MICROBIAL DEGRADATION OF ORGANIC MACROMOLECULES IN ARCTIC FJORDS AND IN THE GULF OF MEXICO

Andrew D. Steen

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Approved by:

Dr. Carol Arnosti

Dr. Daniel B. Albert

Dr. Ronald Benner

Dr. Neil V. Blough

Dr. Christopher S. Martens

ABSTRACT

ANDREW D. STEEN: Microbial degradation of organic macromolecules in Arctic fjords and in the Gulf of Mexico

(Under the direction of Carol Arnosti)

Polysaccharides represent a labile, abundant class of marine dissolved organic matter (DOM), which must be hydrolyzed by extracellular enzymes prior to uptake by heterotrophic microbes. Pelagic microbial communities differ in their ability to access polysaccharides: some communities are completely incapable of accessing certain polysaccharides, which are rapidly hydrolyzed elsewhere. This dissertation approaches the questions of how, when, and why microbial communities express specific sets of extracellular polysaccharide hydrolases using four independent but complimentary approaches. First, degradation rates in seawater of several enzymes are measured, and the factors that control those rates are investigated. These rates will help to clarify when and why production of extracellular enzymes can be a viable strategy for heterotrophic marine microbes. Second, hydrolysis rates of six polysaccharides are measured along horizontal and depth gradients in the Gulf of Mexico and compared to glucose turnover rate constants in order to evaluate the potential for spatial variability in the capabilities of microbial communities with respect to polysaccharide degradation and metabolism. Third, microbial communities in a Svalbard fjord were probed in order to investigate why those communities apparently lack the ability to metabolize

pullulan, a soluble, linear glucose polymer, even when incubated in the presence of relatively high concentrations of pullulan for weeks. Finally, a new assay for polysaccharide hydrolysis rates in environmental aquatic samples is presented, which raises the possibility for novel experiments which would not be feasible using previous methodology. The results presented here indicate that polysaccharide lability in the ocean is not purely a function of intrinsic factors relating to polysaccharides, but is also strongly dependant on the composition and activity of *in situ* microbial communities. These characteristics must therefore also be accounted for in models of the marine carbon cycle.

DEDICATION

This dissertation is dedicated to my grandfather, Dr. Frederick H. Steen, who inspires me to pursue an intellectually rigorous life.

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Chapter 1 Dissertation Introduction

There is a huge quantity of dissolved organic carbon (DOC) in seawater: about 700 Pg, similar to the total amount of atmospheric CO_2. Nevertheless, basic questions about DOC biogeochemistry remain unanswered – what is the composition of DOC, and how did it get that way?

This dissertation explores DOC degradation in the marine water column, focusing on dynamics of polysaccharides and the microbial extracellular enzymes that hydrolyze them. The purpose of this introduction is to review briefly the state of our understanding of DOC chemistry and biology, in order to explain the context and motivation for the research I present.

1.1 Definitions, pool sizes and fluxes

Dissolved organic matter (DOM) is defined operationally, as reduced carbon that is able to pass a filter with a specific pore size (Hedges 2002). Different researchers have used different pore sizes to distinguish particulate organic matter (POM) from DOM, varying typically between ~0.7 μm (the nominal pore size of Whatman GF/F filters) to 0.1 μm, with 0.2 μm being the most common pore size used in the contemporary literature. This operational definition does not correspond perfectly to the chemical definition of a dissolved substance: some amount of colloidal particles (Gustafsson and Gschwend 1997), free viruses

and even viable bacteria (Li and Dickie 1985) may pass through 0.2 μm filters, and sorption of truly dissolved organic matter to filter membranes is always possible. In practice, however, filtration is the only tractable method to separate POM from DOM, so virtually all research on DOM begins with filtration (or the assumption that POM concentrations are negligible).

Seawater concentrations of DOC range from a minimum of about 33 μM C in the most DOC-poor deep seawater to less than 100 μM C in terrestrially-influenced coastal seawater (Benner 2002; Mannino and Harvey 2000). The major sources of DOC to seawater are in situ production by photoautotrophs (primarily pelagic phytoplankton) and input from terrestrial runoff. The size of the terrestrial flux, about 2.5×10^{14} g C $year^{-1}$, is substantial, but low concentrations of markers for terrestrial organic matter in the open ocean imply that terrestrial DOC is degraded two to three orders of magnitude faster than bulk marine DOC (Opsahl and Benner 1997). The flux of DOC from phytoplankton is much harder to constrain because heterotrophic production can be linked quite closely to primary production.

Other sources of DOC are possible, but they are poorly constrained and less likely to be quantitatively important. Sediments might in principle yield fluxes of DOC into seawater, but profiles of DOC concentrations in sediment porewater DOC (Alperin et al. 1999) and in near-bottom seawater (Hansell 2002) do not show strong evidence for this. Hydrothermal vents may in some cases be a source of DOC, but DOC fluxes from hydrothermal fluid appear to be variable and are probably not quantitatively important in terms of the global DOC budget (Lang et al. 2006). They may, however, be important sources for specific classes within DOC (Dittmar and Koch 2006). Chemoautotrophic Bacteria and Archaea are

increasingly viewed as important to deep sea microbial ecosystems (Gasol et al. 2008; Ingalls et al. 2006), but their importance as a source of DOM is not known.

Fluxes of DOM from surface to deep water can be large, comparable to the sinking flux of POC (Carlson et al. 1994). DOM export and POM sinking both have the effect of sequestering carbon from contact with the atmosphere over the thousand-year timescale of ocean circulation, but there is an interesting ecological difference between the two processes. Elemental composition of sinking POM corresponds (roughly) to the Redfield ratio, with C:N of 6.6. DOM is more nutrient poor, with C:N of 9-18 (Benner 2002). Removal of OM from the surface ocean sequesters carbon, but it also sequesters nutrients; when surface water is nutrient-limited this reduces the capacity for phytoplankton to fix new carbon (Eppley and Peterson 1979). Export of nutrients from surface water therefore counteracts export of organic carbon. Since DOM is more nutrient-poor than POM, DOM export sequesters more carbon per mole carbon exported than POM sinking.

1.2 Reactivity of bulk DOC

The radiocarbon content of bulk DOC in deep water is depleted by about -520‰, implying an age of ~6000 years since the carbon was fixed (Williams and Druffel 1988). This age is an average, however, and there exist enormous differences in reactivity within the bulk pool.

DOC may be classified by reactivity as labile, semi-labile, or recalcitrant (Kirchman et al. 1993). The residence times of these fractions (in the absence of advection) are typically considered to be on the order of days, months, and hundreds-to-thousands of years,

respectively. This sort of classification implies that rate laws of DOC degradation can be described with a multiple rate constant model such as the 'multi-G' model (Westrich and Berner 1984). This classification scheme has provided a useful conceptual framework with which to explain measured concentrations and fluxes of marine DOC. However, recent work on the consumption of different classes of DOC by different clades of bacteria (and, via a different approach, in this dissertation) implies that DOC reactivity is also contextual: degradation rates of specific classes of DOC might be critically dependant on the presence of specific classes of microbes or the presence of other chemical factors in the environment (e.g. Cottrell and Kirchman 2000; Elifantz et al. 2008; Malmstrom et al. 2005).

1.3 Analytical challenges in determining the composition of DOC

There are two major impediments to a complete structural characterization of DOM. First, salt concentrations in seawater are three orders of magnitude greater than the bulk concentration of DOC. Since seawater salt interferes with many useful analytical techniques (NMR, mass spectrometry, some detection techniques used in combination with chromatography) it presents a major impediment to analysis. Second, DOM is an exceptionally complex mixture of tens of thousands of distinct molecules, most of which are present in sub-nanomolar concentrations. Accurately determining the concentrations and structures of such dilute components against a tremendous background of other organic molecules and salt is a formidable analytical challenge.

A wide range of analytical techniques have been employed to address that challenge. In general, these involve tradeoffs between yield, structural specificity, and quantitative accuracy or precision.

There are a number of colorimetric and fluorometric techniques to detect broad classes of organic molecules such as carbohydrates or proteins (Bradford 1976; Burney and Sieburth 1977). These methods are typically inexpensive and easy to perform, but give no information about the specific structures present. Because of this non-specificity, it is also difficult to distinguish between accurate concentrations and false positive results due to interfering compounds.

A wide range of gas and liquid chromatographic techniques have been used to identify specific compounds in seawater, including amino acids and various carbohydrates (e.g. Lindroth and Mopper 1979; Mopper et al. 1992). These methods are generally only capable of separating low molecular weight compounds, so they are usually employed either to measure concentrations of free monomers, or distributions of monomers in high molecular weight organic matter after a hydrolysis step. Hydrolysis destroys information on linkages among monomers, so the structures of polysaccharides cannot be reconstructed (Panagiotopoulos and Sempéré 2005).

NMR and mass spectrometry techniques can be extraordinarily powerful techniques to determine structural characteristics of a wide range of molecules simultaneously. NMR studies of DOM most often examine ^{1}H, ^{13}C, or ^{15}N but other nuclei can be examined as well. The great advantage of NMR is that it "sees" the chemical environment of all of the targeted nuclei in the sample simultaneously and quantitatively. However, in typical 1-D analyses, NMR spectra only yield structural information at the level of functional groups (signal

intensity is typically too low for 2-D NMR, precluding the possibility of more detailed structural information). The relative abundance of functional groups can give clues about the relative abundance of classes of chemical structures, but NMR spectra do not unambiguously identify specific structures present in a sample. However, NMR is a highly versatile tool; thousands of distinct NMR 'experiments' (i.e. techniques) exist (A. Simpson, pers. comm.). NMR analysis has revealed a great deal of information about the composition of DOM, and clever combination of NMR analysis with other techniques will likely continue to reveal a great deal about DOM composition (Mopper et al. 2008).

Finally, a wide range of mass spectrometric techniques have been employed to identify structures present in DOM. Among these, ultra-high resolution mass spectrometers, which use extremely high magnetic fields to efficiently separate molecular ions, are extremely promising; Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has been the most useful of these techniques for DOM analysis. FT-ICR-MS spectra of DOM can contain thousands of distinct peaks spanning masses of ~10 to ~5000 Da, each of which can be related to a specific molecular formula (Mopper et al. 2008). The ideal ionization method would quantitatively ionize all structures present in a sample without cleaving covalent bonds, but no such ionization technique exists. As a result, the utility of mass spectrometric techniques as quantitative tools is limited. Electrospray ionization (ESI) has most often been used in analysis of DOM by FT-ICR-MS (Kujawinski et al. 2004), but atmospheric pressure photoionization (APPI) may be capable of ionizing a wider variety of components of DOM (Mopper et al. 2008).

Both NMR and ultrahigh resolution mass spectrometry can provide a wealth of information about chemical structures present in DOM. However, both of these rely on the

concentration and desalting of DOM prior to analysis. The most common ways to achieve this are solid phase extraction and ultrafiltration. These techniques are invaluable, and yet they both function on the principle of concentrating DOM by discriminating among molecules based on their chemical or physical characteristics, and both retain only 10-30% of total DOM (Mopper et al. 2008). A variety of solid phase media exist to extract DOM with different relative affinities for hydrophobicity and other DOM characteristics. Sorption of DOM to solid phase media depends on the enthalpy and entropy changes of sorption. The enthalpy of sorption depends critically on the nature of functional groups within the DOM (Arnarson and Keil 2000), so solid phase extraction media necessarily extract DOM with different characteristics than bulk DOM.

Ultrafiltration is perhaps simpler in principle than solid phase extraction. It aims to retain molecules larger than a certain cutoff size, often 1000 Daltons. Unfortunately high molecular weight (HMW) DOM usually accounts for only around 30% of total DOM in surface water (Amon and Benner 1994), so currently HMW DOM is much better characterized than LMW DOM. The choice of units used to describe the size cutoff is a clue to a second drawback of ultrafiltration: size is not the same thing as mass. Molecular conformation and any possible supra-molecular associations can influence the passage of molecules through ultrafiltration membranes. As a result, ultrafiltration membranes allow passage of some organic matter larger than the molecular weight cutoff, and retain some matter smaller than the cutoff (Burdige and Gardner 1998). Finally, concentration of organic matter might lead to reactions that would not occur in the natural, diluted state. For instance, extracellular enzymes in the concentrated DOM could catalyze hydrolysis of macromolecules at much higher rates than occur naturally, thereby biasing the measured size distribution of

DOM. Conversely, rates of aggregation of DOM molecules might increase greatly on concentration. To my knowledge, such potential effects have received scant attention in the recent literature.

Due to the important limitations of all of the analytical techniques described above, the best studies of DOM composition have combined multiple characterization techniques. One good example of this is the work of Hertkorn et al (2006), who combined a variety of NMR techniques with FT-ICR-MS analysis to identify an abundant, recalcitrant form of DOM they named carboxyl-rich alicyclic material (CRAM). Despite efforts such as these, however, approximately three quarters of DOM is not characterizable as specific compounds (Hedges et al. 2000).

1.4 Known factors related to DOM reactivity

Despite the difficulty of fully characterizing DOM chemically, some chemical indicators have been observed to gauge DOM reactivity. The high reactivity of HMW DOM relative to LMW DOM has been suggested by size fractionation experiments, in which size-fractionated DOM was exposed to inocula of seawater-derived bacteria (Amon and Benner 1994; Covert and Moran 2001), by the lower abundance of DOM in deep water relative to surface water, and by the lower natural abundance of ^{14}C in LMW DOC than HMW DOC (Loh et al. 2004). This finding is somewhat counterintuitive because low molecular weight biomolecules such as monosaccharides and amino acids ought to be extremely bioavailable. These molecules are indeed labile, with residence times in surface seawater of minutes to a few days (Kirchman 2002). However, LMW DOC apparently contains a large quantity of

other structures. The nature of these structures is unclear but CRAM (which was identified in >1 kDa ultrafiltered concentrate, but shown by mass spectrometry to be rich in LMW molecules) is likely highly recalcitrant and abundant in LMW DOC.

The fraction of DOM identifiable as total hydrolysable neutral sugars (THNS) or total hydrolysable amino acids (THAA) decreased during microbial degradation of fresh DOM (Amon et al. 2001; Davis and Benner 2006). Abundances of specific amino acids (Dauwe and Middelburg 1998; Davis et al. 2009) and specific neutral sugars (Aluwihare and Repeta 1999; Kirchman et al. 2001) change in characteristic ways during DOM degradation, such that mole fractions of specific monomers (e.g. glucose) or indices accounting for the abundance of all monosaccharides (e.g. Dauwe and Middleburg's amino acid-based degradation index) can serve as general indicators of the diagenetic state of DOM. These sorts of indicators are useful in assessing DOM quality in samples, but they beg for an explanation: we can predict how bulk DOM changes during degradation, but we do not understand why and how DOM is degraded in the way that it is.

1.5 Importance of extracellular hydrolytic enzymes

The limitations of analytical techniques cited above suggest that approaches to study DOM cycling that are not based solely on chemical composition are necessary. Hydrolysis of HMW DOM by microbial extracellular enzymes is one important mode of transformation. Microbes take up organic matter via uptake porins, through which molecules larger than ~600 Da cannot pass (Benz and Bauer 1988). Bioavailable macromolecules must therefore be hydrolyzed prior to uptake by extracellular enzymes, which may be cell-surface attached

or released freely into the medium (Chróst 1991). Due to the abundance of carbohydrates in high molecular weight dissolved organic matter, the presence of appropriate polysaccharide hydrolases to match the available dissolved organic matter is a prerequisite to the degradation of a large fraction of HMW DOM.

Study of the structure, kinetic properties, and regulatory systems of extracellular enzymes therefore has great potential to inform our understanding of DOC cycling. Three major factors complicate this study. First, concentrations of individual enzymes in seawater are too low to reliably isolate individual enzymes, so that direct investigations of the properties of enzymes present in seawater are generally not possible. Second, the vast majority of microbial species[1] in the environment have no close relatives that have been isolated in pure culture (Rappé and Giovannoni 2003). There have been a number of valuable studies of enzymes produced by isolated marine bacteria (Martinez et al. 1996), but in general it is not possible to confidently extrapolate specific findings from such studies to the full range of marine bacteria and their enzymes. Finally, a single functional enzyme class (β-glucosidases, for example) may contain diverse structures (Domań-Pytka and Bardowski 2004) even within a single environmental sample (Arrieta and Herndl 2002). Low concentrations of diverse isofunctional enzymes produced by microbes of unknown identity are difficult to study rigorously.

1.6 Dynamics of aquatic extracellular enzymes

[1] 'Species' is a loaded word among microbial ecologists. Without wading into the debate as to how best to define microbial species, or whether such a definition even makes sense, I'll use 'species' in an informal way to describe a group of closely-related organisms sharing similar ecological roles.

Extracellular enzymes may be released freely into the medium ('cell-free') or remain cell surface attached. The fraction of enzymes that are cell free may range from <10% (Wetzel 1991) to 100% (Keith and Arnosti 2001); studies in pure culture demonstrate that bacteria can grow rapidly using only substrate liberated by cell-free enzymes (Vetter and Deming 1999). The factors that determine whether enzymes remain as cell-free or cell surface attached are not well understood, but environmental conditions and microbial feeding strategies are likely important factors. One recent model suggests that in aggregates, microbes may receive more hydrolysate by releasing cell-free enzymes which sorb to particles, than by producing cell-surface attached enzymes (Vetter et al. 1998). The characteristic diffusivity of the environment also influences the relative advantage of cell-free versus cell-attached enzymes: 'cheating' organisms, which consume hydrolysate without producing the corresponding enzymes, benefit more relative to enzyme-producing organisms in high-diffusivity environments such as seawater than in low-diffusivity environments such as soils (Allison 2005). Allison et al. concluded that cheaters would outcompete enzyme producers in seawater, and yet substantial cell-free extracellular enzyme activity is sometimes observed in seawater. It is possible that all cell free extracellular enzyme release is 'unintentional', the result of cell lysis or similar processes. However, high fluxes of extracellular enzyme activity from sinking particles (Smith et al. 1992) and the occasional high fraction of cell-free enzyme activity (described above) suggest that in some cases microbes can gain selective advantage by releasing cell-free enzymes. A second possibility is that microbes produce cell-free enzymes as sensors, to locate and then move towards sources of organic matter (Kiørboe and Jackson 2001), rather than as a direct means to obtain

organic substrates. However, this strategy still seems vulnerable to 'cheaters' that would compete with enzyme producing microbes.

Levels of enzyme expression are not the only influence on extracellular enzyme activity in seawater; once produced, enzymes may decay at variable rates. These rates are poorly constrained; the goal of the first chapter of this dissertation is to constrain decay rates of extracellular enzymes in Arctic seawater. Ultraviolet radiation may lead to deactivation of enzymes, which probably occurs via sensitization since enzymes do not absorb strongly in regions of the solar spectrum that reach the Earth's surface (Boavida and Wetzel 1998; Espeland and Wetzel 2001). Sorption to mineral surfaces (Tietjen and Wetzel 2003; Ziervogel et al. 2007) or associations with humic substances (Boavida and Wetzel 1998; Kim and Wetzel 1993) may also decrease the activity of extracellular enzymes. However, both of those processes may also protect enzymes from photodegradation (Tietjen and Wetzel 2003) and hydrolysis by proteolytic enzymes (Steen, Gillis, Arnosti unpublished data). Potential interactions of extracellular enzymes with other forms of organic matter present in seawater, such as transparent exopolymer particles (TEP, Passow 2002) are largely unknown.

The vast majority of investigations of extracellular enzymes in the aquatic environments have been based on measurements of enzyme activity using small chromo- or fluorogenic substrate proxies. These proxies are easy to use, inexpensive, and, in many cases, are extraordinarily sensitive. The simple structure of small substrate proxies, however, does not capture the complexity of macromolecular enzyme substrates, and therefore do not reliably interact with enzymes in the same way as naturally present substances. Enzymes containing substrate binding domains are particularly likely to interact with macromolecules differently than with corresponding small substrate proxies. This is a particular problem for

studies of polysaccharide hydrolases in the environment: polysaccharide hydrolases as a class seem to be particularly reliant on substrate binding domains (Béguin and Aubert 1994), and putative polysaccharide hydrolase genes from the complete genome of a polysaccharide-degrading marine bacterium have a high abundance of substrate binding domains (Bauer et al. 2006). If marine polysaccharide hydrolases typically require substrates to bind to non-catalytic domains prior to hydrolysis, then enzyme activities measured with small substrate proxies will severely underestimate true activities. There is some evidence that this may be the case: commercially available pullulanase, which hydrolyzes the α-linked glucose polymer pullulan, completely fails to hydrolyze methylumbelliferyl-α-glucopyranoside, which is used to measure α-glucosidase activity (Steen, unpublished data).

A second technique to investigate enzyme activity in natural samples relies on measuring size changes macromolecules that have been added to a sample. These techniques can be made considerably more sensitive if the substrates are labeled in some way[2]. Macromolecules can be labeled with fluorescent or spin probes; size detection can be via gel permeation chromatography (Arnosti 1995), high performance liquid chromatography (Pantoja et al. 1997), electron paramagnetic resonance spectroscopy (Steen et al. 2006), or fluorescence anisotropy (Arnosti et al. 2000; this work). These methods are considerably more labor intensive than methods using small-substrate proxies, and their high detection limit for enzyme activity typically requires days-long incubations before activity is observed. When incubations are long relative to microbial generation times, the resulting hydrolysis

[2] In the fields of biochemistry and microbiology it is common to track polysaccharide hydrolysis by the production of reducing ends or free sugars from unlabeled polysaccharides. This is also possible in environmental samples (Hanlon et al 2006), but this method requires the use of inhibitors of unknown efficacy and has not, to my knowledge, been applied successfully in pelagic samples.

rate should not be considered an indication of *in situ* enzyme activity, because organisms would have had time to grow and change gene expression patterns in response to the input of substrate. The resulting rate is more of an indication of the community's ability to respond to a substrate. Nevertheless, such measurements are more realistic than small-substrate proxy-derived activities, and allow investigation of hydrolysis rates at a higher level of substrate structural detail than is possible with small substrate proxies (Arnosti 2002).

Studies with fl-polysaccharides in seawater and sediments have shown that microbial communities in different environments have different capacities to hydrolyze specific polysaccharides. Both absolute hydrolysis rates, and relative patterns of hydrolysis rates varied along estuarine gradients (Keith and Arnosti 2001; Steen et al. 2006), with latitude among globally-distributed samples (Arnosti in prep; Arnosti et al. 2005), with time (Arnosti and Ghobrial unpublished data) and, strikingly, between sediments and overlying seawater (Arnosti 2000; Arnosti 2008). These data demonstrate conclusively that some communities lack the ability to access specific polysaccharides which are readily hydrolyzed by other microbial communities.

1.7 Dissertation synposis

The goals of this dissertation were to investigate controls on the enzymatic degradation of specific polysaccharides, and the role of microbial extracellular enzymes in the water column cycling of DOC.

Chapter 1 seeks to constrain the rates at which extracellular enzymes present in Svalbard fjord seawater are degraded, and to help identify some of the factors that influence

their degradation rates. In this study, phosphatase, β-glucosidase, and leucine aminopeptidase had half-lives of tens of hours or longer, which suggests that natural enzymes may be more stable in seawater than commercially-obtained enzymes which derive from non-marine sources. Enzymes in Svalbard water were degraded under artificial UV illumination, but under typical cloudy conditions photochemical interactions were not an important cause of enzyme degradation.

Chapter 2 reports measurements of polysaccharide hydrolysis, glucose metabolism, bacterial production, and carbohydrate concentrations in depth profiles at three sites in the northern Gulf of Mexico, from a shallow site heavily influenced by the Mississippi River outflow to a more characteristically marine site at 900 m water depth on the continental slope. Systematic differences in polysaccharide hydrolysis rate were observed among the different sites and depths. Such differences could account for the fact that microbial communities have been observed to differ in their ability to process bulk DOC. Polysaccharide hydrolysis rates varied as a function of time, and weren't predictable from in situ rates of glucose metabolism or bacterial production, because microbial communities responded differently to the input of fresh fl-polysaccharides. The responses differed by substrate, however, and these differences yielded clues as to what fraction of the initial microbial community was capable of accessing each substrate.

Chapter 3 investigates the question of why a heterotrophic community might fail to hydrolyze a specific polysaccharide, using pullulan as an example of a polysaccharide which is hydrolyzed rapidly in some environments, but not at all in others. Here I evaluated temperature, carbon or nutrient stress, and absence of specific inducers for enzyme expression as possible reasons that pullulan is usually totally inaccessible to communities in

Svalbard fjord waters. None of these factors explained the absence of pullulan hydrolysis, and I suggest that the most likely explanation is that the microbial communities totally lack genes for pullulanase. This example demonstrates that the observed variation in microbial community composition among ocean provinces is matched by functional variation.

Finally, Chapter 4 evaluates an assay for polysaccharide hydrolase activity in aquatic environmental samples based on changes in fluorescence anisotropy of fluorescently-labeled polysaccharides. A simple model predicts fluorescence anisotropy from size distributions of labeled polysaccharides, demonstrating that potentially confounding factors such as variations in pH and associations with DOM are negligible.

