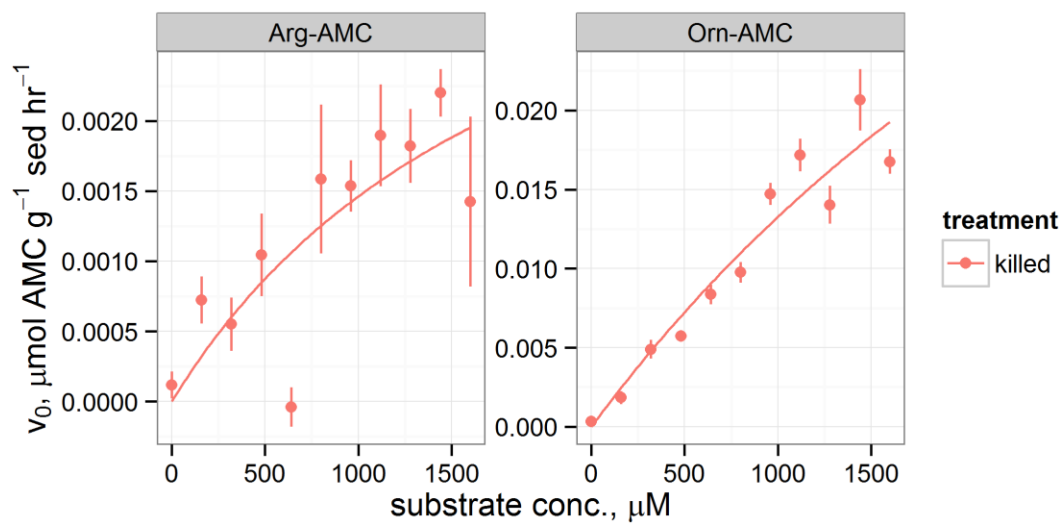


Figure 3-8. Michaelis-Menten Kinetics in Autoclaved Sediment

The Michaelis-Menten equation fit the saturation curves of arginine aminopeptidase and ornithine aminopeptidase created from autoclaved BSB sediment 4.5 mbsf.

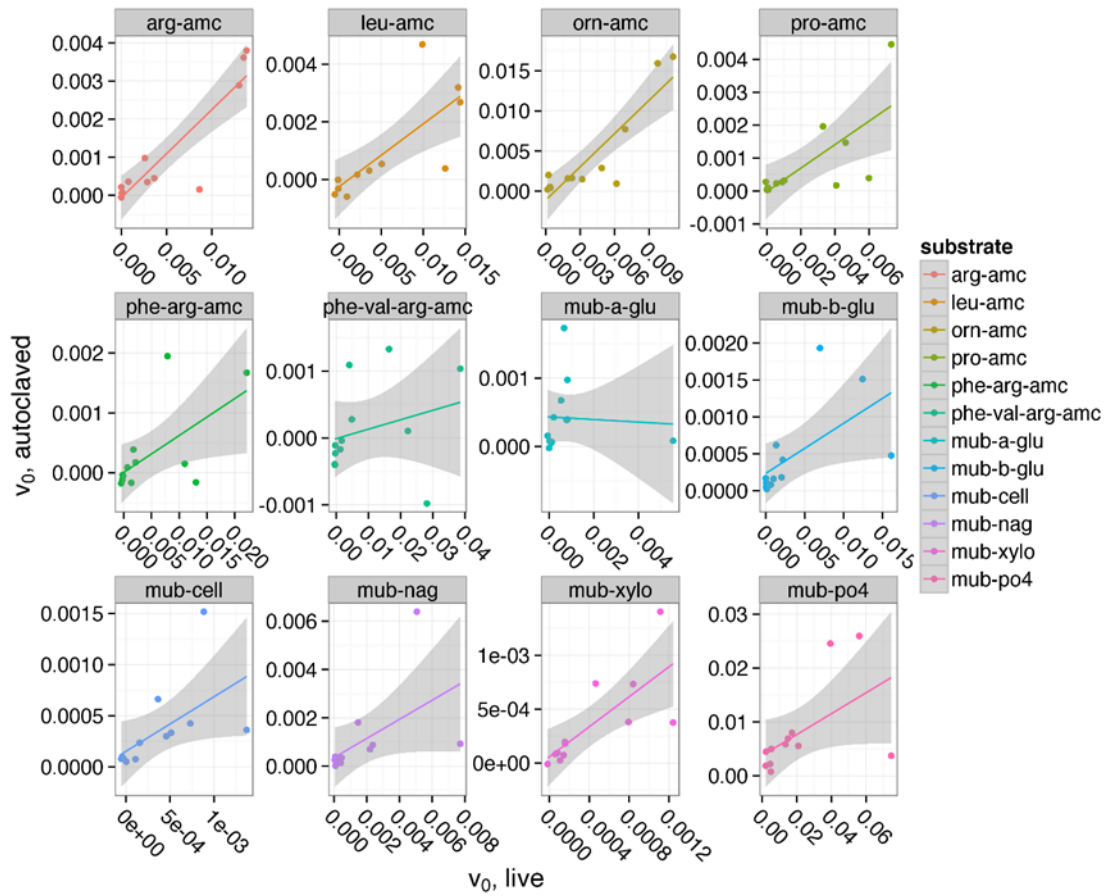


Figure 3-9. V_0 of Untreated Sediment vs. V_0 of Autoclaved Sediment

When separated by substrate, it becomes apparent that different enzymes exhibit varying rates of residual activity after being exposed to extreme heat.