Taken together, these results show that role of microbial communities in the marine carbon cycle varies by location in the water column. Reactivity of DOM is a function of microbial community composition as well as the chemical characteristics of DOM. Functional variability of communities arises from the set of extracellular enzymes that a community is capable of producing, as well as the chemical characteristics of those enzymes. Extracellular enzymes produced by Arctic Ocean communities appear to be more stable than commercially-obtained, non marine enzymes; enzyme stability might be variable within the ocean as well. The factors that drive functional variability among communities will be an important area for future research, and one way to advance that research will be to develop novel methods to probe reactions of DOM.

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Chapter 2 Slow decay of extracellular phosphatase, β-glucosidase, and leucine aminopeptidase in Arctic seawater

Andrew D. Steen and Carol Arnosti

Extracellular enzymes are the conduit through which high molecular weight organic matter enters the microbial loop. The time scales over which enzymes retain functionality in the environment influences the extent to which enzyme-producing microbes can "profit" from them. Here we show that half-lives of phosphatase, β-glucosidase, and leucine aminopeptidase in Arctic seawater are on the order of days or longer, and that naturally-produced extracellular enzymes in seawater may be more robust than enzymes carrying out the same function in non-aquatic environments. Investigation of the effect of ultraviolet radiation on enzyme activity indicates that enzymes can be subject to photochemical degradation, but that typical natural UV intensity is too low for that pathway to be important, at least at our study location. Long enzyme half-lives point to the possibility that sinking particles, on which microbial activity is high, may act as a source of cell-free extracellular enzymes to the deep ocean, decoupled from microbial activity.

2.1 Introduction

Heterotrophic microbes rely on extracellular enzymes to transform high molecular weight organic matter into molecules small enough to cross the cell membrane. In order for production of these enzymes to represent a viable means of obtaining resources, enzyme-producing microbes must 'profit' from enzyme production. Production of enzymes must result in the acquisition of carbon, nutrients and/or energy that results in more growth than would have been possible using the same amount of resources to produce new biomass (Koch 1985). The actual 'economics' of extracellular enzyme production, including the costs of enzymes production and the amount of hydrolysate that production of an enzyme is likely to yield, are poorly constrained (Sterner and Elser 2002).

Mathematical models of extracellular enzyme 'economics' point to the decay rate of extracellular enzymes as a critical factor in determining the profitability of enzyme production (Allison 2005; Vetter et al. 1998). There are, however, almost no data on the actual half-life of enzymes in marine environments. A recent controversy over a mathematical model for the diagenesis of organic carbon in marine sediments hinged in part on estimates of the decay rates of extracellular enzymes in sediments that varied by four-to-eight orders of magnitude (Boudreau et al. 2008; Rothman and Forney 2007). Some purified extracellular enzymes may remain active for weeks when maintained in sterile solution at 25 °C (Griffin et al. 1987), but experimental data from mesocosms and microbial cultures suggest that lifetimes of days or less may be more characteristic of marine environments (Murray et al. 2007).

Investigations of extracellular enzyme degradation in freshwater systems have identified several specific factors likely driving enzyme decay. Scully et al (2003) investigated interactions between light, enzymes, and dissolved organic matter in humic-rich lake water using commercially-obtained phosphatase and glucosidase enzymes. They observed half-lives of 4-8 hours, and argued that the principle decay pathway was a pH-dependent binding to iron. Tietjen and Wetzel (2003) and Boavida and Wetzel (1998) observed deactivation of enzymes on exposure to ultraviolet (UV) radiation, but Boavida and Wetzel also observed that inactive phosphatase bound to humic substances could be reactivated on exposure to mild UV radiation. Observations of the photochemical production of inorganic nitrogen in the ocean (Vähätalo and Zepp 2005) suggest that natural UV causes degradation of proteins, and thus might be a relevant mechanism for enzyme decay in seawater.

Few investigations focusing specifically on enzyme lifetimes have been carried out in seawater, however. The goal of this study therefore is to constrain the decay rates of extracellular enzymes, and to investigate the mechanisms likely responsible for enzyme degradation in marine rather than in freshwater systems. The specific focus is on decay rates and pathways in Arctic seawater. Cold-adapted microbes such as those in Arctic seawater express cold-active extracellular enzymes (Arnosti 2008; Huston et al. 2000) that differ systematically in structure from those expressed by mesophilic microbes (Feller and Gerday 2003). Mechanisms and pathways of enzyme decay may therefore differ in cold versus temperate waters. This distinction is also relevant for the deep ocean, which harbors an extremely large, globally important pool of reduced carbon (Hedges 1992).

In order to measure realistic rates of enzyme degradation, we chose to measure the activities of the enzymes naturally present in seawater, rather than adding enzymes from an external source. This approach has two advantages. First, enzyme decay rates measured using a single enzyme added to the water sample might be unrealistic because isofunctional enzymes, which are likely present simultaneously in a water sample, can have an extremely wide variety of structures (Arrieta and Herndl 2002). Domań-Pytka and Bardowski (2004) point out that the pullulanases, a relatively narrow class of polysaccharide hydrolases, vary in molecular weight by a factor of four, and these varied structures might decay at different rates or via different pathways. Second, decay rates using added enzymes are only realistic if either (a) the enzymes are added in concentrations similar to those naturally present, or (b) decay kinetics approximate a first-order rate law, such that enzyme half-life is independent of enzyme concentration. Some possible decay pathways, such as photochemical decay or thermal decay, are likely to approximate first-order kinetics. However, degradation by proteases is a potentially important decay pathway that would likely follow Michaelis-Menten kinetics rather than first-order kinetics, so using a higher-than-natural concentration of added enzymes would lead to misleading results if those concentrations reached saturating levels.

In order to prevent production of new enzymes that would mask degradation of old enzymes, we measured decay rates of in-situ, cell free enzymes in 0.2 μm-filtered seawater. We minimized the importance of any cells that might have passed through a 0.2 μm filter (Wang et al. 2007) by using short (<36 hour) incubation times. This approach therefore does not measure degradation of cell-surface associated enzymes, or measure effects related to sorption of enzymes on surfaces. A second experiment, comparing degradation rates of

added enzymes in whole versus filtered water was used to constrain the role of particles in enzyme degradation rates in seawater. The fraction of total enzyme activity classified as cell-free (i.e., passing a 0.2 μm filter) is quite variable; Wetzel (1991) reported it to be typically on the order of 10%, with some observations above 50%. Other studies have found substantially greater contributions of dissolved activities to total activities, sometimes approaching 100% (e.g. Hashimoto et al. 1985; Keith and Arnosti 2001; Murray et al. 2007), with the contribution of dissolved enzymes to total hydrolytic activity varying by enzyme, location, and time. Model results (Vetter et al. 1998) suggest that production of cell-free enzymes can in some situations yield more hydrolysate to the enzyme-producing organism than production of cell-associated enzymes. Cell-free enzymes can therefore be an important ecosystem component in their own right, as well as serving as a tractable model for enzyme degradation in this experiment.

2.2 Methods

SAMPLE COLLECTION

Water samples were collected in Kongsfjord, Svalbard (78.93º N, 11.95º E), between August 16 and 22, 2007. Surface water was collected by bucket from the end of a pier immediately prior to performing experiments. In situ temperature was between 4 and 7 °C at time of sampling. Samples were processed in an environmental chamber at 7 °C. In experiments with filtered seawater, seawater was filtered through 0.22 μm Durapore filters under a light vacuum (<50 mm Hg) to prevent the rupture of cells on the filter, which might introduce 'intracellular' enzymes into the samples.

ENZYME ACTIVITIES

Enzyme activities were measured using the small fluorogenic substrate proxies 4-methylubelliferyl-phosphate (MUF-PO_4; to measure phosphatase activity), 4-methylumbelliferyl-α-D-glucopyranoside and 4-methylumbelliferyl-β-D-glucopyranoside (MUF-α-glu and MUF-β-glu; to measure α- and β-glucosidase activity respectively), and L-leucine-7-amido-4-methylcoumarin hydrochloride (leu-MCA, to measure leucine aminopeptidase activity) following King (1986) and Somville and Billen (1983). All substrates were obtained from Sigma-Aldrich. Fluorescence was measured using a Turner Biosystems TBS-380 fluorimeter, with excitation/emission channels set to "UV" (365 nm excitation, 440-470 nm emission). Background fluorescence from CDOM did not vary over the course of the incubation and therefore did not interfere with activity measurements.

The rate of change of fluorescence was measured as follows: 30 µl fluorescent substrate stock solution was added to a disposable methacrylate cuvette followed by 2 ml sample water for a final concentration of 300 µmol l^{-1} (determined from saturation curves to be saturating for all three enzymes) and mixed vigorously with a micropipettor. Fluorescence was measured two to four times over the course of 20-30 minutes (experimental samples) or 1-2 hours (killed controls). Enzyme activity in each replicate was taken as the slope of the regression line of fluorescence vs. incubation time; there were two or three replicate cuvettes per water sample. Fluorescence measurements were calibrated using 4-methylumbelliferone and 4-methylcoumarin dissolved in seawater. Detector stability was monitored using a solid standard.

Abiotic hydrolysis of substrates was measured in whole and filtered water at 7 °C using autoclaved seawater and substrate concentrations of 300 μM.

DECAY OF COMMERCIAL ENZYMES IN ARTIFICIAL SEAWATER

Commercially obtained phosphatase (Sigma P4252, from *Escherichia coli*), β-glucosidase (Sigma 49290, from almonds), and leucine aminopeptidase (Sigma L5006 from porcine kidney) were added to 3 replicates of 100 ml autoclaved artificial seawater (Sigma sea salts, 35.0 g l^{-1}). Initial activities were 28.9 nmol l^{-1} min-1 (phosphatase), 866 nmol l^{-1} min-1 (β-glucosidase) and 71.2 μmol l^{-1} min (leucine aminopeptidase). Enzyme activity was measured periodically as described above.

ARTIFICIAL UV EXPERIMENT

To assess the susceptibility of natural dissolved enzymes to photochemical degradation, 100 ml 0.22 μm-filtered seawater was dispensed into each of six sterile Pyrex beakers, with polyethylene stretched across the beaker mouth. Three beakers were covered in aluminum foil and placed in a 7 °C environmental chamber under fluorescence UV-emitting lamps (Q-Panel UVA-340, 40 Watt, Cleveland, OH). Approximately 15 ml was removed via sterile syringe from each beaker at each timepoint, placed in a clean 20 ml glass scintillation vial, and warmed to 20 °C as described below. Enzyme activities were then measured as described above.

NATURAL LIGHT CONDITIONS EXPERIMENT

To assess the effect of natural light on enzyme decay, nine sterilized polyethylene bags filled with 250 ml 0.2 μm-filtered seawater and placed in low-walled tubs. Three bags were left uncovered in the tubs (full spectrum treatment), three bags were covered in Lexan (visible light only treatment) and three were covered in aluminum foil (dark treatment). Polyethylene absorbs light weakly from 240-280 nm, and is transparent above 280 nm. Lexan is opaque below 400 nm and fully transparent above 440 nm. Temperature of the tubs was kept within a temperature range of 4-7 °C by draining 4 °C water from a cooler through the three tubs. The weather conditions were cloudy for the duration of this experiment; meteorological data was obtained from the Ny Ålesund atmospheric observatory maintained by the Alfred-Wegner-Institute. The bags were periodically sampled through an embedded sampling port by withdrawing ~10 ml from each into a sterile syringe and samples were transferred into a clean glass scintillation vial.

In order to increase the precision of measurement of enzyme activities, enzyme activities for this experiment were measured at 20 °C. To ensure standardized sample temperatures, the scintillation vials were floated for ten minutes in a cooler filled with 20 °C water supplied by a controlled-temperature water system. The samples were placed in a floating rack such that most of the vial was immersed, but the cap was kept dry to prevent contamination of the sample with the temperature-controlled water. Monitoring of the temperature in a "dummy" vial showed that the samples warmed to 20 °C in about seven minutes. Activity measurements were made as described above.

WHOLE WATER VS. FILTERED WATER EXPERIMENT

To assess the importance of particles in degrading dissolved extracellular enzymes, degradation of commercially-obtained phosphatase (alkaline phosphatase from *Escherichia coli*, Sigma P5521) was assessed in whole versus filtered seawater. Six sterile 50-ml centrifuge tubes were each filled with 50 ml unfiltered, freshly-collected seawater, and three were filled with filtered seawater (from the same sample as the unfiltered water; gravity filtered using a 0.22 μm Durapore membrane). Phosphatase was added to a concentration of 0.0122 mg protein L^{-1}. This quantity of phosphatase was selected to yield ~20 times natural phosphatase activity. For this experiment, phosphatase activity was sufficiently high to allow measurement of enzyme activity at 7 °C.

To ensure that the addition of fresh, labile protein in the form of phosphatase did not stimulate growth and subsequent phosphatase production, 0.015 mg L^{-1} α-amylase was added to the last three unfiltered water samples. All nine samples were incubated in the dark at 7 °C; phosphatase activity was measured as described above over the course of approximately 5 hours.

2.3 Results

ENZYME ACTIVITIES AND DARK DEGRADATION

All three commercially-obtained enzymes decayed in artificial seawater in the dark over the course of 12-48 hour incubations. Phosphatase decay was well-fit by an exponential curve (r^2=0.82; no pattern to residuals), with a half-life of 6.8 hours (using raw data, $a_t = 20.14 \pm 1.18 \times e^{-0.1021 \pm 0.020 \times t}$; t is in hours; Fig. 1). Leucine aminopeptidase and β-glucosidase, by contrast, did not appear to decay according to first-order kinetics (aminopeptidase: a_t =

$3.47\pm0.38\times e^{-0.0249\pm0.01573\times t}$, $r^2=0.58$; β-glucosidase: $a_t=10.59\pm0.47\times e^{-0.005631\pm0.00183\times t}$, $r^2=0.56$). Activity of these enzymes decayed rapidly at first and ultimately more slowly than exponential decay.

Natural enzymes incubated at 7 °C in the dark decayed more slowly (Fig. 2). Phosphatase activity did not detectably change after 36 hours of incubation. Leucine aminopeptidase activity decreased to a minimum of 77% of initial activity (t=24 h) while β-glucosidase activity decreased to a minimum of 72% of the initial activity. Activity of leucine aminopeptidase was too variable to make any conclusions about the shape of the decay function, but the decay function of β-glucosidase was comparable to that of commercial β-glucosidase in artificial seawater: a rapid decrease in activity during the first four hours, followed by relatively constant activities for the next 32 hours. Hydrolysis of MUF-α-glu was not observed in any sample of either filtered or unfiltered water.

The activity of leucine aminopeptidase in 0.2 μm filtrate (measured at 7 °C) was 9.8±2.0% of total activity.

ENZYME PHOTODEGRADATION: POTENTIAL AND IN-SITU IMPORTANCE

Activity of phosphatase and leucine aminopeptidase decreased when exposed to UV light, whereas β-glucosidase, if anything, increased (Fig. 3). The results are expressed as the activity of the UV-exposed treatment at each timepoint relative to the activity of the dark treatment at the same timepoint, in order to highlight changes in activity associated specifically with light exposure.

Under natural light conditions, however, none of the enzymes decayed substantially either under full-spectrum light or visible-only light during a 36-hour incubation (Fig. 4).

Mean diffuse downwelling radiation was 59 W m^{-2}. Average UV intensity was 3.5 W m^{-2}. Conditions were cloudy during nearly the entire incubation, which is fairly typical in Svalbard during August. This incubation was performed towards the end of the polar summer, so solar radiation was considerably more intense during the day than at night.

The activity of commercial phosphatase in whole and filtered seawater decreased observably during an approximately 5-hour incubation (Fig. 5). Unlike the incubation in artificial seawater, decay of both of these enzymes appeared linear. The decay rate in unfiltered seawater was 1.9 times faster than in 0.2 μm-filtered water (for unfiltered water, decay rate = -11.4 nM hr^{-2}, r^2=0.98; for filtered water decay rate = -6.00 nM hr^{-2}, r^2=0.95).

In a control sample in which an equivalent concentration of α-amylase was added to autoclaved seawater to test whether the addition of labile protein (as enzyme) might cause microbial growth leading to spurious increases in phosphatase activity, no phosphatase activity was measured.

2.4 Discussion

DECAY RATES OF NATURAL AND COMMERCIAL ENZYMES

The decay rates of natural phosphatase, leucine-aminopeptidase, and β-glucosidase in filtered seawater in the dark were sufficiently slow as to be unmeasurable during the course of a 36-hour outdoor incubation (Fig. 2). Decay of commercially available enzymes in artificial seawater, in contrast, was considerably faster (Fig. 1). Two factors may affect these different behaviors: enzymes in natural seawater may be stabilized by interactions with naturally-occurring dissolved organic matter (DOM), and/or the dissolved enzymes in our

seawater sample may be structurally more stable than those that are commercially available, such that their activity in seawater is constant over the timescale measured. Interactions of protein (ribulose-1,5-bisphosphate carboxylase, RubisCO) with DOM have been shown to protect the protein to some extent from degradation by seawater heterotrophic microbes (Keil and Kirchman 1994). These possible interactions might also protect proteins such as the naturally-occurring enzymes in seawater from degradation.

We are aware of only one previous report of degradation rates of natural extracellular enzymes in seawater (Li et al. 1998), in which phophatase activity in Red Sea water was suggested to be stable for forty days. In that experiment, however, phosphatase activities were nearly constant for forty days, and then declined by about 60% during the subsequent ten days. These unusual decay kinetics imply that enzyme degradation was driven by biological rather than purely chemical processes, and indeed it is not surprising that heterotrophic microbes would be active in 0.2 μm-filtered seawater incubated for forty days (Li and Dickie 1985). The presence of an active microbial population suggests in turn that production of new phosphatase could mask decay of old phosphatases, so the slow degradation rates of phosphatases in Red Sea water suggested by Li and colleagues may not be reliable.

The dissolved enzymes occurring in seawater are very likely considerably different in structure from enzymes that are commercially available. Databases such as CAZY (Carbohydrate Active enZYmes; www.cazy.org) and BRENDA (www.brenda-enzymes.org) demonstrate the vast structural diversity of enzymes that hydrolyze the same polysaccharides and proteins. Since enzymes derived from marine organisms are vastly underrepresented among those sequenced to date, the structures of most of the hydrolytic enzymes at work in

marine systems are entirely unknown. Moreover, multiple isofunctional enzymes can exist within the same organism (Bauer et al. 2006) and as many as eight distinct β-glucosidases have been observed to be active in a single aquatic sample (Arrieta and Herndl 2002). It is therefore impossible to determine with certainty the extent and nature of the differences between the enzymes present in samples described here and other prokaryotic and eukaryotic enzymes whose structures have already been determined. The limited comparisons that can be made (in particular, comparisons between prokaryotic cold-active enzymes and mesophilic enzymes targeting the same substrates) demonstrate that the cold-adapted enzymes are more physically flexible than their mesophilic counterparts (D'Amico et al. 2002). The observed differences in enzyme stability between the naturally occurring and externally-added enzymes are thus likely also a function of intrinsic structural and resultant biochemical differences.

Scenarios under which release of free enzymes may be ecologically relevant have been discussed in several studies. Vetter et al (1998) use a model of enzyme production in aggregates to show that microbes might maximize the flux of hydrolysate they receive by producing cell-free enzymes, since enzymes that diffuse away from the cell and sorb to surfaces can experience locally high concentrations of substrate if those enzymes are sufficiently long-lived. This model, however, did not account for competition between different microbes. A competition-based model indicates that in a medium such as seawater in which diffusivity is high (relative to soil or sediments), 'cheaters' – microbes that take up hydrolysate without producing the corresponding extracellular enzymes will outcompete enzyme producers, because cheaters reap the benefits of enzyme production without paying the costs (Allison 2005). Diffusivity of the medium and enzyme lifetime are the critical

factors in determining the relative success of enzyme producers and cheaters in a system with cell-free extracellular enzymes; in marine aggregates diffusivity is ~90% that of pure water (Ploug and Passow 2007). The enzyme half-lives observed here would certainly be long enough for dissolved enzymes to diffuse far enough from their source (~1 mm) for their transport to be dominated by turbulent advection, which would mean that the enzyme would be effectively separated from its parent microbe.

The production of cell-free enzymes that are active much longer than they can be useful to organisms that produce them seems somewhat paradoxical. Dissolved enzymes may have multiple sources, however: enzymes may be freely released in response to a signaling cue (e.g. quorum sensing, Gram et al. 2002; Keller and Surette 2006), and thus they may still provide 'community benefit'. Moreover, dissolved enzymes may also result from cell lysis due to viral attack or sloppy feeding. If most cell-free enzymes originate from cell lysis due to viruses or grazing, the growth of microbes only using carbon released by cell-free extracellular enzymes observed by Vetter and Deming (1999) would by atypical in pelagic ecosystems. Observations of the selective nature of cell-free enzymes (e.g., high levels of dissolved laminarinase activities simultaneous with low levels of dissolved xylanase activities; Keith & Arnosti 2001; Murray et al. 2007), however, suggest that at least in some environments at specific times, release of free enzymes is highly selective, implying more control than would be likely if grazing or lysis were the principle routes of enzyme release,

Cell-free extracellular enzymes may also be produced primarily in areas of high substrate concentration (for instance particles or aggregates) or in response to large inputs of fresh organic matter where they can rapidly return a "profit", and their long active lifetime is of little immediate consequence to the parent cell. One possible consequence of the

apparently long half-lives of enzymes is that cell-free extracellular enzymes might be able to diffuse or be advected from areas of high microbial activity to adjacent areas of low microbial activity. Longer half-life is a potential mechanism for extracellular enzyme activity to become decoupled from microbial activity, especially which may be particularly relevant to sinking aggregates or marine snow. Porous sinking aggregates leave behind a plume of dissolved constituents from the aggregate (Kiørboe et al. 2001). Such aggregates typically have much higher microbial activity and extracellular enzyme activity than the surrounding seawater (Ploug et al. 2008; Smith et al. 1992; Ziervogel and Arnosti 2008). Since production of cell-free enzymes may be an optimal foraging strategy for heterotrophs living in aggregates (Vetter et al. 1998), aggregates likely act as a source of enzyme activity to the surrounding seawater. If these enzymes remain active on a timescale of days or weeks, aggregates could act as a source of extracellular enzyme activity to the deep ocean. Spatially patchy phytoplankton blooms and sea ice algal communities are further examples of areas of high microbial activity in close proximity to areas of low microbial activity, and thus might be loci for the diffusion of extracellular enzymes between environments.

UV EFFECTS ON ENZYME HALF-LIFE

UV exposure shortened enzyme lifetimes of phosphatase and leucine aminopeptidase, but not β-glucosidase. Photodegradation of enzymes could in principle take place via two mechanisms: direct photolysis or photosensitization of other photoactive compounds in seawater, which then in turn react with the dissolved enzymes. Amino acids absorb poorly in UV-A and UV-B (the regions of the UV spectrum for which there is substantial solar flux at the Earth's surface) with the exception of the aromatic amino acids tryptophan, tyrosine, and

phenylalanine. Any direct photochemical decay of enzymes must therefore begin by excitation of one of those three residues. It follows that enzymes which are richer in those residues would be more vulnerable to photochemical decay. Photosensitization reactions are likely to be more random, since decay would depend on the encounter of an enzyme with a photoactive compound. The different rates of degradation under UV observed here for the different enzymes may therefore be due to direct photochemical decay rather than to sensitization.

Under natural lighting conditions, however, neither UV nor visible light had any apparent effect on rates of enzyme decay. Conditions were cloudy during virtually the entire experiment, which is not unusual on Svalbard in August. These results are consistent with the conclusions of Scully et al (2003), who suggested that although UV radiation below 290 nm (as may be emitted from broad spectrum UV lamps such as the one used in this study) may be capable of degrading phosphatase (as observed by Boavida and Wetzel 1998), the quantum yield for phosphatase activation above ~300 nm is close to zero, so natural sunlight at the Earth's surface is unlikely to be sufficiently intense to directly degrade extracellular enzymes *in situ* at a fast enough rate to be environmentally relevant. Espeland and Wetzel (2001), however, observed UV-A (320-400 nm) and UV-B radiation (300-320 nm in their experimental setup) were each responsible for substantial decreases in activity of α-glucosidase, β-glucosidase, and phosphatase. Given that Espeland and colleagues did not expose their enzymes to light below ~300 nm and low quantum yield above 300 nm of enzymes observed by Scully et al, it would seem that the inactivation observed by Espeland et al must have been via photosensitization. In that case, enzyme susceptibility to UV radiation in natural environments would be as much a function of the concentration and

nature DOM and other dissolved constituents present in the medium as it is a function of the enzymes themselves.

DEGRADATION OF COMMERCIAL ENZYMES IN WHOLE SEAWATER VERSUS FILTERED SEAWATER

All of the experiments described above were performed in 0.2μm-filtered seawater. If factors associated with cell surfaces are important in degrading enzymes (for instance, via the action of proteases bound to cell surfaces), then enzyme degradation would be faster in whole seawater than in filtered seawater. To test the influence of cell surfaces in degradation rates, commercially-obtained phosphatase was added to whole seawater and to 0.2-μm-filtered seawater. Phosphatase activity decreased approximately twice as fast in whole seawater than in filtered seawater, indicating that in whole seawater about half of the decay was attributable to particles (including organisms). Interestingly, the decay of the commercial enzyme was measurable over a five-hour incubation, whereas naturally-present phosphatase activity decreased by much less over a much longer (36-hour) incubation, suggesting that the commercially-obtained phosphatase was less robust than natural phosphatase. Since DOM concentrations should have been identical in the unfiltered and in the filtered seawater, differential protection from degradation via interaction with DOM is unlikely. We hypothesize that the faster decay of the commercially-available phosphatase relative to the naturally-present phosphatase is due to intrinsic differences in enzyme stability.

We note, finally, that decay of commercial phosphatase in both whole and 0.2 μm-filtered water was apparently linear with time. This is consistent with a zero-order rate law, which could arise from decay primarily due to saturated proteases. Other likely degradation

pathways, such as photochemical or thermal degradation, would approximate first-order kinetics. The quantity of phosphatase added, 12.2 μg protein L^{-1}, corresponds to about 0.08 μmol peptide bonds L^{-1}, two-to-three orders of magnitude smaller than the measured saturation constant for leu-MCA hydrolysis of 23 ± 17 μM leu-MCA [data not shown; error is the 95% confidence interval of the nonlinear regression fit]). This suggests that the added phosphatase may have been primarily hydrolyzed by proteases which do not hydrolyze leu-MCA (not all proteases hydrolyze leu-MCA; Konopka and Zakharova 2002) and which have a lower saturation constant, or that phosphatase was degraded primarily by some other, unidentified zero-order process.

Previous investigations have used commercially available enzymes to explore the dynamics of natural enzymes in aqueous systems (Boavida and Wetzel 1998; Scully et al. 2003). The data presented here indicate that extracellular enzymes produced naturally in seawater may feature structural differences that make them more robust than enzymes found in other environments or inside the protoplasm. As pointed out by Vetter et al (1998), the robustness of an enzyme is in principle subject to selective pressure. The long half-lives of cell-free enzymes in seawater, however, would seem to increase the advantage of 'cheaters' over enzyme-producing heterotrophs, since a large fraction of an enzyme's active lifetime might occur while the enzyme is too far away from the parent cell to return an appreciable flux of hydrolysate. Nevertheless, two scenarios would seem to resolve the apparent contradiction that cell-free extracellular enzyme activity is often observed in the field. First, it may be that most production of cell-free enzymes is "unintentional"; the result of cell lysis or excretion of enzymes from animal guts. Second, the fact that some marine heterotrophs can grow rapidly solely using cell-free extracellular enzymes indicates that cell-free enzyme

production might occur primarily in areas or times when fresh, high-molecular weight organic matter is plentiful, and organisms compete most effectively by maximizing growth rate rather than efficiency of resource utilization. One possible strategy might be for microbes to produce cell-free extracellular enzymes while sensing gradients of hydrolysate, and then use chemotaxis to move towards areas that are rich in enzyme-labile organic matter. Sinking particles and aggregates, in which labile organic carbon is abundant and microbial activity is rapid (Ploug et al. 2008; Skoog et al. 2008) probably represent environments in which this strategy might be appropriate, and so could represent a source to the deep ocean of extracellular enzyme activity decoupled from microbial activity.

2.5 Figures

FIGURE 2-1: ACTIVITY OF COMMERCIALLY OBTAINED ENZYMES DISSOLVED IN STERILE ARTIFICIAL SEAWATER (35‰); INCUBATED IN THE DARK AT 7 °C

Activities were measured at 7 °C. Activity of each enzyme is expressed relative to the initial activity of that enzyme. Error bars are +/- one standard deviation of triplicate measurements.

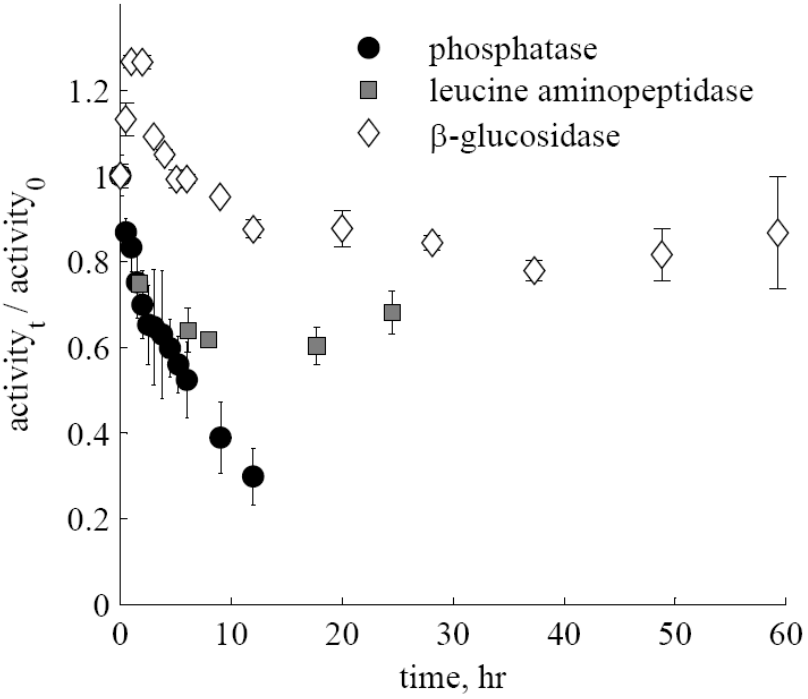


FIGURE 2-2: ACTIVITY OF NATURAL CELL-FREE ENZYMES IN 0.2 µM-FILTERED SEAWATER, INCUBATED IN THE DARK AT 7 °C

Activities were measured at 20 °C. Activity of each enzyme is expressed relative to the initial activity of that enzyme. For purposes of clarity, leucine aminopeptidase data points are offset to the right by 0.25 h, and β-glucosidase activities are offset by 0.5 h, from their correct values. Error bars are +/- one standard deviation of triplicate measurements for phosphatase and β-glucosidase, leucine aminopeptidase activities were not measured in replicates.

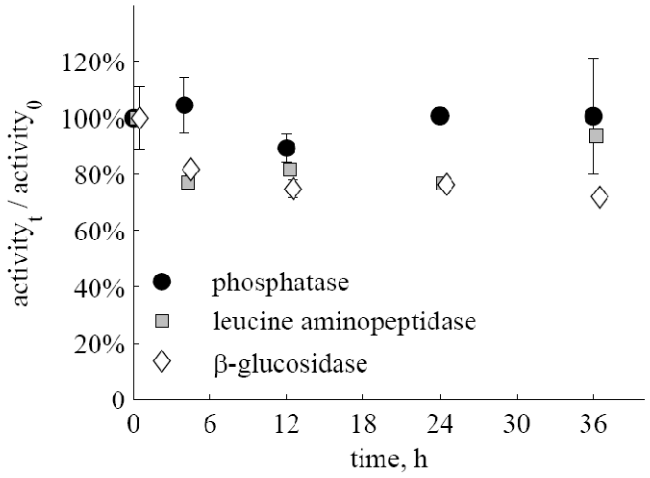


FIGURE 2-3: ACTIVITY OF NATURAL CELL-FREE ENZYMES IN 0.2 µM-FILTERED SEAWATER EXPOSED TO AN ARTIFICIAL UV SOURCE AND MAINTAINED AT 7 °C

Activities were measured at 7 °C. Activity of each enzyme is represented relative to the activity of the same enzyme maintained in the dark at the corresponding timepoint. Error bars are +/- one standard deviation of triplicate measurements for phosphatase and β-glucosidase, leucine aminopeptidase activities were not measured in replicates.

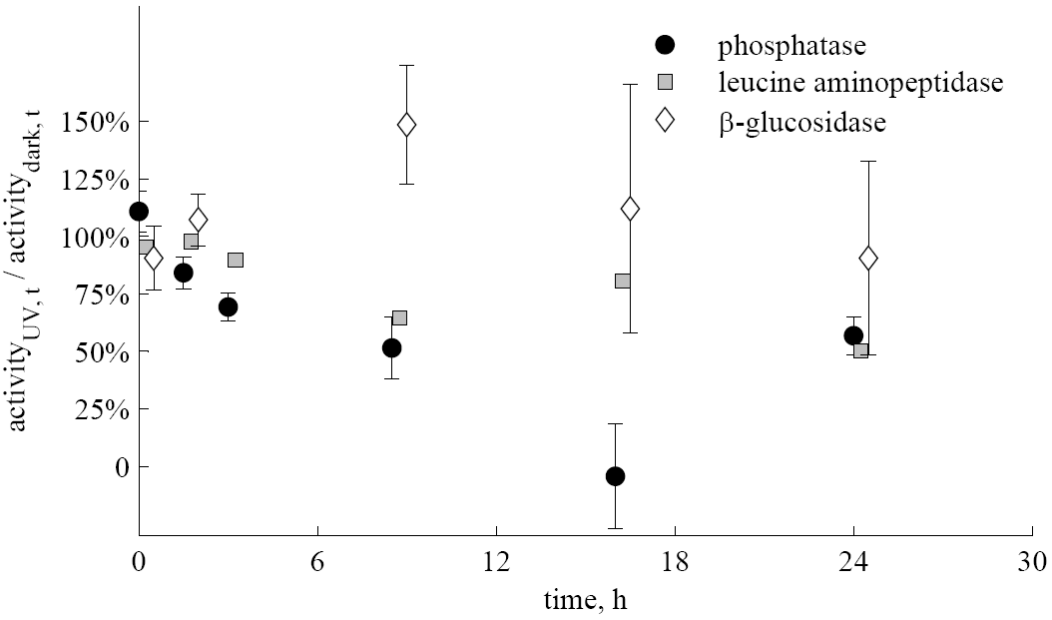


FIGURE 2-4: ACTIVITY OF NATURAL CELL-FREE PHOSPHATASE (A), LEUCINE-AMINOPEPTIDASE (B) AND β-GLUCOSIDASE (C)

Activities measured in 0.2 μm-filtered seawater incubated at 4-7 °C in the dark (filled circles), under visible-light only (Lexan shield; gray squares) or in full spectrum natural sunlight (open diamonds). Activities were measured at 20 °C. Error bars are +/- one standard deviation of triplicate measurements for phosphatase and β-glucosidase, leucine aminopeptidase activities were not measured in replicates.

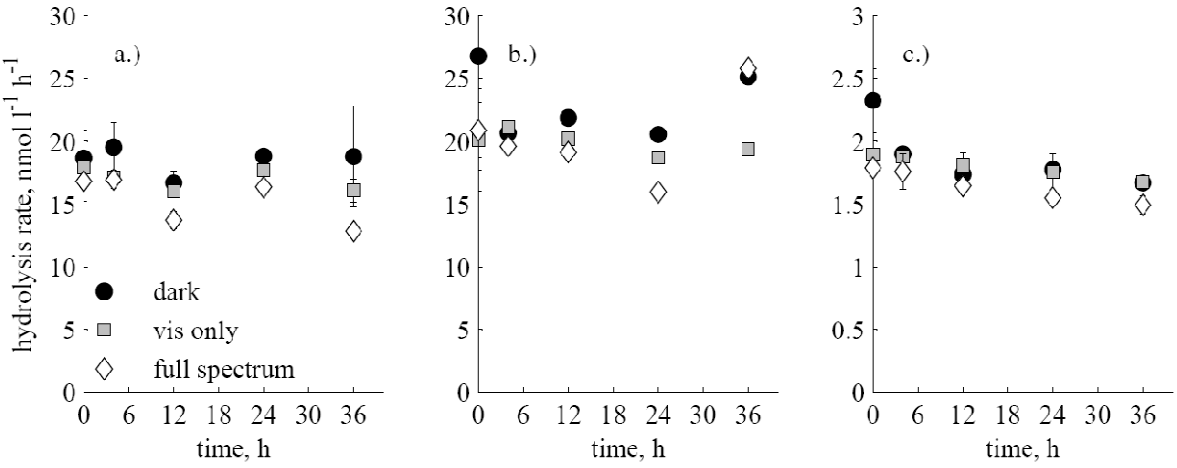
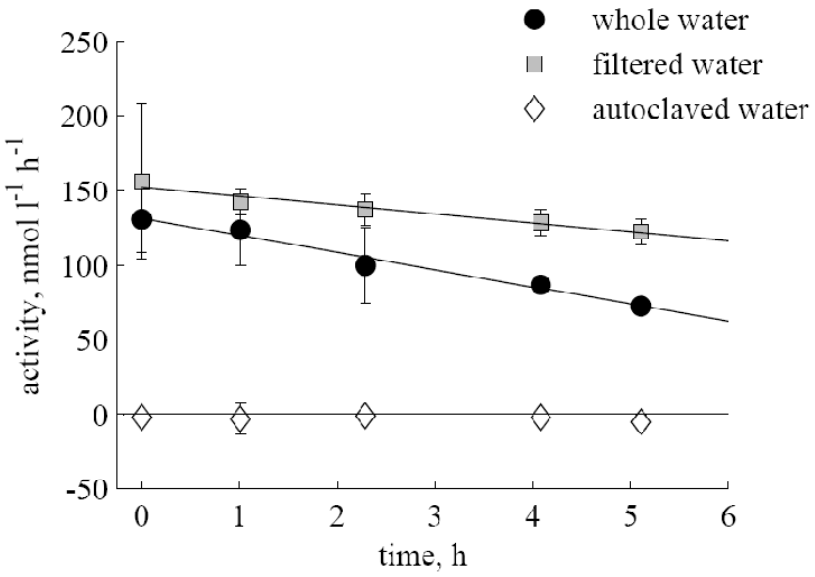


FIGURE 2-5: ACTIVITY OF COMMERCIAL PHOSPHATASE IN WHOLE SEAWATER

Phosphatase activity in whole seawater amended with commercially-obtained phosphatase (filled circles), 0.2 μm-filtered seawater amended with commercially-obtained phosphatase (gray squares), or whole seawater amended with commercially obtained α-glucosidase. Error bars are +/- one standard deviation of triplicate measurements.



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Chapter 3 Gradients in pelagic microbial community function over short spatial scales in the Gulf of Mexico

Andrew D. Steen, Kai Ziervogel, Carol Arnosti

The lability of dissolved organic carbon seems to be determined by interactions between molecular structure and the composition and metabolic capabilities of the microbial community. Changes in metabolic capabilities over horizontal or vertical gradients therefore may influence the lability of specific dissolved organic molecules. We measured hydrolysis rates of specific polysaccharides in three depth profiles along a continental shelf-to-slope transect in the northern Gulf of Mexico in order to determine the extent to which microbial community function varies over short spatial scales in coastal seawater. The community's ability to hydrolyze specific polysaccharides varied with depth and among stations. This variability was not predictable from measurements of bacterial productivity or glucose incorporation rate constants. These measurements suggest that, when high molecular weight dissolved organic carbon persists over seasonal timescales in seawater, the cause may not be intrinsic structural factors but rather the failure of the microbial community to produce the necessary extracellular hydrolases.

3.1 Introduction

Dissolved organic carbon (DOC) in the ocean is typically divided into three categories based on the decay rates: labile DOC, which has half-lives on the order of days or less, recalcitrant DOC, which appears to persist for thousands of years, and semi-labile DOC, which persists on a seasonal timescale (Kirchman et al. 1993). Fluxes of semi-labile DOC from surface water can be substantial: at the Bermuda Atlantic Time Series, the flux of semi-labile DOC from the euphotic zone to the mesopelagic (100-250 m water depth) exceeded the flux of particulate organic carbon (Carlson et al. 1994).

The nature of semi-labile DOC, however, is poorly constrained. Based on the concentration and composition of DOC in depth profiles (Aluwihare et al. 2002) and following phytoplankton blooms (Kirchman et al. 2001) semi-labile DOC is enriched in HMW carbohydrates, likely polysaccharides. Ratios of neutral sugar content in dissolved polysaccharides change during diagenesis: mole fraction of mannose+xylose decrease, while mol fraction of glucose increases, indicating that some polysaccharide structures are considerably more labile than others (Goldberg et al. 2009; Kirchman et al. 2001; Skoog and Benner 1997). Differences in reactivity can be dramatic even among polysaccharides with very similar structures: pullulan and laminarin are both linear glucose polymers, but laminarin is typically readily available to pelagic communities whereas pullulan is often completely inaccessible (Arnosti 2000; Arnosti et al. 2005).

The metabolic capabilities of marine microbial communities are also variable, such that the same DOC might be labile in some environments and semi-labile in others. Microbial communities from below the thermocline at the Bermuda Atlantic Time Series (BATS) were more capable of remineralizing surface water DOC than communities actually

present in the surface water from which the DOC originated (Carlson et al. 2004; Goldberg et al. 2009). Microbial communities differ systematically among surface and mesopelagic waters at BATS (Morris et al. 2005) and elsewhere (Fuhrman et al. 2006; Fuhrman and Steele 2008), suggesting that functional differences among those two communities led to the greater ability of the mesopelagic community to consume surface water DOC. Some of these differences can be traced to individual microbial species or clades. Cytophaga-like bacteria, for instance, seem to specialize in consuming high-molecular weight OM, whereas SAR-11 seems to specialize in low-molecular weight compounds (Kirchman 2002; Malmstrom et al. 2005). The ability to take up different classes of dissolved organic matter (including polysaccharides, glucose, and protein) varied among classes of estuarine and marine bacteria (Elifantz et al. 2008; Elifantz et al. 2005).

The purpose of this study was to investigate differences in microbial community function with respect to polysaccharide hydrolysis, glucose assimilation, and bacterial production, over short spatial scales in the Gulf of Mexico. Previous investigations in the northern Gulf of Mexico have shown that enzymatic hydrolysis rates are rapid in surface waters (Arnosti et al. 2005), and that there are differences in enzyme activities with depth (Arnosti et al. in prep). The extent to which measures of hydrolytic activity are related to other aspects of microbial activity is unknown, however, as are the spatial scales upon which enzyme activities vary. To examine in concert microbial activity and carbohydrate dynamics in the water column, we measured hydrolysis rates of six polysaccharides as well as rates of glucose incorporation, glucose respiration, bacterial production and carbohydrate concentrations at three stations (and two, four, and five depths, respectively) in the coastal Gulf of Mexico. The water masses sampled crossed a gradient from water on the continental

shelf heavily influenced by the Mississippi River plume to a more typically marine station on the continental slope, providing the opportunity to investigate the extent to which microbial activities and carbohydrate dynamics vary across this changing gradient.

3.2 Methods

STUDY SITES AND SAMPLING

Water samples were collected via Niskin bottle mounted on the conductivity-temperature-depth (CTD) rosette from the R/V *Cape Hatteras* at three sites in the northern Gulf of Mexico on 27 and 28 September 2007. Station 1 (28° 51.131' N, 89° 29.810' W; depth 41 m; Fig. 1) was most influenced by the Mississippi River plume (particularly in the surface water sample, which was considerably fresher than the deep sample), Station 2 (28° 32.041' N, 89° 21.503' W; depth 285 m), and Station 3 28° (16.369' N, 89° 21.772' W, depth 925 m). At stations 2 and 3, a pycnocline separated the two shallowest ('epipelagic') and the deeper ('mesopelagic') samples. Concurrent with sample collection, vertical profiles of temperature and salinity were obtained. All samples were processed immediately after collection.

POLYSACCHARIDE HYDROLYSIS RATES

Hydrolysis rates of fluorescently-labeled arabinogalactan, chondroitin sulfate, fucoidan, laminarin, pullulan, and xylan were determined according to the method of Arnosti (1996; 2003). Briefly, polysaccharides were obtained from the sources as listed in Steen et al (2008). These were fluorescently labeled according to the method of Glabe (1983) as

modified by Arnosti (1996; 2003). To measure fl-polysaccharide hydrolysis rates, fl-polysaccharides were added to 50 mL seawater to a concentration of 3.5 μmol monomer L^{-1} (2.8 μmol L^{-1} for xylan), which was then divided into three, 16.7 mL replicate samples stored in 20 ml combusted glass scintillation vials with plastic caps. Additionally, sample water was autoclaved to serve as a killed control, and prepared in the same way except that only a single incubation was used. One, approximately 2 ml subsample was immediately withdrawn from each subsample, filtered using 0.2 μm surfactant free cellulose acetate (SFCA) syringe filters into combusted glass vials, and frozen. Incubation vials were stored in the dark at temperatures listed in Table 1. Subsamples were withdrawn in the same way after 2, 4, 6, 11, 14, and 26 days. Fl-polysaccharide size distributions were determined using gel permeation chromatography, and size distributions were used to determine hydrolysis rates, as per Arnosti (1996; 2003), with the modification that the reference sample for each timepoint was the killed control sample for the corresponding timepoint, rather than the live time zero sample, so that the calculated hydrolysis rates do not include any possible abiotic changes.

Measured hydrolysis rates are negatively influenced by competitive inhibition from compounds present in the DOC pool. However, these interferences are probably quite small, because the quantity of polysaccharide added (3.5 μmol monomer L^{-1}, or 17.5 – 24.5 μmol C L^{-1}) are comparable to or larger than typical concentrations of total dissolved carbohydrates (10-25 μM C), HMW DOC (~35-70 μM C), and total hydrolysable neutral sugars (2-6% of DOC) in surface seawater (Benner 2002 and references therein). Concentrations of any individual polysaccharide that might competitively inhibit hydrolysis added fl-

polysaccharides are therefore likely to be low relative to the concentrations of fl-polysaccharides.

HYDROLYSIS RATE DATA ANALYSIS

In order to visualize the differences in hydrolysis rate patterns among samples, a cluster analysis approach was employed. The maximum hydrolysis rate of each substrate was expressed as a fraction of the sum of maximum rates in that sample. Fractional hydrolysis rates in each sample were expressed as a vector, with each row corresponding to a different polysaccharide. These vectors were used to calculate Euclidean distances between each sample using the pdist function in MATLAB R2008b. A dendrogram expressing those distances was then generated using the linkage and dendrogram functions. This dendrogram therefore expresses the similarity in patterns of hydrolysis rates among sites, without being influenced by the absolute magnitudes of hydrolysis rates.

CARBOHYDRATE CONCENTRATIONS

Samples for carbohydrate concentration measurements were collected in glass jars rinsed first in 10% HCl and then triple-rinsed with sample. Seawater samples were then filtered using 10% HCl-rinsed, 0.1 μm pore size Durapore membrane filters (Millipore VVLP). Filtrate was collected in a combusted, acid-rinsed flask which was rinsed three times with filtrate prior to storing samples. Samples were stored in VWR TraceClean vials with fluoropolymer-lined caps and frozen until analysis.

Carbohydrate concentrations were determined using the TPTZ method (Myklestad et al. 1997), with the modification that the sample and reagent volumes were all halved.

Glucose dissolved in artificial seawater (Sigma Sea Salts, 35.0 g L^{-1}, with 0.2% [w/v] NaN_3) served as a concentration standard. Seawater samples were measured in duplicate for monosaccharides, as described above. Triplicate samples were analyzed for total carbohydrates after hydrolysis following Burdige (2000). Briefly, 3 mL sample was mixed with 333 μL H_2SO_4, sealed in glass ampoules, and heated 100 °C for 3 hours in a heater block. Samples were then neutralized with 12.1 M NaOH and analyzed as described above. Glucose standards for the total carbohydrates measurements were also subjected to the hydrolysis procedure. The slope of the calibration curve of glucose subjected to the hydrolysis procedure, corrected for dilution by the reagents, was 81% of that of unhydrolyzed glucose. Hydrolysis efficiency, assessed using laminarin and pullulan standards (at 10 μM monomer-equivalent concentration), was 76±1% for laminarin and 73±2% for pullulan.

We note here that some other workers have reported heating glass ampoules in an oven (Grzybowski and Dudzińska 2004; Hung and Santschi 2001), a procedure that we have found to be unsafe: if ampoules are packed close to each other in an oven, the explosive failure of a single ampoule due to increased internal pressure can set off a chain reaction, resulting in a powerful explosion. The use of a heating block isolates ampoules from each other and therefore is considerably less hazardous.

MICROBIAL CELL COUNTS

Ten ml of seawater samples from each depth were fixed with 0.2-μm filtered formaldehyde (2% v/v final concentration) and stored at 4º C until further treatment. One slide per depth was prepared by staining two to five ml of samples with 4', 6-diamidino-2-

phenylindole (DAPI, 0.1 mg l^{-1} final concentration) using the method of Porter and Feig (1980). Cells were microscopically examined using an epifluorescence microscope (Olympus, magnification x1000) equipped with a digital camera (Olympus TH4-100); 20 pictures or at least 1000 cells were counted per sample.

RADIOTRACER DATA

Leucine incorporation rates were determined using ^{3}H-labeled leucine (Sigma L-5897, 92.6 Ci $mmol^{-1}$, diluted by a factor of 5 with unlabeled L-leucine) according to Kirchman (1993) with the exception that precipitated protein was collected on 0.2 μM cellulose acetate filters. Final leucine concentration was 20 nmol L^{-1} in a 10 ml sample. Incubations were carried out at the same temperature as the fl-polysaccharide incubations. Incubation times were approximately 3 hours (approximately 6 hours for the 125 m, 250 m, 700 m, and 900 m depths at station 3); precise incubation times were used to calculate rates.