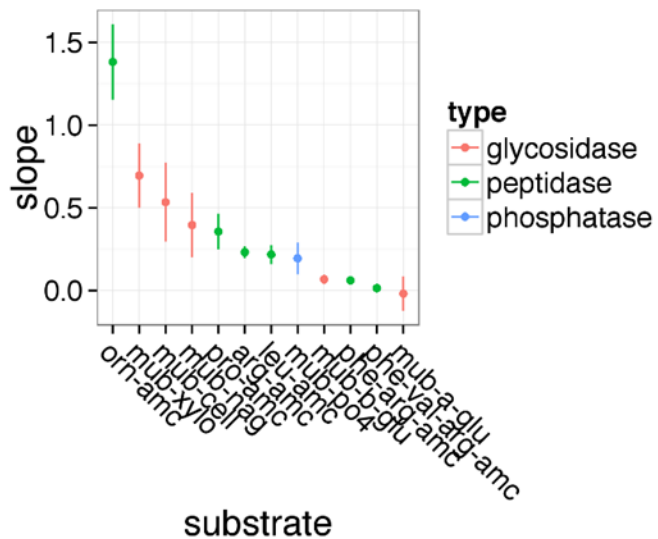


Figure 3-10. Percentage of Residual Activity in Autoclaved Sediments

Slopes from the best fit lines in Figure 3-9 showing the percentage of residual activity for each enzyme in decreasing order. There does not seem to be a correlation between enzyme type and autoclave survival.

CHAPTER IV
CONCLUSION

The role extracellular enzymes play in the marine carbon cycle is central to carbon remineralization. Extracellular enzymes released by microorganisms in water and sediment could be acting as intermediaries between organic carbon and oxidized carbon dioxide in marine environments. Furthermore, extracellular enzymes could be providing a means for heterotrophic microorganisms inhabiting thousand-year old sediment to metabolize recalcitrant organic matter.

The objectives of this thesis were to 1) develop reliable methodology for microbial extracellular enzyme assays in aquatic sediments; and 2) measure the activities of multiple carbon-degrading extracellular enzymes in Baltic Sea subseafloor sediment.

An accurate and precise method for measuring potential maximum hydrolysis rates of extracellular enzymes in aquatic sediments was established by revisiting the methodology and fluorimeters used for sediment enzyme assays to see if a different method would provide higher resolution results. A preliminary sediment enzyme assay performed with a BioTek Cytation 3 96-well plate reader yielded results that were not precise enough to provide information useful to this study. Problems observed included instrumental drift over time, intra-well variation among replicates, and settling of slurry homogenate over long incubations. A series of assays involving fluorimeter comparisons, drift experiments, and saturation curve production performed on a BioTek Cytation3 96-well plate reader and a Promega Glomax multi JR single-cuvette reader showed that using the single-cuvette method was the more precise technique.

Manually measuring fluorescence one cuvette at a time leads to little instrumental drift, less variation among replicates, and a consistent shading effect that does not alter final results. Saturation curves created with the Promega Glomax multi JR obeyed Michaelis-Menten kinetics which confirmed this was a reliable method to measure extracellular enzymatic activity in aquatic sediment.

Activities of 12 different organic carbon-degrading extracellular enzymes were measured in Baltic Sea sediment down to 80 meters beneath the seafloor using the single-cuvette method described in Chapter II. Bulk enzymatic activity decreased with depth, and observed enzymatic activities followed organic carbon content. With the exception of alkaline phosphatase, all enzymatic activity dropped to zero when the organic matter content dropped to below 1 wt%.

Enzymatic activity was detected in all 6 peptidases that were assayed. Gingipain and clostripain showed the highest potential activities and their higher $V_{\max}$ may indicate that the microbial community accesses degraded organic matter by endo-hydrolysis. Ornithine aminopeptidase exhibited high activity in autoclaved sediments suggesting that it could be a highly stable enzyme that has adapted to recalcitrant organic matter. β -D-glycosidase and N-acetyl- β -D-glucosaminidase were the only polysaccharide hydrolases we assayed that exhibited detectable enzymatic activity which implies that the microbial community spends energy to acquire nitrogen and phosphorus before spending energy to acquire carbon. Alkaline phosphatase exhibited the highest activity out

of all the enzymes assayed. These data support the hypothesis that microbes in the Baltic Sea subsurface utilize extracellular enzymes to acquire organic carbon, nitrogen, and phosphorus. The microbial community in the Baltic Sea deep biosphere seems to be seeking nitrogen and phosphorus via amino acids and phosphate more than it is looking to acquire carbon because of the paucity of observed polysaccharide hydrolase activity.

Cell-specific peptidase activities measured here ranged from 0 to 150 amol substrate per cell per hour. $V_{\max}$ per cell decreased faster than bulk $V_{\max}$ suggesting that enzymes become less important to heterotrophic metabolism with depth. The hypothesis that an enzyme's affinity for its substrate would increase with depth was not supported. These data suggest either that clostripain is not adapted to lower substrate concentrations at depths, or that all adaptation has occurred at depth shallower than 4.5 mbsf.

Residual enzyme activity in autoclaved sediments implies that some enzymes are extremely stable and possibly long living. If an enzyme is capable of surviving a 60 minute autoclave cycle reaching 121° C, that enzyme might be capable of surviving in the 4° C subsurface for hundreds or thousands of years. We hypothesize that mineral grains are providing physical protection for the extracellular enzymes that inhabit deep subsurface sediment and suggest further research on this concept.

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VITA

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Jenna's journey through graduate school was not easy and came with many trials along the way. However, perseverance is everything. Jenna does not regret a single moment and truly believes that she has grown as a scientist and as a person over the past two years. Jenna's goals as she looks for employment are to make an impact on her two passions: education and the environment. She wishes to thank the wonderful Volunteer spirit at UTK for inspiring her every day.