Glucose incorporation and respiration was measured using methods modified from Rich et al (1996) and Skoog et al (1999) as well as techniques and equipment developed by D. Albert (pers. comm.). Prior to sample collection, 5.0 pmol ^{14}C-labeled glucose (Sigma G-5020, 262mCi mmol-1) was added to 20 mL scintillation vials with plastic caps (for $PO^{14}C$ measurement) or PTFE septum caps (for $DI^{14}C$ measurement). Vials serving as killed controls were also amended with 526 μl 100% trichloroacetic acid (TCA) for a final concentration including sample) of 5% (v/v) TCA. Within ten minutes of sample collection, 10 mL water sample were dispensed into each of six scintillation vials to serve as live incubations, and two vials containing TCA to serve as killed controls. Samples were mixed

vigorously and incubated for approximately one hour at the same temperature as the fl-polysaccharide incubations; precise incubation times were used to calculate rates.

Samples for $DI^{14}C$ measurement were killed with the addition of 200 µL, 1 M NaOH through the septum of the incubation vial, enough to cause precipitation of $CaCO_3$. $DI^{14}C$ was trapped in modified Woeller's solution, which serves both to trap CO_2 and as a scintillation cocktail (Woeller 1961, modified by D. Albert), consisting of 50% ScintiVerse II, (Fisher Scientific), 25% β-phenethylamine, and 25% methanol. Immediately prior to trapping DIC, the sample was connected to a sparging apparatus (Fig. 2) consisting of the sample vial, a liquids trap consisting of a septum-capped 20 ml scintillation vial containing 10 ml 1 M HCl, and a 20 ml glass scintillation vial containing 15 ml modified Woeller's solution connected in series via syringe needles, Luer-lok fittings, and plastic tubing. The input to these systems was an N_2 bottle connected to a 4-way gas line splitter, so that 4 samples could be processed at once. The target gas flow rate was 10 mL min^{-1}, slow enough that gas passed through the liquid phase as a stream of discrete bubbles rather than a continuous flow, although actual flow rates were variable. Samples were sparged for 30 min. $^{14}CO_2$ yield was assessed by injecting radiolabeled bicarbonate into seawater and then processing in the same way that the samples were processed; recovery of $DI^{14}C$ was 105±4% compared to radiolabeled bicarbonate injected directly into modified Woeller's solution.

Samples for $PO^{14}C$ measurement were killed with 810 µL, 37% formaldehyde and filtered onto 0.2 µm white nitrocellulose filters (25 mm diameter, Millipore). After rinsing the membrane was placed in a second scintillation vial, dissolved in 0.5 ml ethyl acetate. 15 ml modified Woeller's solution was then added and samples were radioassayed in a Perkin-Elmer scintillation counter.

Glucose turnover rate constants were calculated as the sum of the rate constants of glucose assimilation and remineralization expressed as a fraction of the glucose initially added. Glucose growth efficiency (GUE) was calculated as the rate of glucose assimilation divided by the sum of glucose assimilation and respiration (Kirchman et al. 2001). GUE is analogous to bacterial growth efficiency (BGE) in the sense that both measure the amount of substrate incorporated into biomass as a fraction of the total amount of substrate incorporated. However, I do not use GUE as a substitute for BGE: the two measures usually differ in magnitude by a large amount (Del Giorgio and Cole 1998). In the past GUE has been measured as a (very poor) proxy for BGE; my purpose here is to examine specifically the metabolism of carbohydrates and to eliminate the effect that differing DOM composition may have on assimilation efficiency.

3.3 Results

PATTERNS OF MAXIMUM HYDROLYSIS RATES

Polysaccharide hydrolysis rates were calculated for each timepoint of each incubation. The maximum hydrolysis rate among all timepoints in a given water sample is taken as a measure of the maximum possible rate the microbial community can access a specific polysaccharide. The incubation times are sufficiently long so that both enzyme production and microbial growth are possible reactions to substrate addition.

Maximum hydrolysis rates of each polysaccharide were in almost all cases greater in all of the epipelagic samples than in any mesopelagic sample (Fig. 2 a, b, c.). (The exceptions are fucoidan and pullulan, which were hydrolyzed slightly faster at Station 2, 150

m depth than in any other sample; variation among replicates in each of those cases were also large). Accordingly, the summed maximum hydrolysis rates decreased considerably in the mesopelagic relative to the epipelagic. Surface water maximum hydrolysis rates were slightly greater at Stations 1 and 2 than Station 3.

Patterns of hydrolysis of each substrate relative to the summed rates in each sample were variable (Fig. 2 d, e, f). Laminarin hydrolysis was quite constant relative to the summed rates, averaging 25%±6.7% of the total. Other polysaccharides accounted for a much more variable fraction of summed hydrolysis: chondroitin sulfate, for instance, accounted for 50±8% of the total rate at station 3 (700 m), but only 24±13% at station 3 (250 m) and 17±0.2% at station 2 (150 m). Similarly, fucoidan hydrolysis was 22±10% of the total at station 3 (125 m) but decreased to 9±6% at 250 m depth and was undetectable at 700 m

CLUSTER ANALYSIS OF HYDROLYSIS RATES

Cluster analysis reveals that all of the mesopelagic samples clustered together, indicating that patterns of hydrolysis rates among mesopelagic samples were more similar to each other than they were to epipelagic samples (Fig 4). Epipelagic samples also clustered together, with the exception of Station 3 surface, which fell as an outgroup, probably due to the uniquely high rate of arabinogalactan hydrolysis in that sample. There was much more diversity in hydrolysis rate patterns among epipelagic samples than among mesopelagic samples, indicating that microbial community function with respect to polysaccharide hydrolysis was much more variable in surface waters than in deeper water.

TIMECOURSES OF HYDROLYSIS RATES

The timecourse of hydrolysis rates indicate how the microbial community present at the time of sampling reacted to the input of fresh polysaccharide; these timecourses varied considerably among polysaccharides. Broadly, patterns of hydrolysis rate as a function of incubation time fell into three qualitative categories: substrates for which the hydrolysis rate was maximum at the first timepoint, substrates which reached a maximum rate after a lag time, and substrates for which there was not a strong relationship between incubation time and hydrolysis rate or for which no hydrolysis was observed (Fig. 3, Table 2). In some stations laminarin, chondroitin, and xylan were hydrolyzed most rapidly before the first timepoint. Most substrates fit the pattern of increasing rate over a period of between 2 and 11 days. This period of increasing hydrolysis rates was typically shorter (but not always) in shallower waters with higher maximum rates. Arabinogalactan was unusual in that there was not a strong relationship between hydrolysis rate and time in most samples, even in cases in which most of the substrate was hydrolyzed by the end of the incubation.

CARBOHYDRATE CONCENTRATIONS AND CELL COUNTS

Total carbohydrate concentrations ranged from 15.1±0.5 to 1.8±0.5 μmol L^{-1} glucose-equivalent, with greater values in epipelagic samples and in stations closer to the Mississippi River plume (Table 3). Monosaccharide concentrations ranged from 6.65±0.5 μmol L^{-1} to below the limit of quantification (~0.7 μmol L^{-1}), and in many cases were a large fraction of the total carbohydrate concentrations. We interpret the monosaccharide data generated using the TPTZ measurement with caution, however. Concentrations of monosaccharides (properly, reducing ends of sugars, often interpreted as concentrations of reducing sugars) of

greater than 1 μM in seawater have commonly been reported using the TPTZ method. This is at odds with results using HPLC methods, which typically detect glucose as the most abundant monosaccharide, with concentrations typically <20 nmol L^{-1} (Kirchman et al. 2001; Rich et al. 1996; Rich et al. 1997; Skoog and Benner 1997). The TPTZ method relies on the reduction of Fe(III) to Fe(II) by reducing sugars, which introduces two potential sources of systematic error. First, this method would in principle be sensitive to the reducing ends of oligosaccharides. Second, other substances in seawater may be capable of reducing Fe(III) under the reaction conditions used in this reaction. Humic substances isolated from the Gulf of Gdańsk have been shown to produce a positive response using the TPTZ method at a ratio of 0.61-0.67 μmol glucose mg^{-1} humic substance (Grzybowski and Dudzińska 2004), and it is possible that other constituents of marine DOM, like carboxyl-rich alicyclic material could also be capable of reducing Fe(III) under the reaction conditions (Hertkorn et al. 2006).

GLUCOSE UPTAKE: ASSIMILATION, REMINERALIZATION AND GROWTH EFFICIENCY

The rate constants for both assimilation of glucose into biomass and respiration of glucose decreased considerably with depth and distance to shore (Table 3). Daily turnover of glucose was extremely high in the surface waters of Stations 1 and 2, ranging from 1.45±0.10 to 2.5±0.2 day^{-1}. Turnover was much lower at Station 3 (although turnover in surface water at Station 3 was still relatively high, 0.83±0.05 day^{-1}). Turnover decreased by an order of magnitude or more in the mesopelagic relative to the epipelagic.

GUE values varied in a much narrower envelope than glucose uptake rate constants. GUE decreased considerably in the mesopelagic at Station 3 relative to surface values at all

three stations (33±3%-39±4%), but the two mesopelagic values at Station 2 (52±6% and 42±5%) were comparable to the range of surface values at all three stations (38±3%-54±5%).

3.4 Discussion

PATTERNS OF ENZYME ACTIVITY, BACTERIAL PRODUCTION AND GLUCOSE INCORPORATION

The differences in maximum hydrolysis rates shown in Fig 1 (a, b, c) demonstrate that the water samples we obtained contained microbial communities with different capacities to access polysaccharides. Previous work comparing sites along estuarine gradients (Keith and Arnosti 2001; Steen et al. 2008) and among globally-distributed sites in the surface ocean (Arnosti et al. 2005) have shown different patterns of polysaccharide hydrolysis rates among different sites; the data presented here show that marine microbial communities separated by short (tens of km) horizontal distances, and tens to hundreds of meters in depth, also vary in their capacity to access specific fractions of HMW DOC. The fact that these differences cannot be accounted for by simple factors such as kinetic effects due to incubation at different temperatures is most easily seen from variations in the contributions of each polysaccharide to the summed hydrolysis rates (Fig 2 d-f). At Station 3, the chondroitin sulfate hydrolysis rate was nearly equal in all mesopelagic samples, despite the fact that incubation temperatures in those samples varied from 6-16 °C. In contrast to the insensitivity of chondroitin hydrolysis to temperature and depth at Station 3, the deepest samples at Station 3 completely lacked the ability to hydrolyze both pullulan and arabinogalactan. Again, this does not appear to be due purely to scaling factors: at Station 2,

arabinogalactan hydrolysis decreased relatively modestly with depth so that it represented a greater fraction of summed hydrolysis rates in deeper samples than in shallower samples.

These functional differences did not necessarily correspond to gross measurements of heterotrophic activity based on radiolabeled glucose and leucine incorporation. The variation in radiotracer rates was considerably greater than variation in summed maximum hydrolysis rates (Table 2, Fig. 2). Station 1 illustrates these disparities: bacterial production was 78% lower, and glucose incorporation rate constants were 21% lower in the bottom water sample than in the surface water sample. By contrast summed hydrolysis rates were slightly higher in the bottom water sample, suggesting an inactive community that was able to become active once fresh polysaccharides were added. This inactive community was apparently quite large, since cell counts were 5 times higher in bottom water than in surface water.

In general, lower GUE corresponded to lower bacterial productivity and lower glucose assimilation rate constants. GUE values here were generally lower than typical values observed elsewhere (see Chapter 3 for a more detailed review of GUE values in seawater). Lower GUEs imply preferential use of glucose for catabolic rather than anabolic pathways, which may be due to higher maintenance energy requirements brought on by environmental conditions that prevent growth (Del Giorgio and Cole 1998). This is somewhat surprising in that the Mississippi River plume often contains high nutrient concentrations, and the bacterial productivity values are within normal ranges for coastal environments (Ducklow 2000).

Plots of hydrolysis rates as a function of time illustrate the dynamic response of microbial communities to the input of fl-polysaccharide. For many substrates in many samples, the hydrolysis rate was essentially zero after the first (two day) timepoint, suggesting that at the start of the incubation, enzyme activity for those substrates was near zero, and that it took several days for the community to begin producing those enzymes in measurable quantities. In some cases, for instance with chondroitin in the epipelagic samples, the community was able to hydrolyze the substrate quite rapidly after the lag period, so that that the presence of the lag period did not imply that the community was slow to hydrolyze the substrate in the long run.

Broadly speaking, a rapid increase in enzyme activity could be explained in two ways: either by induction of an enzyme that can be produced by many members of the microbial community, or by growth of a small fraction of the microbial community that is able to express the enzyme until that fraction is abundant enough to produce measureable amounts of enzyme. The timescale of days between the input of polysaccharide and the observation of hydrolysis is more consistent with the second hypothesis, suggesting that each specific polysaccharide in this study could only be accessed by a small fraction of the microbial community. Using genomic analysis of tagged DNA, Mou et al (2008) concluded that most members of a coastal pelagic microbial community were able to metabolize dimethylsulfoniopropionate and vanillate, two substances of which there is a substantial flux into the DOC pool in coastal seawater. The results presented here indicate that these polysaccharides may typically only be available to a small fraction of the community. This would agree with the finding that some microbial taxa are more adept than others at accessing extracellular polymeric substances (Elifantz et al. 2008; Elifantz et al. 2005) and

that some clades apparently specialize in low molecular weight substrates while others prefer high molecular weight DOC (Cottrell and Kirchman 2000). Microbial community response to xylan and laminarin was usually rapid, with maximum hydrolysis rates often reached by the first timepoint. In these cases, it is equally possible that there was substantial activity of the appropriate enzymes in the water prior to sampling, or that suitable enzymes were expressed fast enough to lead to considerable hydrolysis of the polysaccharide after 2 days. This timescale is consistent with a larger fraction of the population being able to express xylanases and laminarinases.

In some cases, arabinogalactan and fucoidan hydrolysis rates were measurable from the first timepoint, but did not increase much with time (Fig 3 column c). This is consistent with low-level constitutive expression of those extracellular enzymes as is common in many regulation systems for polysaccharide hydrolases (Béguin and Aubert 1994; Ensor et al. 1999), however, the fact that hydrolysis rates did not increase indicates a malfunction in that system. Another possibility is that the observed hydrolysis was mediated by enzymes already which were present in the sample at the start of the incubation and were not associated with living cells. Extracellular enzymes in 7 °C water (Chapter 1) and 28 °C water (unpublished data) can have half-lives of tens to hundreds of hours, meaning that cell-free enzymes could potentially remain active long enough to be advected far from their site of production.

3.5 Conclusions

The microbial loop recycles 10-20×10^{15} g C worth of DOM per year, more than the annual flux of anthropogenic CO_2 to the atmosphere (Denman et al. 2007; Hedges 2002). Labile DOM is is by definition quickly respired back to CO_2, but semi-labile carbon may persist long enough to be exported below the thermocline and remain out of contact with the atmosphere over human-relevant timescales.

The reactivity and high carbohydrate content of bulk HMW DOM point to polysaccharides as an important carbon source for marine heterotrophic microbes. Genomic analysis of *Gramella forsetti*, a member of the Bacteroidetes phylum isolated from North Sea water which devotes a large fraction of its genome to glycosyl hydrolases, underscores the importance of polysaccharide hydrolysis to at least some species of marine bacteria (Bauer et al. 2006). The genome of *Sacchrophagus degradans*, a polysaccharide degrading bacterium isolated from the Chesapeake Bay, also devotes a large fraction of its genome to polysaccharide hydrolases. These are apparently highly modular, may have been acquired by horizontal transmission, and imply complex regulation systems in which multiple enzymes work in concert to hydrolyze structurally complex polysaccharides. The ability of bacteria such as *G. forsetti* and *S. degradans* to access a wide range of polysaccharides, their apparent ability to share genes for polysaccharide hydrolysis, and the observed importance of polysaccharides as a carbon source to heterotrophic bacteria, all contrast with the poor ability of some marine microbial communities to access some of the relatively structurally simple, soluble polysaccharides used in this study. The reasons for this apparent discrepancy will need to be elucidated in order to predict fluxes of semi-labile DOC among marine reservoirs.

3.6 Tables

TABLE 3-1: SAMPLE CHARACTERIZATION

	Notable features	Salinity	In-situ temp, oC	Incubation temp, oC
Station 1 2 m	surface	~26 (changing rapidly)	28	28
Station 1 35 m	~6 m above bottom	35.3	28	28
Station 2 2 m	surface	34.5	29	28
Station 2 17 m	just above pycnocline	35.5	29	28
Station 2 150 m		36	16	16
Station 2 280 m	~5 m above bottom	35.4	11	11
Station 3 2 m	surface	35.5	29	28
Station 3 47 M	Chlorophyll maximum	36.5	25	28
Station 3 125 m		36.3	18	16
Station 3 350 m	Oxygen minimum	35.2	9.8	11
Station 3 700 m		34.9	6.4	6-7
Station 3 905 m	~4 m above bottom	34.9	5.4	6-7

TABLE 3-2: TIME OF MAXIMUM HYDROLYSIS RATE

	ARA	CHN	FUC	LAM	PUL	XYL
st 1 d 1	4,6,11,14,26	4	6	2	14	2
st 1 d 2	26	4	11	2	4,6,11,14	2
st 2 d 1	4	2	11,14	2	2	4
st 2 d 2	14	2	11,14	2,4,6	26	4
st 2 d 3	2	4,6,11	2	6	no hydrolysis	6
st 2 d 4	2	11	no hydrolysis	6,11,14	no hydrolysis	11,14
st 3 d 1	11	4	11	4	26	4
st 3 d 2	2,4,11,14	4	6,11	4	6	4
st 3 d 3	26	11,14,26	4	11,14	4	14
st 3 d 4	2,6	6,11,14,26	no hydrolysis	6	4	6,11
st 3 d 5	26	6,11,14	no hydrolysis	11	no hydrolysis	11,14,26
st 3 d 6	26***	6,11,14	26	11,14	no hydrolysis	11,14

*: only one measurement was made

TABLE 3-3: CARBOHYDRATE CONCENTRATIONS, RADIOTRACER MEASUREMENTS AND CELL COUNTS

	MCHO, uM carbohydrate	TCHO, uM carbohydrate	Glucose incorporation, hr^{-1}	Glucose respiration, hr^{-1}	Glucose turnover, day^{-1}	Glucose growth efficiency	Leucine incorporation, nM hr^{-1}	Cell counts, $\times 10^6$ mL^{-1}
St 1, 2 m	6.65±0.5	15.1±0.5	0.0494±0.0054	0.0554± 0.0032	2.52±0.18	47%	0.32 ±0.03	2.2
St 1, 35 m	5.12±2.1	5.57±0.3	0.0317±0.0036	0.0515± 0.0037	2.00±0.14	38%	0.069 ±0.018	11.
St 2, 2 m	2.98±1.2	7.16±0.5	0.0465±0.0047	0.0397± 0.002	2.07±0.15	54%	0.076 ±0.063	n. d.
St 2, 17 m	2.44±0.2	8.49±0.1	0.0289±0.0002	0.0317± 0.0019	1.46±0.10	48%	0.039 ±0.0498	7.2
St 2, 150 m	3.44±1.1	8.37±0.8	0.0021±0.0001	0.0019± 0.0003	0.0970±0.010	52%	0.0007 ±0.002	1.6
St 2, 285 m	1.31±0.3	5.43±0.3	0.0012±0.0016	0.0017± 0.0003	0.0708±0.008	42%	-0.0001 ±0.001	n. d.
St 3, 2 m	0.73±0.9	3.83±0.5	0.0142±	0.0203± 0.0011	0.828±0.055	41%	0.022 ±0.010	n. d.
St 3, 45 m	2.39±0.4	5.06±0.8	0.0042±0.0005	0.0061± 0.0003	0.247±0.016	41%	0.0073 ±0.0024	6.9
St 3, 125 m	-0.36±0.4	1.81±0.5	0.0013±0.0002	0.0021± 0.0001	0.0808±0.005	39%	0.0008 ±0.0002	2.9
St 3, 250 m	0.35±0.7	3.79±0.2	0.0006±0.0001	0.0015± 0.0001	0.050±0.003	28%	-0.0004 ±0.0002	1.2
St 3, 700 m	1.34±0.2	n. d.	0.0006±0.0001	0.0017± 0.0001	0.055±0.003	26%	-0.0006 ±0.0004	1.2
St 3, 905 m	0.58±0.2	n. d.	0.0007±0.0001	0.0013± 0.0001	0.048±0.003	33%	-0.0006 ±0.0001	1.8

n.d. indicates not detected

3.7 Figures

FIGURE 3-1: MAP OF SAMPLING SITES

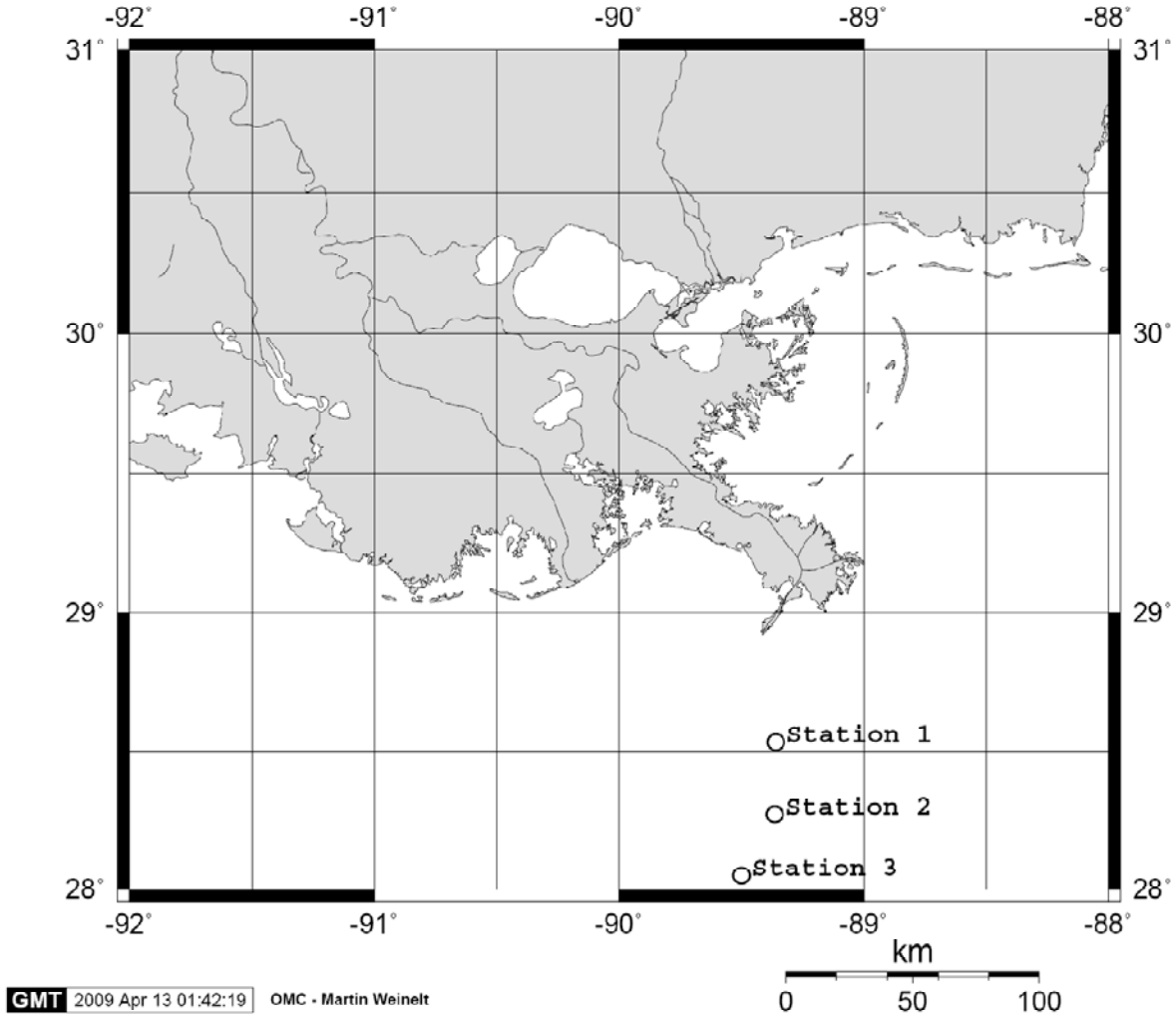


FIGURE 3-2: MAXIMUM ABSOLUTE (A-C) AND RELATIVE (D-F) HYDROLYSIS RATES

Abbreviations are: LAM, laminarin; XYL, xylan; CHN, chondroitin sulfate; FUC, fucoidan; PUL, pullulan, ARA, arabinogalactan. Error bars are the standard deviation of replicates.

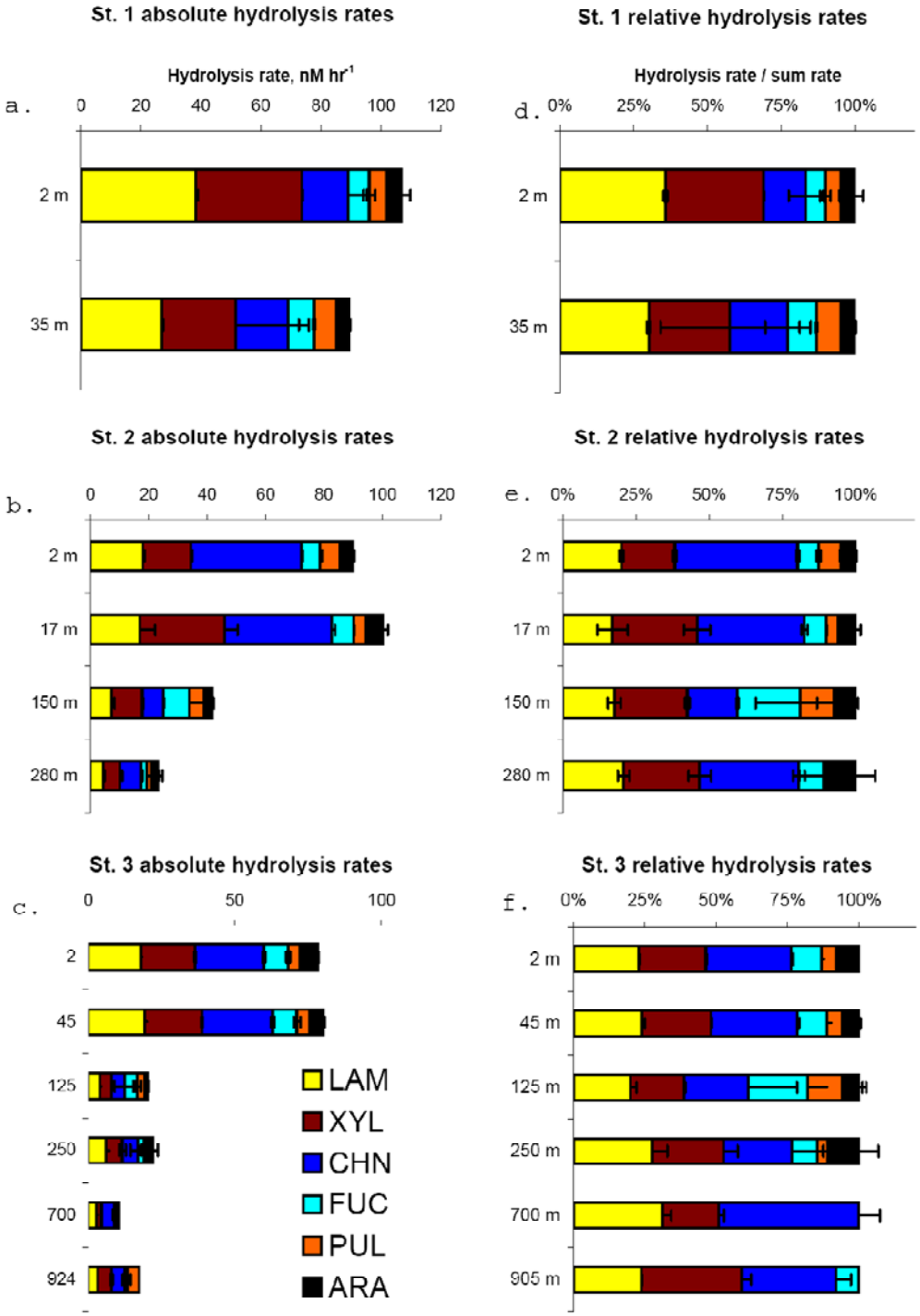


FIGURE 3-3: DENDROGRAM OF HYDROLYSIS RATE PATTERNS

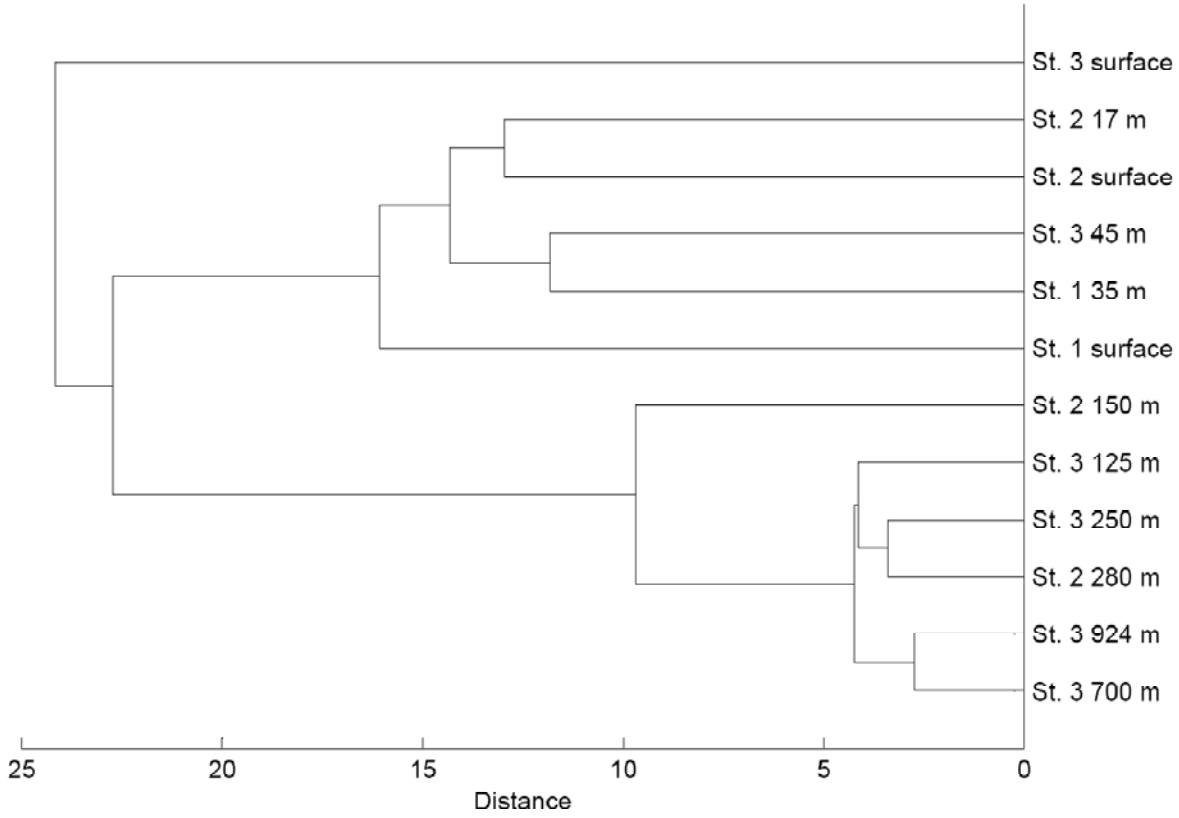
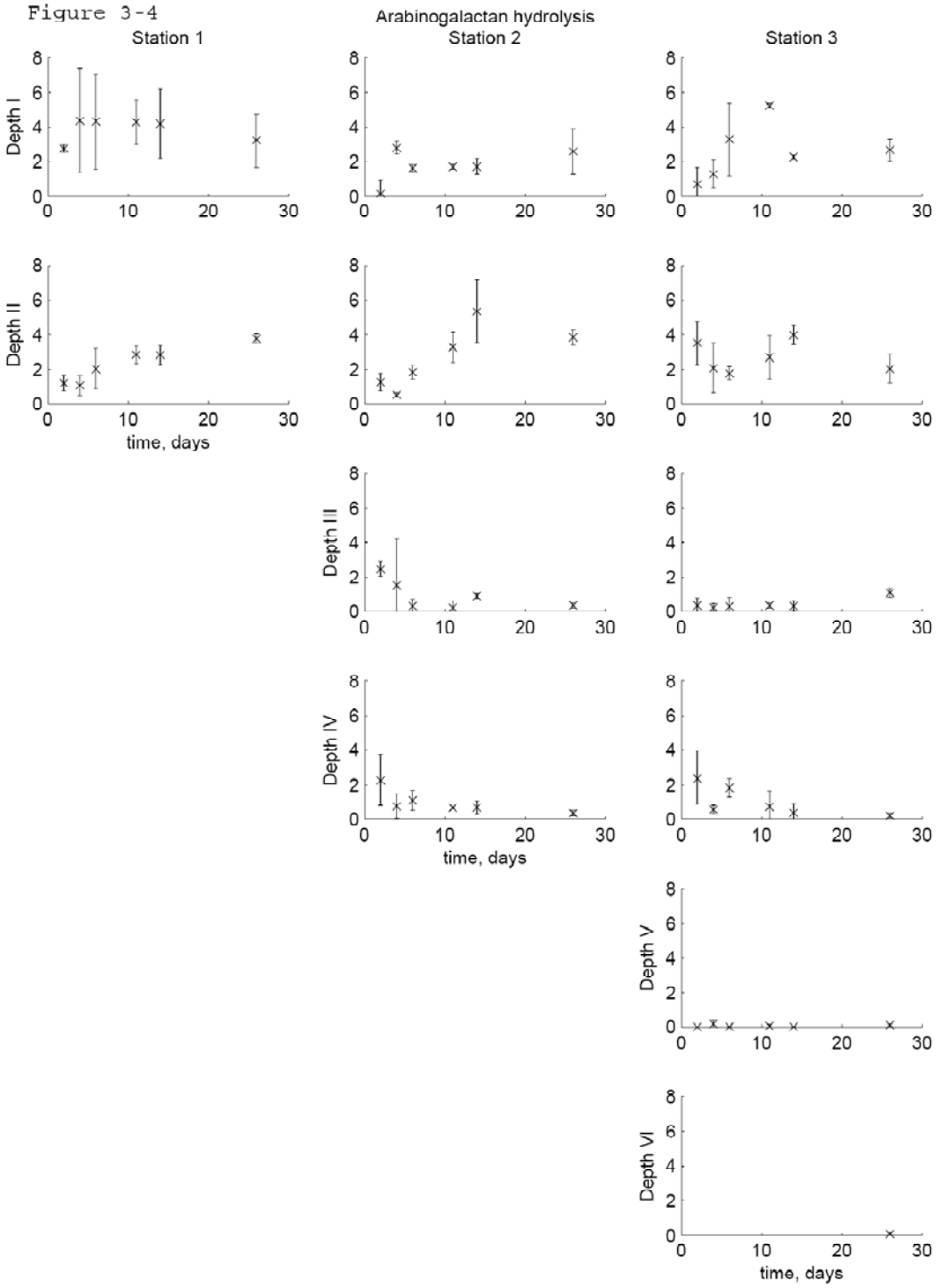


FIGURE 3-4: TIMECOURSES OF HYDROLYSIS RATES FOR EACH POLYSACCHARIDE IN EACH WATER SAMPLE

Depths are listed as I-VI because the precise depths vary among stations. Precise depths at each station are listed in Table 1.



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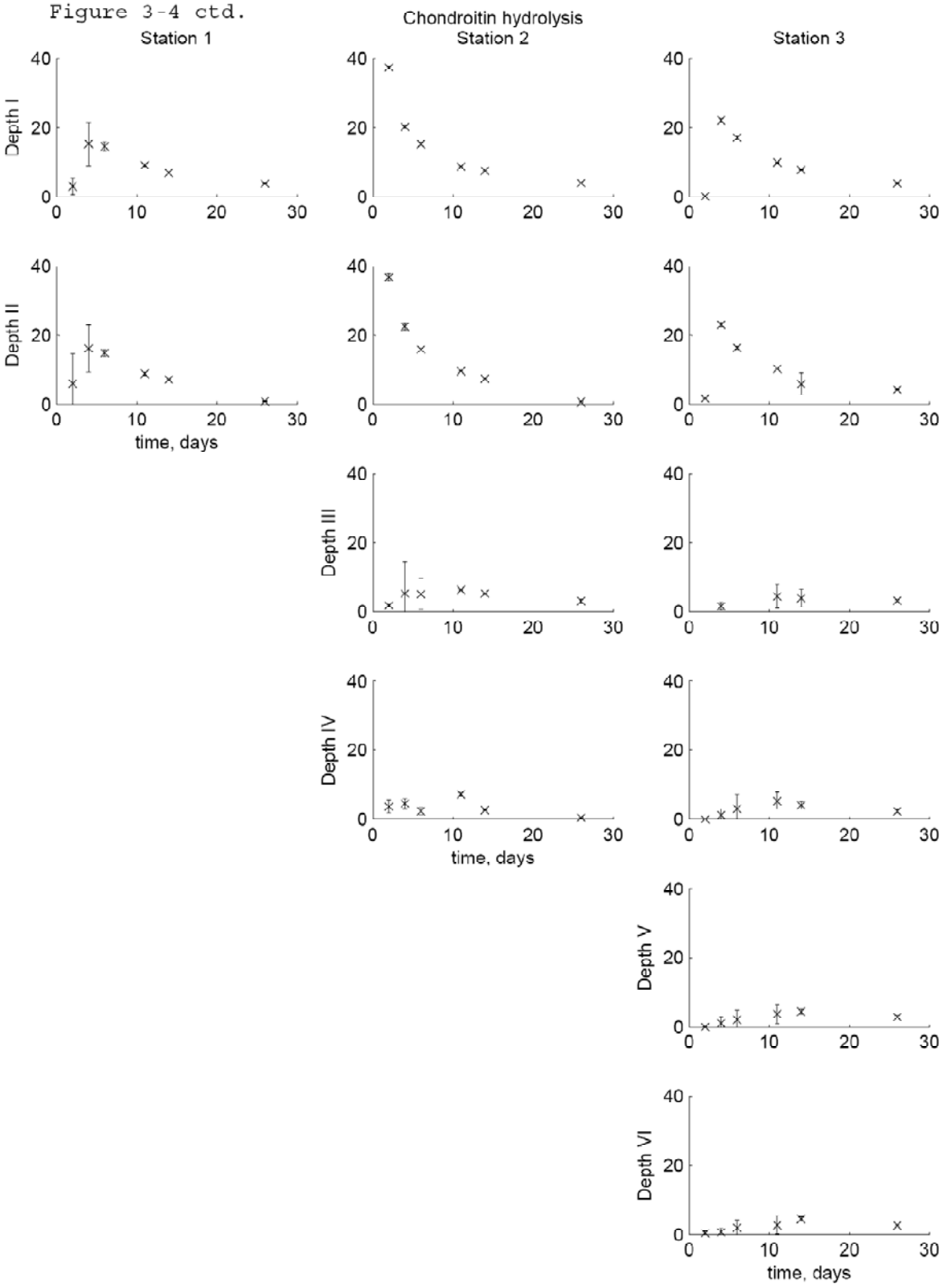

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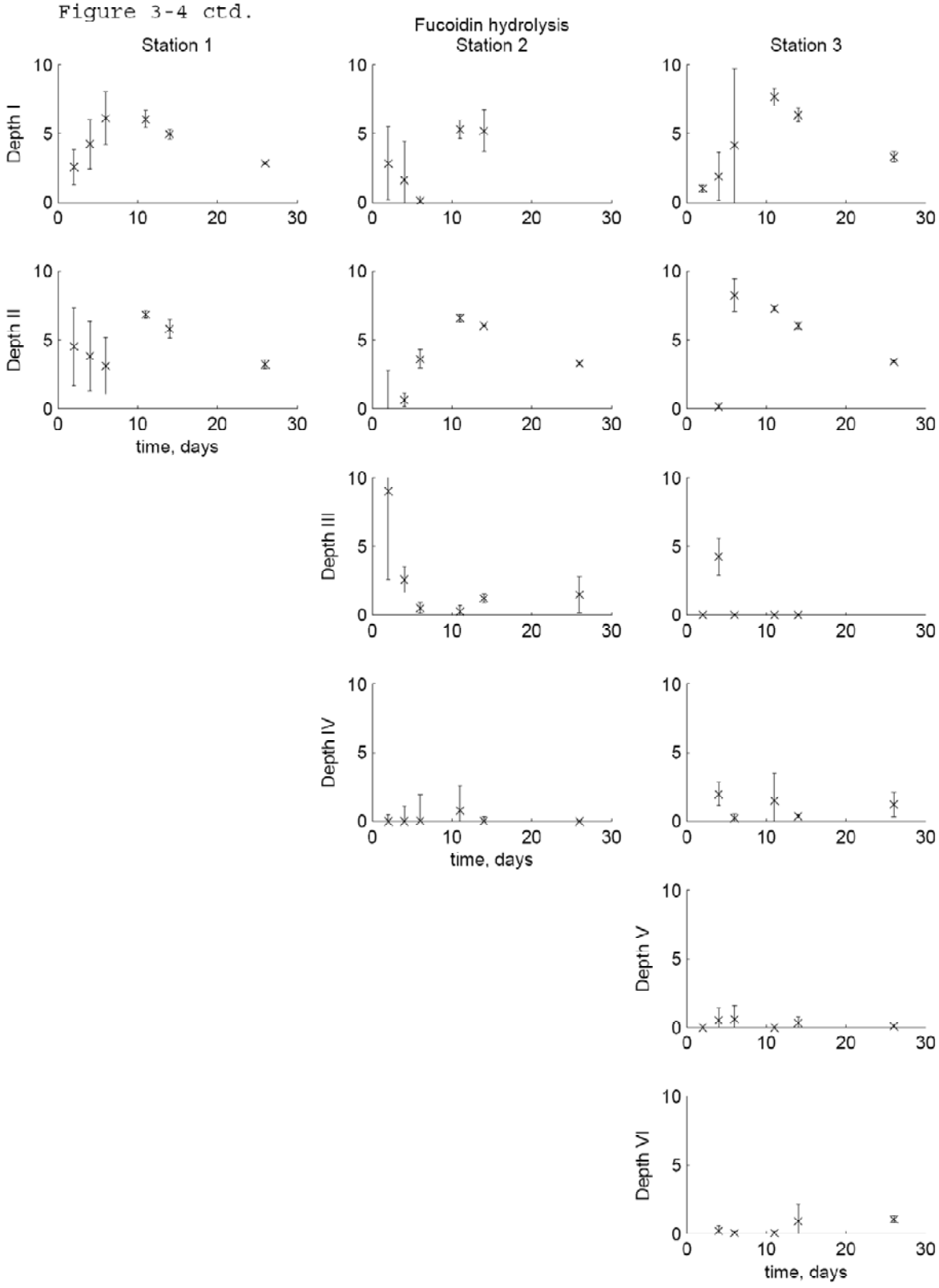

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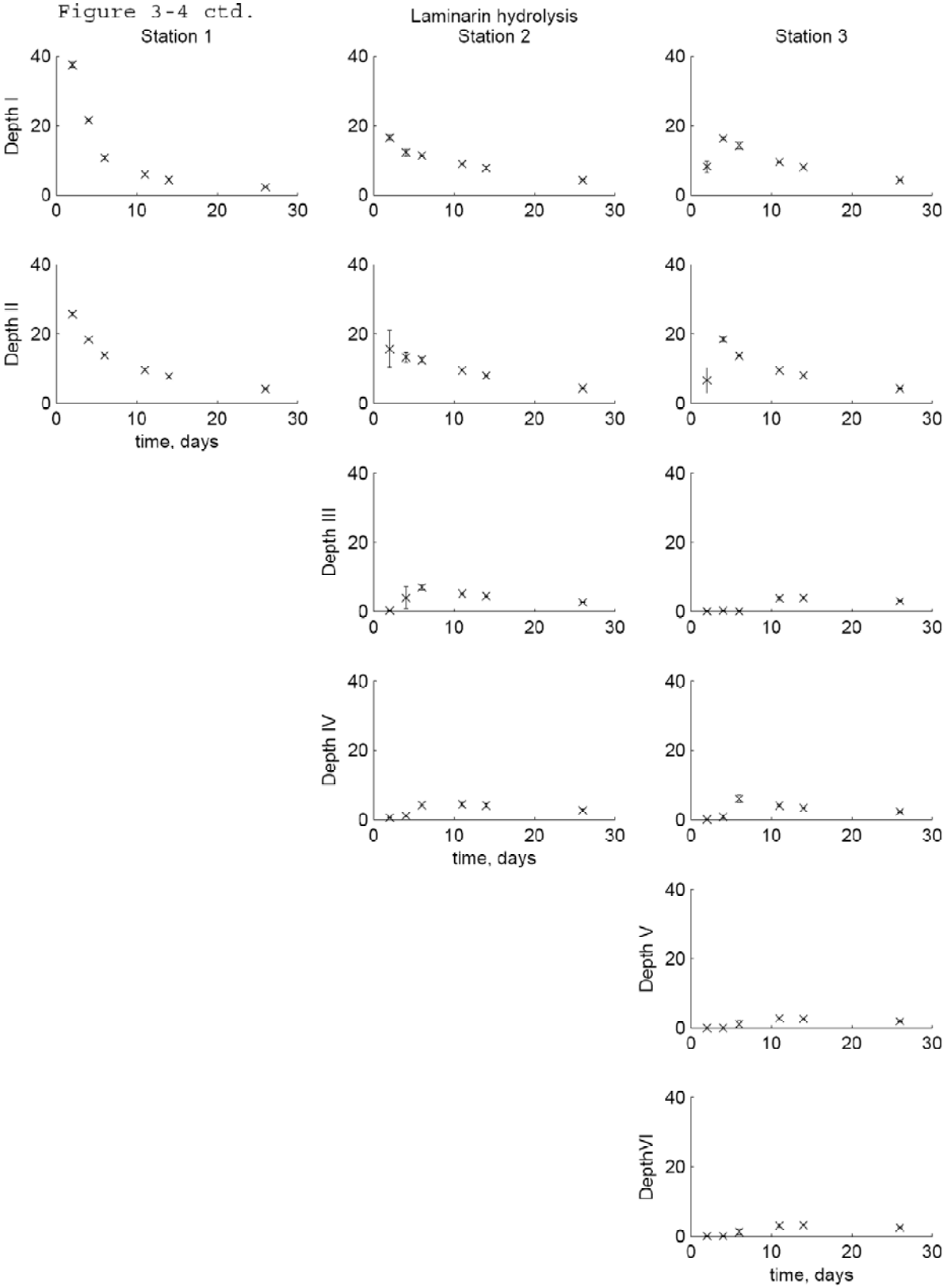

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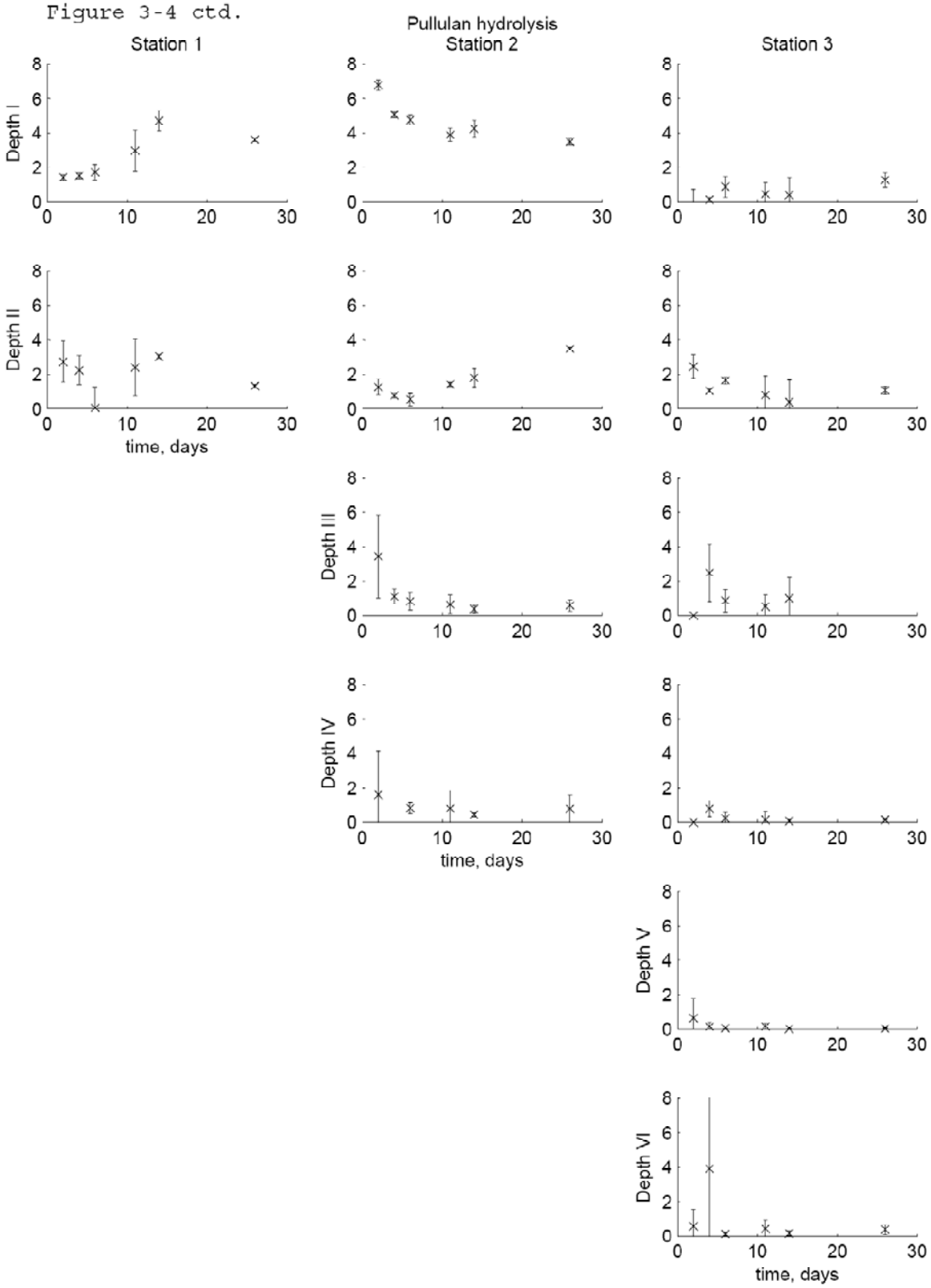

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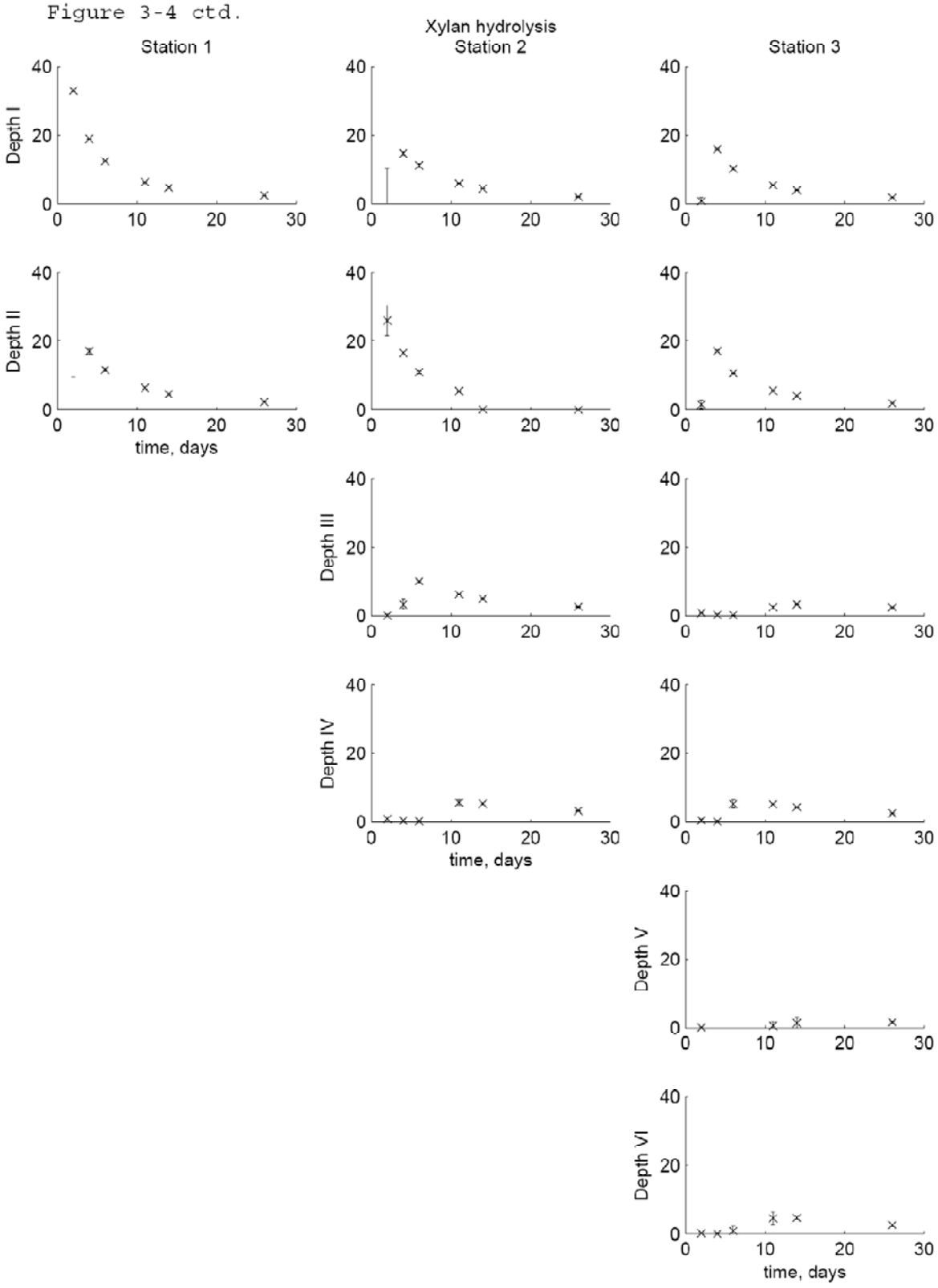

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Chapter 4 Picky, hungry eaters in the cold: investigating the failure of Arctic pelagic heterotrophs to access soluble polysaccharides

Andrew D. Steen and Carol Arnosti

Microbial communities in the Arctic Ocean typically are able to access a narrower range of polysaccharides than marine communities in lower latitudes, even after long exposure to high concentrations of polysaccharides for which there is no chemical barrier to hydrolysis. It is not clear why a heterotrophic community would fail to utilize one polysaccharide, when the same community hydrolyses similar polysaccharides rapidly. We attempted to isolate factors that might cause communities in a Svalbard fjord to fail to access pullulan, a soluble linear glucose polymer, by comparing hydrolysis rates among sediments and in seawater and by amending water samples with a range of compounds likely to induce pullulanase expression. Rapid rates of pullulan hydrolysis in sediments demonstrated that low temperatures alone are not a barrier to pullulanase production. Exposure to maltotriose, a subunit of pullulan likely involved in pullulanase induction pathways, led to very low level production of pullulanase in one out of four experiments; no other compounds induced pullulanase expression in any experiment. These results suggest that members of the microbial communities we sampled totally lacked the ability to express pullulanase. The

ability of some communities in other locations to hydrolyze a broader range of substrates is likely due to the presence of more versatile heterotrophic microbes.

4.1 Introduction

The remineralization of high-molecular-weight dissolved organic carbon (HMW DOC) in seawater begins with hydrolysis by microbial extracellular enzymes. The number of different polysaccharides that can be hydrolyzed varies among surface seawater communities: investigations with fluorescently-labeled polysaccharides show that some communities appear capable of accessing a wide range of substrates, whereas others can access only a few of the fl-polysaccharides tested (Arnosti 2000; Arnosti et al. 2005a; Steen et al. 2008). A compilation of fl-polysaccharide hydrolysis rates from 35 globally distributed sites indicates that, in general, microbial communities in warmer, lower-latitude waters are consistently capable of accessing a wider range of polysaccharides than those in colder, more poleward locations (Arnosti et al, *submitted*). Measurements from Svalbard, in particular have shown that only a narrow spectrum of polysaccharides is accessible to the pelagic community there (Arnosti 2004; Arnosti 2008). If this trend, identified with six specific polysaccharides, extends to the range of macromolecules comprising marine high-molecular weight dissolved organic carbon (HMW DOC_, then a fraction of DOC which would be semi-labile or recalcitrant in the presence of Arctic microbial communities would be labile in more temperate seawater.

Broadly, four possible scenarios could explain the failure of a microbial community to hydrolyze polysaccharides that are readily hydrolyzed in other environments. First, cold temperatures could directly impose a limit on the diversity of enzymes a community might be able to produce. Secondly, a severe shortage of carbon, nutrients, or some other environmental stressor might result in a malfunctioning loop (Thingstad et al. 1997) that is

unable to access polysaccharides which would otherwise be labile. Pelagic heterotrophs are typically assumed to be limited by organic carbon, but nutrient limitation is also possible (Church 2008; Thingstad et al. 2005). Severe nutrient or carbon limitation – as indicated by low bacterial growth efficiency – was associated with lower extracellular β-glucosidase expression in eutrophic lake water (Middelboe and Søndergaard 1993). Third, chemical inducers of specific extracellular enzymes might be absent, leading to a situation in which communities have the genetic potential to express specific extracellular enzymes, but that potential is not actually reached in the environment (Arnosti 2004). Finally, different microbial clades appear to have different capacity to assimilate specific high-molecular weight compounds (Elifantz et al. 2008). It is therefore possible that microbial communities which cannot hydrolyze some polysaccharides simply do not contain any members with the genes required to produce the necessary hydrolytic enzymes.

The goal of this project was to test the four hypotheses listed above in Svalbard seawater. In order to evaluate the effects of cold temperatures, hydrolysis rates in seawater were compared with those in the permanently cold sediments of Smeerenburgfjord. Bacterial production, turnover time of glucose in the water column, and the efficiency of glucose conversion into biomass were assessed as broad indicators of the functioning (or malfunctioning) of the heterotrophic microbial community.

It is not currently possible to conclusively test for the presence of genes or necessary inducers for polysaccharide hydrolases, since most genes identified in marine metagenomic libraries are of unknown function (Mou et al. 2008; Venter et al. 2005) and the full range of biochemical systems controlling extracellular polysaccharide hydrolase expression are far from being known. However, one broad regulatory scheme for polysaccharide hydrolase

expression appears to be very common: polysaccharide hydrolases are produced constitutively, with further expression induced by oligomers of the relevant polysaccharide and repression by corresponding monomers (Béguin and Aubert 1994). This scheme has been observed in the polysaccharide-degrading marine bacterium *Sacchrophagus degradans* (Weiner et al. 2008; Whitehead et al. 2001). Svalbard water samples were therefore amended with maltotriose, the trimeric repeating unit of pullulan, as well as with a range of organic carbon and nutrient sources, in order to determine whether the frequently-observed absence of pullulanase activity was due to the lack of specific inducers or to low concentrations of organic carbon or nutrients.

4.2 Methods

STUDY SITE AND SAMPLING

Seawater and sediments for the hydrolysis rate measurements and radiotracer measurements were obtained from Station J (Fig. 1, 79° 42.8' N, 011° 05.2'E, 218 m water depth) in Smeerenburgfjord, Svalbard, on 15 August 2008. Smeerenburgfjord, located on the north-west coast of Spitsbergen, is open on two ends. To the best of our knowledge, no detailed studies have been made of water circulation through the fjord. Nonetheless, surface flow (depending on the wind direction, from the north or from the east) is observable through Smeerenburgfjord, so surface water is likely more heavily influenced by continental shelf water than would be typical in a fjord with only a single outlet, although flow from the east is restricted by a sill ~20m deep. Continental shelf water near Smeerenburgfjord is influenced by the West Spitzbergen Current, derived from Atlantic water flowing north from the Fram

Strait and is typically trapped against the shelf break in the vicinity of Smeerenburgfjord (Aagaard et al. 1987).

A surface-water sample (~2 m depth) and a bottom water sample (~10 m above bottom) of Smeerenburgfjord water were obtained via Niskin bottle and stored in 3x-sample-rinsed plastic carboys, kept in coolers filled with surface water at approximately *in situ* temperature, for approximately 12 hours during transit to the laboratory at Ny Ålesund. Sediment samples were obtained by Haps corer, and were stored in gas-tight bags, homogenized, and maintained at near in situ temperature during transit prior to the start of experiments. "Surface" sediments came from approximately 0-3 cm depth, and "deep" sediments came from approximately 3-9 cm depth.

On separate expeditions, surface seawater for the pullulanase induction experiments was obtained from Kongsfjorden, Svalbard (78° 16.55' N, 15° 10.1' E, 245 m water depth) on 29 June 2005 and on 3 August 2006. Samples were kept on ice, dark, in coolers until during transit to the Ny Ålesund lab, about 48 hours.

POLYSACCHARIDE HYDROLYSIS RATES

Polysaccharide hydrolysis rates were measured by the method of Arnosti (1996; 2003). Arabinogalactan (mixed galactose-arabinose polysaccharide), chondroitin sulfate (sulfated polymer of alternating N-acetyl-glucosamine and glucuronic acid), fucoidan (sulfated fucose polysaccharide), and xylan (β[1,4]-linked xylose polysaccharide) were obtained from Fluka; laminarin (β[1,3]-linked glucose) and pullulan (α[1,6]-linked maltotriose [α[1,4]-linked glucose])were obtained from Sigma-Aldrich. Polysaccharides

were labeled with fluoresceineamine according to the method of Glabe (1983) as modified by Arnosti (1996; 2003).

To measure hydrolysis rates in seawater, fluorescently labeled (FL-) polysaccharides were added to 50 mL water samples to a final concentration of 3.5 μmol monomer L^{-1} (2.8 μmol monomer L^{-1} in the case of xylan). These 50 ml samples were divided into three replicate incubations of ~17 ml each. FL-polysaccharides were also added at the same concentrations to a killed control consisting of a single replicate of autoclaved seawater. Each replicate incubation was subsampled immediately after the addition of polysaccharides and again at 3, 7, 10, 15, and 40 days of incubation. Samples were incubated at 4 °C in a temperature-controlled room, except during transit from Svalbard to Bremen, Germany (day 9) and during transit from Bremen to Chapel Hill (day 17) when samples were kept in a cooler surrounded by ice packs frozen at -20 °C. To subsample the incubations, about 2 ml of the incubation was withdrawn via sterile syringe and filtered through 0.2 μm pore size syringe filters into combusted glass vials, which were immediately capped and frozen until analysis.

To measure hydrolysis rates in sediments, FL-polysaccharides were added to 20 ml homogenized sediment samples, which were gassed with N_2 and incubated in N_2-filled gas tight bags to a final concentration of 175 μmol monomer L^{-1} sediment (surface sediments) and 350 μmol L^{-1} sediment (deep sediments), and then were mixed thoroughly with a spatula. To subsample, sediments were centrifuged in a refrigerated centrifuge and ~1.5 ml porewater was withdrawn, filtered through a 0.2 μm filter and frozen. After withdrawing the sample the sediments were homogenized and gassed with N_2 again. Subsamples were removed after 0, 1, 2, 3, and 5 days of incubation.

Hydrolysis rates were calculated from molecular weight distributions, determined by gel permeation chromatography (GPC) as described in Arnosti (1996; 2003). Briefly, samples were separated on columns of Sephadex G-50 and G-75 and detected by fluorescence (excitation, 490 nm; emission, 530 nm). The extent of hydrolysis (equivalent to the number of free reducing ends produced via enzymatic hydrolysis) at each timepoint was calculated from changes in molecular weight distribution from time zero, less the changes in molecular weight distribution of the killed controls. In all cases, rates are expressed as the number of enzymatic cuts (i.e. number of reducing ends produced, "extent of hydrolysis") divided by the total incubation time, which gives the average hydrolysis rate during the entire incubation. This method causes an underestimate of the instantaneous hydrolysis rate in cases when hydrolysis rate increased with time, for instance when there was a significant lag phase prior to the onset of hydrolysis.

GLUCOSE ASSIMILATION AND REMINERALIZATION

Glucose assimilation rates were determined from the rate of incorporation of ^{14}C-labeled glucose into particulate organic carbon (POC). Remineralization was determined from the rate of production of radiolabeled dissolved inorganic carbon (DIC), using a modification of the method of Hobbie and Crawford (1969). 7.5 pmol uniformly ^{14}C-labeled glucose (Perkin-Elmer, 200.6 mCi mmol^{-1}) was added to 15 ml seawater in 20 ml glass scintillation vials with plastic caps for a final concentration of 1 nmol L^{-1} glucose. Vials for killed controls also received trichloroacetic acid (TCA, 100%) prior to addition of seawater to a final concentration of 5%. For the seawater samples, samples for both PO^{14}C and DI^{14}C

measurement were incubated at 4 °C and killed after 3, 8, or 24 hours (with triplicate incubations plus one killed control for each incubation time).

Glucose incorporation and remineralization were measured as described in Chapter 2, with the exception that the CO_2 trapping/scintillation cocktail consisted of 40% ScintiSafe 3 (Fisher), 25% β-phenethylamine, and 35% methanol. Trapping efficiency of this solution measured with $NaH^{14}CO3$ added to seawater was 105±4% compared to radiolabeled bicarbonate injected directly into the trapping cocktail.

One caveat for these measurements is that they rely on the assumption that glucose is added at tracer level concentrations, i.e. <10% of in-situ glucose concentrations Here the glucose addition of 1 nM was chosen because it was close to the smallest amount possible for which respiration and assimilation at the predicted rates would be measureable. Free glucose concentrations were not measured in this study, and it is possible that 1 nM may be more than 10% of in situ concentrations: free glucose concentrations averaged 5±1.5 nM in surface waters and 2±1.1 in the pycnocline of the open Kara sea (Meon and Amon 2004). By contrast, free glucose concentrations in the Central Arctic were in excess of 50 nM (Rich et al. 1997).

Glucose respiration in surface sediments was measured similarly. Five sediment slurries were made from 2.5 ml, 0.2 μm-filtered seawater at 4 °C, and approximately 2.5 ml surface sediment, in chilled 20 ml glass scintillation vials with a septum cap. 1 ml 37% formaldehyde was added to one vial to serve as a killed control. Vials were weighed before and after addition of sediment, although the precise mass of sediment does not affect the calculation of fractional glucose respiration. 30 pmol radiolabeled glucose were added to each vial for a concentration of 12 nmol glucose per L sediment (or 6 nmol glucose per L

slurry). The slurries were incubated under N_2 for 20 minutes and then killed with 1 ml 37% formaldehyde. Shortly thereafter the sample vials were connected to the sparging system described above. 3 ml 37% HCl was added and DIC was trapped as described above.

Glucose assimilation and remineralization rates were calculated as

rate = $dpm_t / (t \times dpm_0)$

where dpm_t is the activity of $PO^{14}C$ or $DI^{14}C$, dpm_0 is the initial activity of ^{14}C-glucose added to the incubation, and t is incubation time.

Glucose utilization efficiency (GUE) was calculated as the rate of glucose assimilation divided by the sum of glucose assimilation and respiration as in Chapter 2.

BACTERIAL PRODUCTIVITY

Bacterial productivity was determined from the rate of incorporation of ^{3}H-labeled leucine (Kirchman et al. 1985). L-[3,4,5 ^{3}H(N)]-leucine (Perkin-Elmer), was diluted 1:20 with nonradioactive L-leucine to a specific activity of 5.77 Ci mmol^{-1}. 150 pmol total leucine was added to glass scintillation vials to which 15 ml seawater was added. Killed controls (one per sample source and incubation time) were amended with 835 µL 100% TCA prior to the addition of seawater. Triplicate samples for each water source were incubated for 3, 8, or 24 hours. The incubation was stopped by the addition of 835 µL 100% TCA. Vials were then heated to 80 °C in a heating block for 15 minutes to precipitate proteins, and air-cooled. The precipitate was then filtered onto 0.2 µm, 25 mm diameter nitrocellulose filters shortly thereafter. The sample vials and filter tower were rinsed twice with 3 ml ice-cold 5% TCA, twice with 3 ml, ice-cold ethanol, and then the tower was removed and the filter was rinsed with 1 ml cold ethanol (Kirchman 1993). The nitrocellulose filters were then transferred to

new glass scintillation vials, dissolved in 0.5 ml ethyl acetate, and mixed with 10 ml ScintiSafe. Vials were allowed to "rest" at room temperature for 8 days to maximize counting efficiency, and then radioassayed.

PULLULANASE INDUCTION EXPERIMENTS

Upon return to Ny Ålesund, water samples from Isfjorden were divided into sample-rinsed plastic containers (~4 L). Pairs of cubitainers (one set for the '24-hour' pulse; another set for the '5-day' pulse) were amended with either nothing or one of several amendments. In 2005, the amendments were glucose (0.875 μM), maltotriose (0.875 μM monomer-equivalent), KNO_3 (40 μM), Na_2HPO_4 (16 μM), or both KNO_3 and Na_2HPO_4 (40 μM and 16 μM respectively). In 2006, amendments were glucose (0.875 μM), maltotriose (0.875 μM monomer equivalent), unlabeled pullulan (0.875 μM monomer equivalent) a mixture of the 22 protein-amino acids (5.25 μM-C), and KNO_3 plus Na_2HPO_4 (40 μM and 16 μM respectively). The 0.875 μM concentration of carbohydrate additions was chosen because it is relatively small (¼) compared to the monomer-equivalent concentration of fl-pullulan added.

After 24 hours, 50 ml was removed from each of the '24 hour pulse' containers. Two 50 ml aliquots were removed from the control container. Fl-pullulan was added to these to a concentration of 3.5 μmol monomer L^{-1}, and the 50 ml aliquots were divided into three incubation vials (glass scintillation vials). These were incubated at 7 °C and subsampled immediately, and after 3, 8 and 21 days (3, 7, and 14 days in 2006). 5 days after the addition of 'pulses' (3 days in 2006), fl-pullulan incubations were started in the other set of pulsed

containers in the same manner, except that subsampling occurred after 3, 12 and 21 days (3, 7, and 14 days in 2006).

4.3 Results

COMPARISON OF HYDROLYSIS RATES IN THE WATER COLUMN AND SEDIMENTS

The maximum hydrolysis (the maximum rate calculated for all timepoints for a specific substrate in a specific sample) is an indication of the maximum rate at which a microbial community can access a substrate, given time to change composition or gene expression. Patterns and magnitudes of maximum hydrolysis rates were broadly similar among surface and bottom seawater samples (Fig 2 a-c), but there were a few notable differences: laminarin hydrolysis was 3.6 times faster in surface than in deep water (0.93 nmol L^{-1} hr^{-1} vs. 0.26 nmol L^{-1} hr^{-1}), and xylan hydrolysis was 3.3 times faster in bottom water than in surface water (2.97 nmol L^{-1} hr^{-1} vs. 0.90 nmol L^{-1} hr^{-1}). Pullulan and arabinogalactan hydrolysis was barely detectable in deep seawater after 40 days of incubation, and was undetectable in surface seawater. Chondroitin hydrolysis dominated the total hydrolysis in both surface and deep water, accounting for 59±2% of summed maximum hydrolysis rates in surface water and 59±1% in deep water.

Summed maximum hydrolysis rates were considerably faster in sediments than in seawater (360-570×), but the patterns of hydrolysis rates differed strongly between sediments and seawater (Fig. 2 d-f). In sediments, all fl-substrates tested were readily hydrolyzed. This was in strong contrast to the seawater communities, which were completely unable (in surface water) or only very weakly able (bottom water) to access arabinogalactan and

pullulan. For example in surface sediments, the slowest-hydrolyzed polysaccharide (pullulan) accounted for 10±2% of total hydrolysis, while laminarin, the fastest-hydrolyzed polysaccharide, accounted for 26±8% of total hydrolysis. In surface seawater, by contrast, pullulan was not detectably hydrolyzed and arabinogalactan hydrolysis contributed to 0.9±1.4% of hydrolysis, while chondroitin hydrolysis accounted for more than half of the summed hydrolysis rates.

TIMECOURSE OF HYDROLYSIS RATES

Seawater hydrolysis rates typically increased to a maximum following a lag time, after which hydrolysis rates decreased as the concentration of unhydrolyzed substrate decreased (Fig 3). The duration of this lag time was short for chondroitin sulfate in surface water, a linear extrapolation of the first four data points implies that the rate of hydrolysis was zero until about one day of incubation. Most substrates had longer lag times. For instance, substantial hydrolysis of xylan in bottom water did not begin until 7-10 days of incubation, the lag time for fucoidan was 10-15 days, and detectable hydrolysis pullulan in bottom water began between 15 and 40 days of incubation.

In sediments, the timecourse of polysaccharide hydrolysis for some substrates resembled those in seawater, with a lag time followed by an increase in hydrolysis rates (Fig 4). For instance, hydrolysis of chondroitin in surface sediments was not observable after 3 days of incubation, but a considerable fraction of the polysaccharide was hydrolyzed between 3 and 5 days. Lag phases for enzyme activity in sediments were also evident in surface sediments for arabinogalactan, and chondroitin sulfate, and in deep sediments for xylan (for some substrates it was difficult to evaluate whether there was a lag phase, because early

timepoint chromatograms were not usable[3]). Other polysaccharides, however, were hydrolyzed at relatively constant or decreasing rates throughout the experiment. In surface sediments, laminarin, pullulan, and xylan were hydrolyzed at the 1-day timepoint, and hydrolysis continued throughout the experiment.

GLUCOSE INCORPORATION AND BACTERIAL PRODUCTION

^{3}H-leucine uptake was approximately linear with time, with leucine uptake rates of 35.3-43.8 nmol L^{-1} hr^{-1} in surface water, and 11.7-12.1 nmol L^{-1} hr^{-1} in deep water (data not shown). Henceforth I will use the rates measured in the 8 hour incubation, because rates at that timepoint had the lowest coefficient of variation (standard deviation of replicates / mean rate). Conversion factors of 1500 – 3100 g C mol^{-1} leucine have been accurate to calculate bacterial productivity from leucine incorporation in a wide variety of environments (Simon and Azam 1989). Using those conversion factors on our samples, bacterial productivity was between 110±12 and 219±23 μg C L^{-1} day^{-1} in surface water and 32.5±2.8 and 65.0±5.5 μg L^{-1} in deep water (Table 1).

Production of DI^{14}C and PO^{14}C were also fairly linear with time. Again, I will focus on the 8 hr rates. Production of DI^{14}C was nearly equal in surface and bottom waters: 0.7±0.11% hr^{-1} and 0.62±0.03% hr^{-1} respectively. Production of PO^{14}C was greater in surface water, 1.83±0.36% hr^{-1} versus 1.08±0.24% hr^{-1} in deep water (Fig. 5). Together,

[3] In sediments, it is not uncommon for time-zero and early-timepoint chromatograms to show a dominance of low-molecular material and low values of integrated fluorescence (i.e. the total area under the chromatogram, a proxy for the total amount of fl-polysaccharide). It is not clear why this happens; insufficient mixing of the sediment after addition of the fl-polysaccharide may be one reason. Nevertheless, this pattern is fairly easy to identify in chromatograms, and chromatograms that looked this way were thrown out.

these rates indicate glucose turnover times of 40 and 59 hours, respectively. GUE was 72±2.9% in surface water and 63±3.1% in deep water.

Sediment respiration of glucose was 11.9±0.3% hr^{-1}, implying a turnover time for dissolved glucose in porewater due to respiration of 8.4 hr. This is an upper bound on the actual turnover time for dissolved glucose in porewater, because some amount of additional glucose was incorporated into biomass. Assuming GUE in sediments of 50% to 66.7%, glucose turnover time in porewater was 4.2-5.6 hr.

PULLULANASE INDUCTION

Heterotrophs capable of accessing polysaccharides were active in the pullulan induction experiment, as shown by maximum xylan hydrolysis rates of 4.3 nM L^{-1} hr^{-1} in the 24-hour controls and 6.8 nM L^{-1} hr^{-1} in the 5-day controls, comparable to or faster than rates observed at Station J in 2008. As with Station J samples, pullulan hydrolysis was very close to zero in the unamended controls. In all treatments except for the 5-day +maltotriose treatment, chromatograms show a very slight shift towards lower molecular weight fl-pullulan in the 21-day incubation samples (Fig. 6). This shift is not unequivocally distinguishable from the effects of column compaction, so it is not clear whether this represents real (very slight) hydrolysis or merely an artifact of changes in chromatography column characteristics.

In the 21-day timepoint for the 5-day +maltotriose treatment, production of lower-molecular weight fl-pullulan is clearly visible. The quantity of this lower-molecular weight organic matter is considerably larger than in any other sample, indicating that this is due to enzymatic hydrolysis rather than column compaction.

Maltotriose addition induced pullulanase expression in the 5-day pulse experiment, but such induction was not detectable in the 24-hour pulse experiment. This was probably not due to random variation, since chromatograms were very similar among replicates in both treatments (this is visible from the size of the error bars in Figure 7). One possible explanation is that enzyme expression did not begin until the last few days of the incubation period; if that were the case then the beginning of hydrolysis would have occurred after the last sample was taken in the 24-hour pulse samples.

The putative extent of hydrolysis in all experiments is shown in Figure 7. These values are not corrected for column compaction, and I do not argue that they necessarily represent real hydrolysis. These data indicate the magnitude of the effect of maltotriose treatment compared to all other treatments.

In 2006, no hydrolysis was observed for any treatment.

4.4 Discussion

The differences in patterns of hydrolysis rate between sediments and seawater demonstrate that the seawater communities sampled here had fundamentally different biogeochemical function than sediment communities. Arnosti (2008) observed similar differences between hydrolysis rate patterns in surface seawater and surface sediments at Station J, and pointed out that those differences cannot be due solely to scaling factors such as cell density.

The large decrease in the measures of microbial activity with respect to simple substrates (leucine, glucose) from surface water to bottom water, combined with the slight

increase in summed maximum hydrolysis rates, shows that, in these samples at least, hydrolytic potential is not well-predicted by in situ rates based on simple substrates. The many-day incubations of fl-polysaccharides, combined with the relatively high concentrations of polysaccharides relative to likely in-situ fresh DOM, measure the ability of the microbial community to react to the input of new DOM (as, for instance, might happen during a phytoplankton bloom). The low rates of in-situ activity in bottom water, combined with high ability to respond to input of organic matter over a timescale of days, suggest a dormant community in deep water that had low in-situ activity but was capable of quickly responding in response to input of fresh organic matter.

Cold water communities tend to hydrolyze a narrower spectrum of polysaccharides than warmer-water communities (Arnosti et al 2005, Arnosti et al in prep, also compare data presented in this chapter to chapter 2). Low temperatures *per se* cannot, however, explain this effect, because the permanently cold sediment communities observed here were fully capable of hydrolyzing a broad range of polysaccharides (Arnosti 2008; Arnosti et al. 2005b; Arnosti and Jørgensen 2003).

GUE in both water samples were similar to those reported in a wide variety of warmer-water marine environments, approximately 60-75% (Table 2). Thermal stress decreased GUE for microbes in pure culture, as well as for heterotrophs in an algal mat and a lake sediment community (Tison and Pope 1980). Bacterial production in surface water, 3.2 μg C L^{-1} day^{-1}, falls within the envelope of production rates Rich et al (1997) measured in a transect of the central Arctic Ocean (1-4 μg C L^{-1} day^{-1}; two stations in the Chukchi Sea had production of 7-8 μg L^{-1} day^{-1}) and is very close to the average value for surface water in the open Kara sea reported by Meon and Amon (2004; 45 pM hr^{-1} or 3.24 ug C L^{-1} day^{-1} using

the conversion factors used in this work). This production is slightly greater than the average value in the open North Atlantic observed in a north-to-south transect of 33 pmol L^{-1} hr^{-1} (2.4 μg C L^{-1} hr^{-1}) and slightly less than the average for the South Atlantic (4.5 μg C L^{-1} hr^{-1}). Daily fractional turnover of glucose of 0.61 (surface) and 0.41 (deep) were comparable to measurements from the open Kara Sea (0.39±0.2 in surface waters, 0.52±0.23 above the pycnocline)(Meon and Amon 2004) as well as the upper range of observations from the Equatorial Pacific (0-0.6 day^{-1}, with the subset taken north of the equator in August and September in the range of 0.3-0.6 day^{-1} (Rich et al. 1996). These rates are somewhat greater than values observed in Gulf of Mexico surface water (0.2-0.5 day^{-1}; Skoog et al. 1999) and in the Central Arctic (0.17-0.26 day^{-1}; Rich et al. 1997)

The radiotracer measurements therefore showed no evidence of a microbial loop that was impaired in any sense relative either to microbial communities in the Arctic or in more temperate latitudes. This is perhaps not surprising in light of the fact that in the Arctic, microbial extracellular enzymes, bacterial production, and growth rates are well adjusted to cold temperatures (Huston et al. 2000; Kirchman et al. 2005; Rivkin et al. 1996). The narrow spectrum of extracellular enzymes expressed by the community was therefore not due to gross malfunctioning of the microbial loop.

Although heterotrophic microbes in the ocean are often assumed to be limited by the supply of organic carbon, nutrient limitation of bacterial production has also been observed (Thingstad et al. 2008; Thingstad et al. 2005). Production of extracellular enzymes demands a considerable investment in carbon and nutrients (Allison 2005); one could imagine that in a severely carbon- or nutrient- limited system, extracellular enzyme activity would decrease. Additions of both labile carbon, organic N (as amino acids), and inorganic nutrients failed to

stimulate production of extracellular pullulanase. Addition of maltotriose induced pullulanase in the 2005 experiment, but only in one treatment, and then only a very small amount of pullulanase was hydrolyzed between 12 and 21 days. Maltotriose did not clearly induce pullulanase expression in either of the 2006 treatments, and a previous experiment using Smeerenburgfjord seawater, in which fl-pullulan was added simultaneously with maltotriose at 1% of the monomer-equivalent pullulan concentration, also did not show evidence of pullulanase induction (Arnosti 2004). In all of these experiments, microbial communities in the incubation vial co-existed with a large (21 μmol C L^{-1}) concentration of fresh pullulan, showing that the presence of pullulan itself did not induce pullulanase activity. The capacity to express pullulanase is either typically absent in Svalbard pelagic communities, or if pullulanase genes are present, they are only induced under specific conditions; mere presence of pullulan or its intermediate breakdown products are not sufficient to induce it.

Hydrolysis of most polysaccharides in Svalbard water is predictable: chondroitin sulfate and laminarin are reliably hydrolyzed, whereas pullulan hydrolysis has never been observed. Fucoidanase, however, seems to be sporadically present: no hydrolysis of fucoidan was observed in July of 1999 or June of 2000 (Arnosti et al. 2005a), but fucoidan readily hydrolyzed in samples taken in September of 2001 (Arnosti 2008), August of 2007 (unpublished data) and August of 2008. The occasional failure of Svalbard pelagic communities to access fucoidan is particularly surprising because fucoidan is the major energy storage product for brown algae of the genus Fucus (Painter 1983), which are abundant in Svalbard fjords (Hop et al. 2002). It seems very likely that fucoidan is present in

Svalbard DOC, but the microbial community there is not consistently able to access it even after incubations of as long as 35 days.

4.5 Conclusions

Despite recent advances in our understanding of heterotrophic cell activity (Del Giorgio and Gasol 2008), techniques to determine and evaluate marine metagenomes (Moran 2008), and a new examination of the physical structuring of marine microbial communities (Azam and Malfatti 2007), the factors that control population dynamics and functional characteristics of marine microbial communities remain poorly understood. A recent study using genomic techniques suggested that, among coastal bacteria, the population capable of assimilating two model compounds, dimethylsulfoniopropionate and vanillate was not detectably different from the total population, suggesting that (with respect to those two compounds at least) the ecosystem was composed of generalist species, all with roughly equal capacity to assimilate DOM (Mou et al. 2008). The results presented here suggest that the ability to assimilate specific polysaccharides is much less evenly distributed across microbial species, and that entire communities can predictably lack the ability to hydrolyze specific polysaccharides.

Analysis of marine metagenomic data, combined with ecological theory, suggest that some bacterial species act as 'opportuni-trophs' with flexible genomes and metabolic capabilities that vary with space and time (Polz et al. 2006). Höfle et al (2008) suggest that rare species act as a "seed bank" which stores specific metabolic capabilities, and resupplies the broader community with those capabilities when necessary. The failure of the

communities in this study to respond to the presence of several polysaccharides indicates that, in the seawater around Svalbard, either the rare microbes that "store" the capacity to hydrolyze pullulan, and sometimes fucoidan, are either sufficiently rare as to be absent from the milliliter-scale incubations used here, or are sufficiently dormant as to not respond for a period of weeks, even as their capabilities would have conferred access to the large amount of polysaccharide, which was inaccessible to the bulk of the microbial community.

4.6 Tables

TABLE 4-1: BACTERIAL PRODUCTION IN SEAWATER MEASURED DURING INCUBATIONS OF DIFFERING LENGTHS

	Assuming 1.5 kg C mol leucine^{-1}				Assuming 3.0 kg C mol leucine^{-1}			
	mean bacterial production, µg C L^{-1} day^{-1}		standard deviation, µg C L^{-1} day^{-1}		mean bacterial production, µg C L^{-1} day^{-1}		standard deviation, µg C L^{-1} day^{-1}	
incubation time	surface	bottom	surface	bottom	surface	bottom	surface	bottom
3 hr	1.33	0.42	0.41	0.05	2.66	0.85	0.81	0.11
8 hr	1.58	0.43	0.17	0.04	3.15	0.87	0.35	0.08
24 hr	1.27	0.42	0.78	0.05	2.54	0.84	1.56	0.10

TABLE 4-2: GUE VALUES FOR PELAGIC COMMUNITIES REPORTED ELSEWHERE

Environment	GGEs reported	Notes	reference
Coastal Bahamas, 0-50 m depth	mean 58%	n=9	(Williams and Yentsch 1976)
Lake Kinnaret, Isreal, 0-10 m depth	61.5%	n≈95	(Berman et al. 1979)
Duplin River Estuary, GA (salt marsh estuary)	70-90%	n≈76	(Hanson and Snyder 1980)
Bay of Marseilles	mean 57.3%	n=6	(Garabétian 1991)
Offshore California, Point Sur to Point Arena	60%-75%	n≈72; These are values from the control samples of an enrichment experiment	(Kirchman et al. 2000)
Equatorial Pacific	40-75%	n=7	(Rich et al. 1996)

4.7 Figures

FIGURE 4-1: MAP OF SVALBARD WITH SAMPLING LOCATIONS

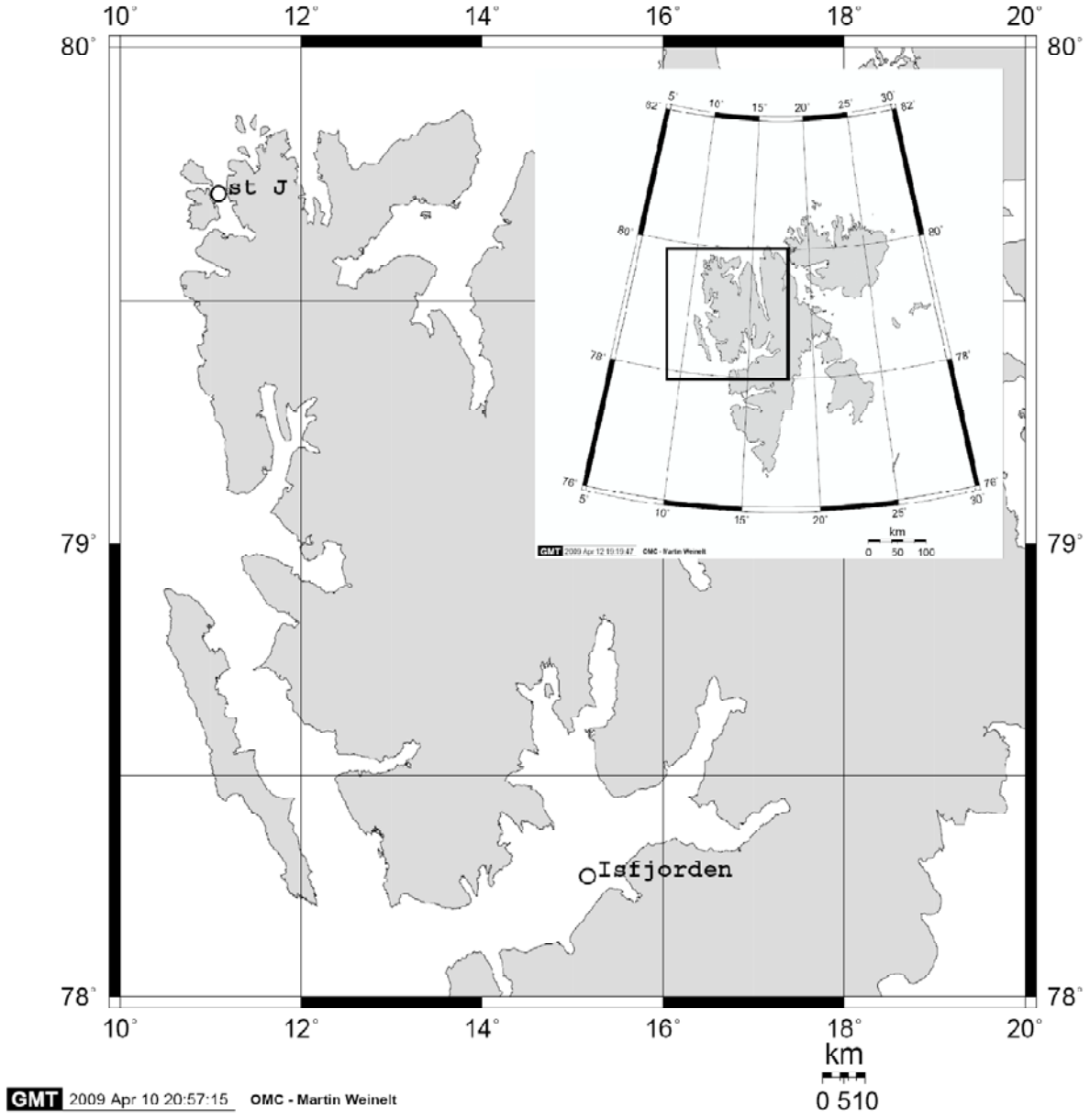

FIGURE 4-2: SEAWATER AND SEDIMENT MAXIMUM HYDROLYSIS RATES (ABSOLUTE AND RELATIVE TO THE SUM OF MAXIMUM RATES).

Substrate abbreviations are: ARA, arabinogalactan, CHN, chondroitin sulfate, FUC, fucoidan, LAM, laminarin, PUL, pullulan, XYL, xylan

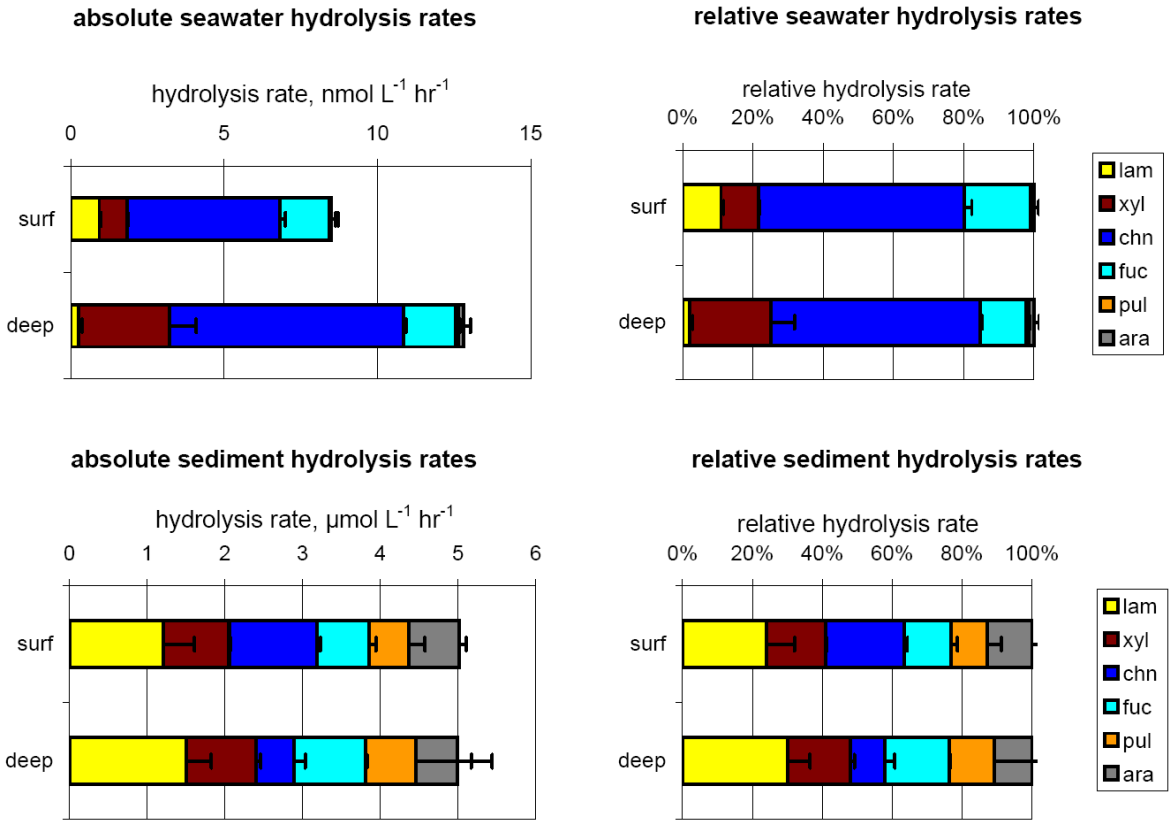

FIGURE 4-3: SEAWATER HYDROLYSIS TIMECOURSES IN SURFACE AND BOTTOM WATER.

For clarity, arabinogalactan and pullulan were omitted. Neither substrate was hydrolyzed in surface water, and both were hydrolyzed only after 40 days in bottom water.

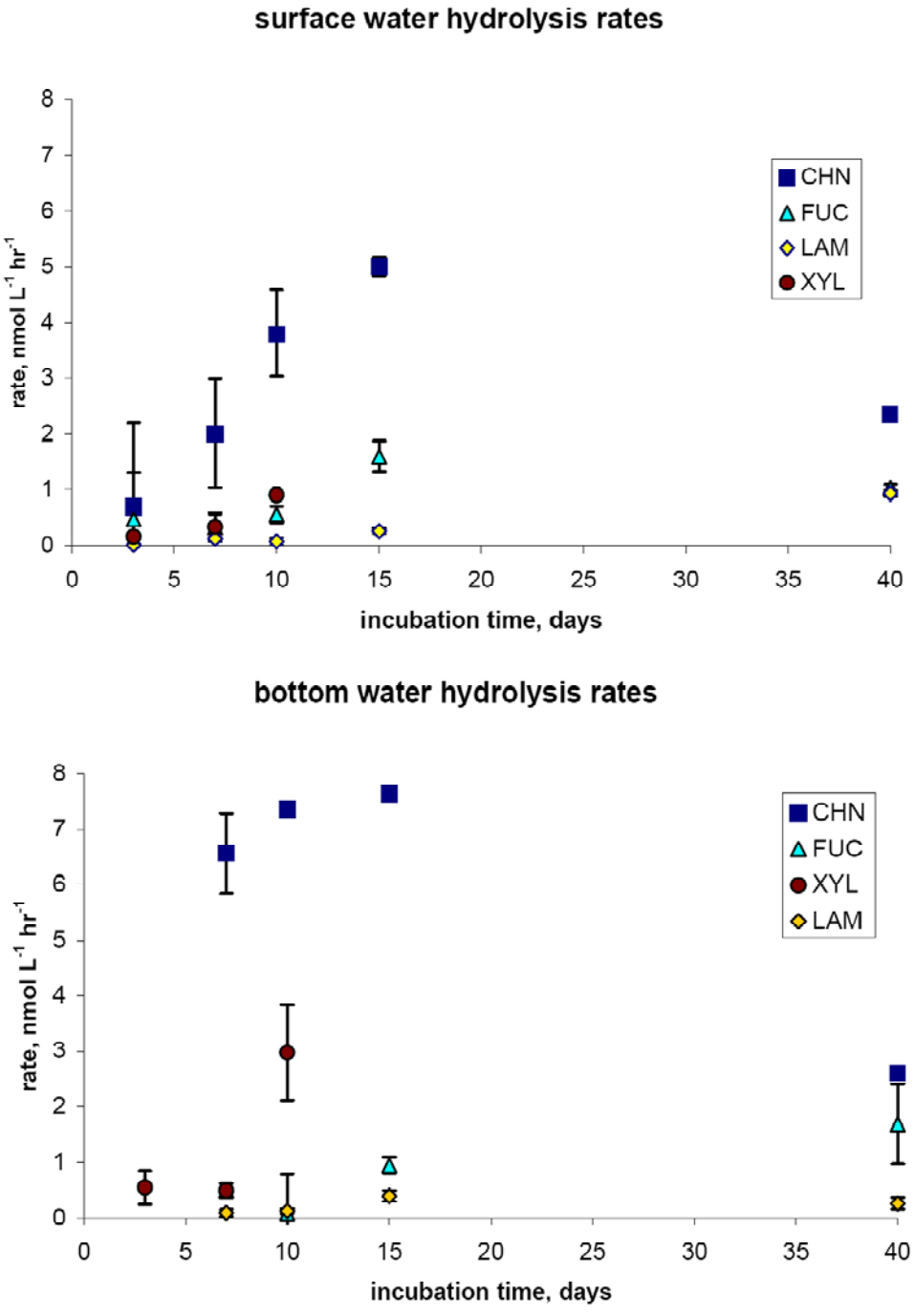

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FIGURE 4-4: HYDROLYSIS TIMECOURSES IN SURFACE AND DEEP SEDIMENT.

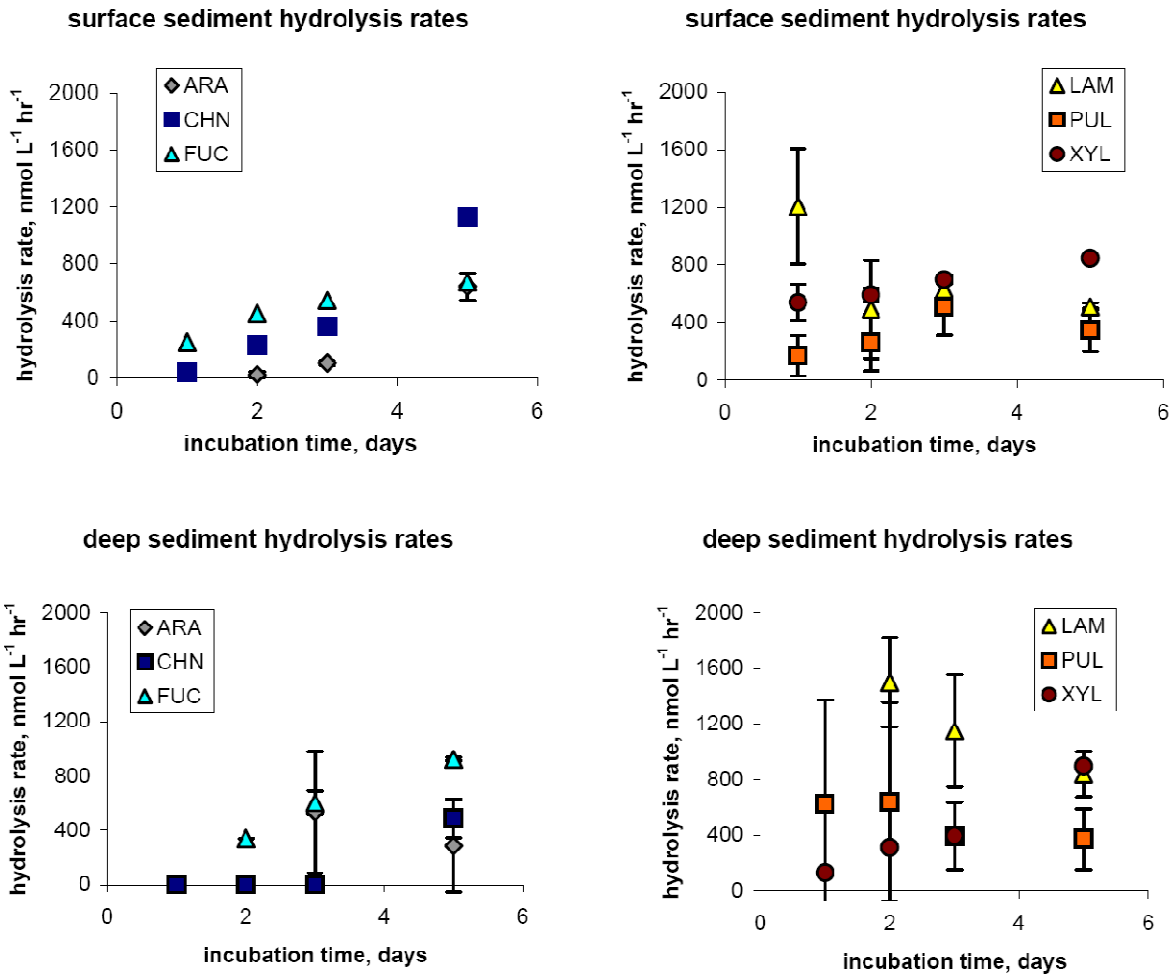


FIGURE 4-5: GLUCOSE ASSIMILATION, MINERALIZATION, AND UTILIZATION EFFICIENCY IN SEAWATER.

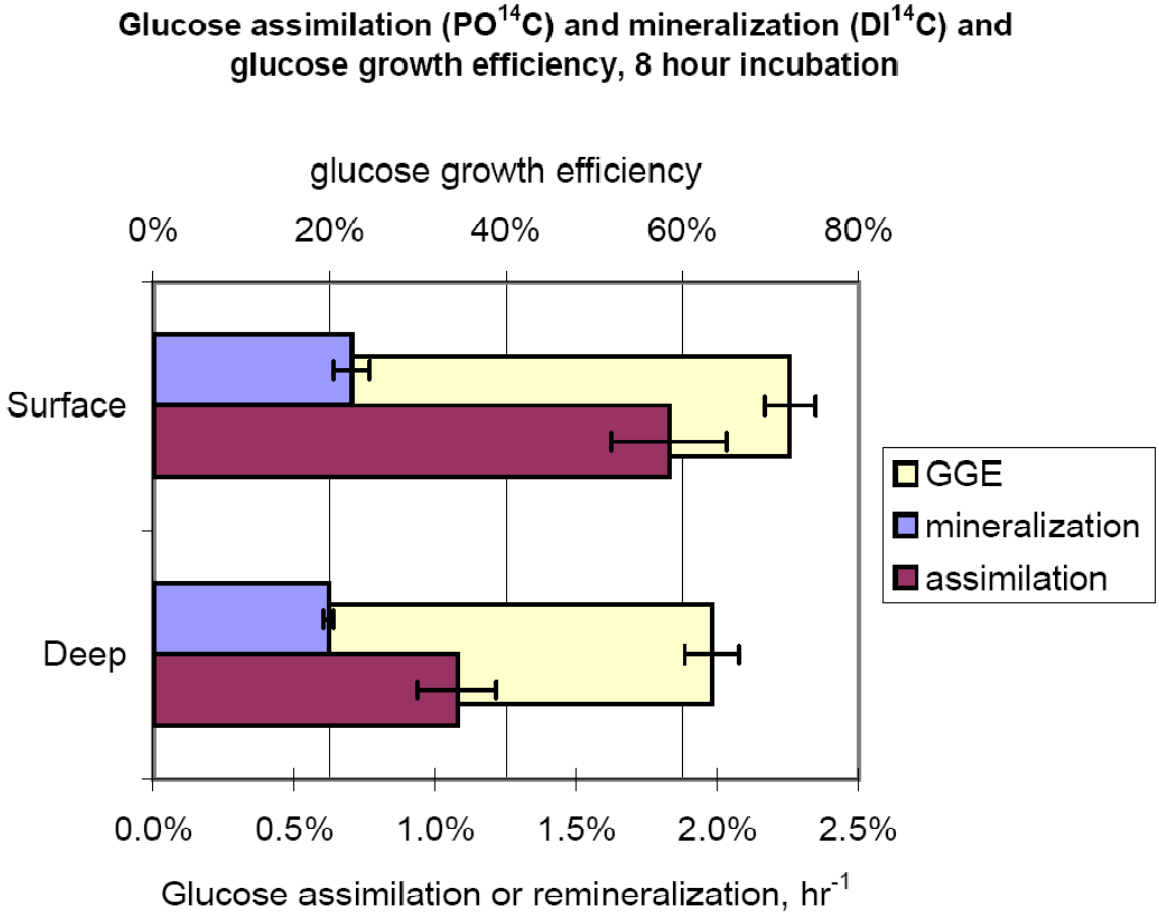


FIGURE 4-6: REPRESENTATIVE CHROMATOGRAMS FROM THE PULLULANASE INDUCTION EXPERIMENTS (2005)

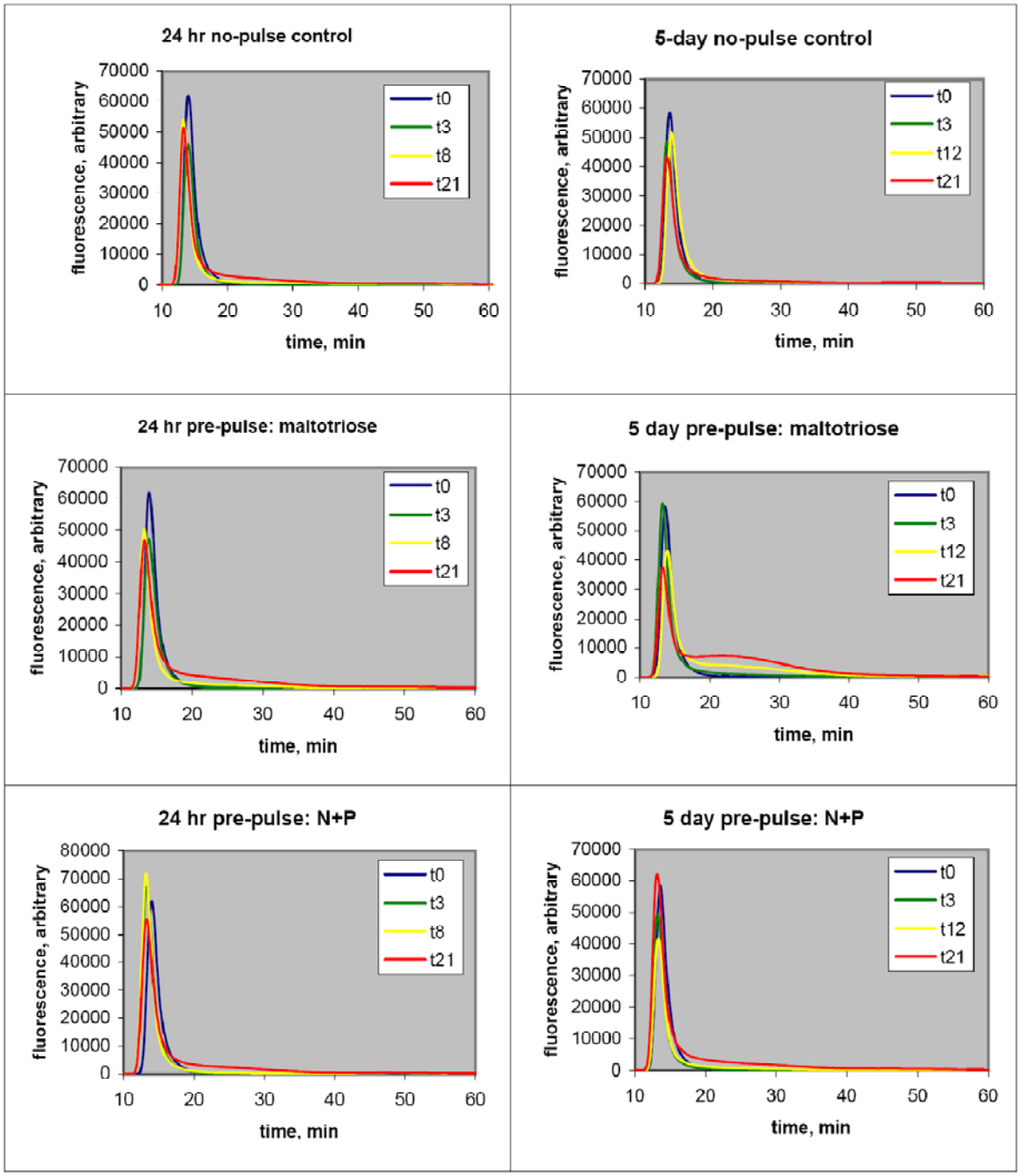
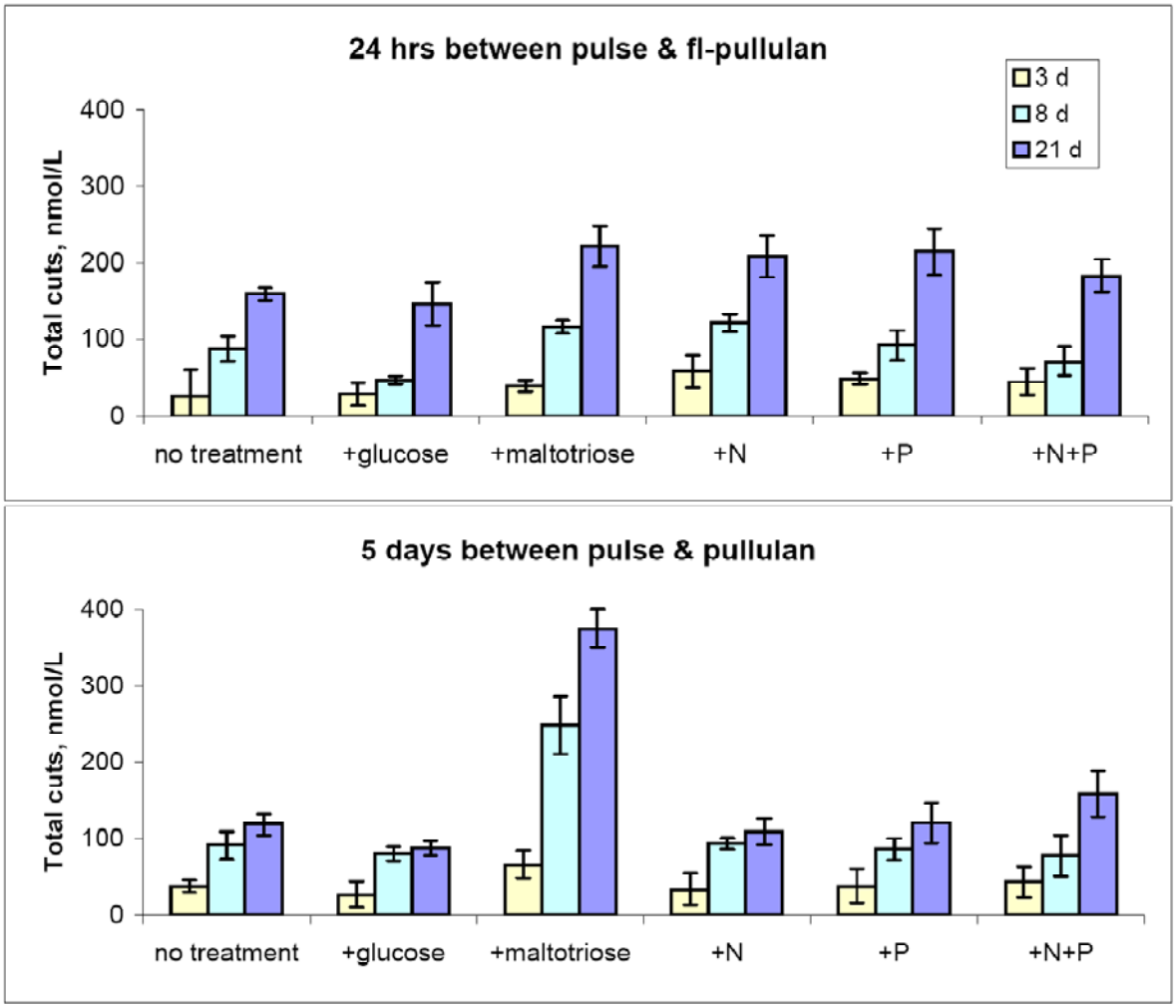


FIGURE 4-7: PUTATIVE EXTENT OF HYDROLYSIS IN THE PULLULANASE INDUCTION EXPERIMENTS (2005).



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Chapter 5 Fluorescence anisotropy as a means to determine extracellular polysaccharide hydrolase activity in environmental samples

Andrew D. Steen[1*], Pramila Gururaj[2], Jiahai Ma[2], Neil V. Blough[2], Carol Arnosti[1]

[1]Department of Marine Sciences, University of North Carolina at Chapel Hill

[2]Department of Chemistry and Biochemistry, University of Maryland

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Current approaches to measure the activities of microbial extracellular enzymes in aquatic environments are hampered by slow throughput or by differences between the structure of simple substrate proxies and macromolecules. Here we show that measurements of fluorescence anisotropy can be used to determine the hydrolysis rate of two fluorescently-labeled polysaccharides, laminarin and xylan, in environmental samples. A simple analysis suggests that the anisotropy of these fluorescently-labeled polysaccharides can be approximated using a modification of the Perrin equation.

5.1 Text

The rates and structural specificities of microbial extracellular enzymes help control the bioavailability and turnover of high molecular weight dissolved organic matter (DOM) in natural waters (Azam and Malfatti 2007). The most widely used method to assess these enzyme activities is based on small substrate proxies (e.g. 4-methylumbelliferyl-β-D-glucopyranoside (King 1986) and l-leucine-7-amido-4-methylcoumarin (Somville and Billen 1983)) that consist of a monomer bound to a fluorophore which fluoresces when the fluorophore-monomer bond is cleaved. This method is inexpensive, precise, and rapid, but the structural differences between macromolecules and small substrate proxies mean that many aspects of enzyme structural selectivity cannot be explored, and the hydrolysis rates obtained with these substrate proxies may differ from those measured with the corresponding polymer (Konopka and Zakharova 2002). Furthermore, small substrate proxies can diffuse into cellular periplasm, so they may record a combination of extracellular and periplasmic enzymatic activity (Martinez and Azam 1996). An alternative approach is to measure directly, either by chromatographic analysis (Arnosti 1996; Arnosti 2003; Pantoja et al. 1997) or by electron paramagnetic resonance (EPR) spectroscopy (Steen et al. 2006), changes in the size of labeled macromolecules due to enzymatic hydrolysis. This approach has the advantage of using macromolecules rather than low molecular weight proxies, and has been used to demonstrate that structurally similar polysaccharides may be hydrolyzed at very different rates in the same water sample (Arnosti 2000; Arnosti et al. 2005; Steen et al. 2008). However, chromatographic analysis for these approaches is relatively slow (30-90 minutes), and lack of portability of an EPR spectrometer precludes direct application in field

studies. Here we demonstrate the utility of fluorescence anisotropy measurements as a rapid (<2 minutes per sample) means to measure microbial extracellular enzyme activities through a comparison of the dependence of fluorescence anisotropy on molecular weight as determined by gel chromatography, permitting real-time data collection in the field. Analysis of the results using a simple model suggests that fluorescence anisotropy can be predicted reasonably accurately for a given molecular weight distribution in samples from fresh, estuarine, and marine environments.

Arnosti et al (2000) demonstrated that the anisotropy of fluorescently labeled (fl)-polysaccharides decreases as fl-polysaccharides are enzymatically hydrolyzed, due to the decrease in anisotropy with decreasing molecular weight. This approach was used to track the hydrolysis of two fl-polysaccharides (fl-laminarin and fl-xylan) incubated in pore waters from sediments of the Chesapeake Bay. This previous study did not quantitatively compare anisotropy changes with chromatographic analysis of hydrolysis, however.

Laminarin (6 kDa) and xylan (8 kDa) were obtained from Fluka and labeled with fluoresceinamine by the method of Glabe (1983) as modified by Arnosti (2003). Surface water samples were collected from freshwater at the head of the Chesapeake Bay, USA, two estuarine sites in Chesapeake Bay, and a coastal ocean site, as described in ref. (Steen et al. 2008). Fl-polysaccharides were added to triplicate, unfiltered water samples to a concentration 3.5 μmol l^{-1} (monomer-equivalent) in samples intended for GPC analysis, and 12.4 μmol l^{-1} (fl-laminarin) or 2.4 μmol l^{-1} (fl-xylan) in samples for fluorescence anisotropy analysis. The samples were incubated at 22 °C for ten days. Subsamples were periodically withdrawn, 0.2-μm filtered, and immediately frozen.

The extent of hydrolysis, equal to the number of free reducing ends produced via hydrolysis, was calculated from GPC chromatograms as in Arnosti (1996). (The GPC data and conditions are reported in Steen et al. 2008). To calculate extent of hydrolysis, each chromatogram was divided into five molecular weight 'bins' spanning the range from 150 kDa to free fluorophore. The amount of carbohydrate in each size "bin" was assumed to be proportional to the fluorescence signal integrated across that bin. The extent of hydrolysis was calculated as the minimum number of enzymatic 'cuts' required to produced the observed chromatogram at each timepoint, from the distribution observed at the initial timepoint.

Fluorescence anisotropy measurements were made using an ISS K2 frequency domain spectrofluorometer in steady-state counting mode as described in Arnosti (2000) with excitation and emission set to 498 and 530 nm, respectively, and measurement time of 100 s. Fluorescence liftetime measurements were acquired with the same instrument employing procedures described previously (Kaiwar et al. 1997).

Fluorescence anisotropy of each substrate was well correlated to the extent of hydrolysis determined by GPC (laminarin: $r^2 = 0.951$, xylan: $r^2 = 0.909$; Fig. 1a, b). One fl-laminarin sample was excluded from this comparison due to high variability among replicates in the GPC measurement. Representative timecourses of GPC chromatograms from the coastal ocean sampling station showing the progression from unhydrolyzed polysaccharide to monomeric (or near-monomeric) form are shown in Figs. 1c and d.

The roughly linear decrease of anisotropy with respect to the GPC-measured extent of hydrolysis and anisotropy is somewhat surprising, since the observed anisotropy arises from an ensemble of polysaccharides with a range of sizes. The relationship between anisotropy

and molecular weight for a single polysaccharide is nonlinear (see below), and the relationship between the size of a polysaccharide and the number of enzymatic cuts needed to arrive at that molecular weight is also nonlinear, so this result was not expected. Nevertheless, this linear relationship held across the entire range of molecular weights for the polysaccharides studied here.

To examine this relationship in more detail, the following analysis was employed. Fluorescence anisotropy *r* of a rigid sphere is given by the Perrin equation (Lakowicz 1999),

$$r = \frac{r_0}{1 + \frac{\tau}{\theta}} \qquad \text{(eq. 1)}$$

where r_0 is the fundamental anisotropy (0.375 for fluorescein; Lakowicz et al. 1985), τ is fluorescence lifetime, and θ is rotational correlation time. θ is given by

$$\theta = \frac{\eta M(\bar{v} + h)}{RT} \qquad \text{(eq. 2)}$$

where η is solvent viscosity, M is molecular weight, $\bar{v}$ is specific volume of the solute and h is hydration. Fluorescence lifetime data for the labeled polysaccharides, collected at pH 8.4 in 50 mM borate buffer, were well fit by a bi-exponential decay: for xylan, fluorescence lifetimes were 3.4 ns (53.7 % of steady-state intensity) and 0.76 ns (46.3%); for laminarin, 3.8 ns (86.3%) and 0.94 (13.7%) The dual lifetimes suggest two distinct populations, likely arising from fluorophores in differing environments within the polysaccharide. The specific volume of a range of neutral carbohydrates is close to 0.649 ml g^{-1} (Adachi and Matsuno 1997), and assuming that half of hydroxyl groups in the polysaccharide are bound to a water molecule (Gekko 1980), we estimate *h* to be 0.22 for laminarin and 0.20 for xylan.

The observed anisotropy for a mixture of fluorophores is given by

$$r = \sum_i f_i r_i \qquad \text{(eq. 3)}$$

where f_i represents the fraction of steady-state fluorescence intensity having anisotropy r_i. Anisotropy as a function of molecular weight was therefore modeled as

$$r = \sum_i \frac{f_i r_0}{1 + \frac{\tau_i}{\theta}} \qquad \text{(eq. 4)}$$

with f_i as the steady-state intensity for each observed lifetime, η= 0.94 cP, ν= 0.649 ml g^{-1}, h= 0.42 ml g^{-1}, and T=300 K. For an ensemble of polysaccharides of varying molecular weights M, therefore,

$$r = \sum_M f_M \sum_i \frac{f_i r_0}{1 + \frac{\tau_i}{\theta_M}} \qquad \text{(eq. 5)}$$

where f_m is the mass fraction of the ensemble having molecular weight M. f_M for each chromatogram was determined by assigning a molecular weight to each elution time (the fluorimeter returned measurements at a frequency of 5 Hz) based on a fit of elution time to the logarithm of the molecular weight of fl-dextran size standards, with a minimum possible MW set to 180 Da.

The anisotropies calculated according to equation 5 are well correlated with observed anisotropies (laminarin: $r_{observed} = 1.01 \pm 0.18 \times r_{calculated} - 0.244 \pm 0.064$, $r^2 = 0.925$; xylan: $r_{observed} = 1.16 \pm 0.28 \times r_{calculated} - 0.300$, $r^2 = 0.895$, reported error is 95% confidence interval). The slope of each of these relationships is close to one, but in both cases observed anisotropy was considerably lower than calculated anisotropy. Fluorescently-labeled macromolecules almost always show lower anisotropy than would be calculated using a rigid-sphere approximation, because internal flexibility of the macromolecule and motion of the

fluorophore relative to the macromolecule cause faster rotational diffusion than would be predicted for a rigid sphere (Yguerabide et al. 1970).

The results of equation 5 were corrected for the offset between calculated and observed anisotropy of the unhydrolyzed polysaccharide,

$$r = r_{calculated} - (r_{calculated,\ unhydrolyzed} - r_{observed,\ unhydrolyzed}) \qquad \text{(eq. 6)}$$

This correction yields a fit line that passes approximately through the origin (laminarin: intercept = -0.003±0.021; xyl: intercept = -0.021±0.021; fig 2). The fact that the offset between calculated and measured anisotropy is fairly constant with respect to molecular weight is consistent with a model in which depolarization arises mainly from two sources: rotation of the polysaccharide as a whole, approximating rigid body rotation, and rotation of the fluorophore independent of the polysaccharide (for instance rotation about the fluorophore-saccharide bond.

The success of this model indicates that equation 6 reasonably approximates the anisotropy of a disperse ensemble of fluorescently-labeled polysaccharides, oligosaccharides, and monomers, in freshwater, estuarine, and marine environments. A complete theoretical description of the anisotropy of such an ensemble would need to account for a great many variables, including pH and ionic strength of the medium, internal flexibility of polysaccharides, and potential changes in fluorescence lifetime as a function of molecular weight. The success of the simple model here indicates that those complicating factors may be ignored in planning experiments that use fluorescence anisotropy to measure hydrolysis rates of labeled macromolecules. The speed and ease of measuring fluorescence anisotropy, combined with the fact that a spectrofluorometer may be paired with a multiwall plate reader and carried to a field station or used aboard a research vessel suggests that fluorescence

anisotropy measurements may open the door to experiments probing the controls on enzyme activities in the environment involving large numbers of samples and real-time data analysis.

5.2 Figures

FIGURE 5-1: ANISOTROPY VS. EXTENT OF HYDROLYSIS FOR PARTIALLY-HYDROLYZED FL-LAMINARIN (A) AND XYLAN (B) AS A FUNCTION OF TIME.

Error bars are +/- one standard deviation for replicate samples. Representative chromatograms from each timepoint at the marine station for fl-laminarin (c) and fl-xylan (d).

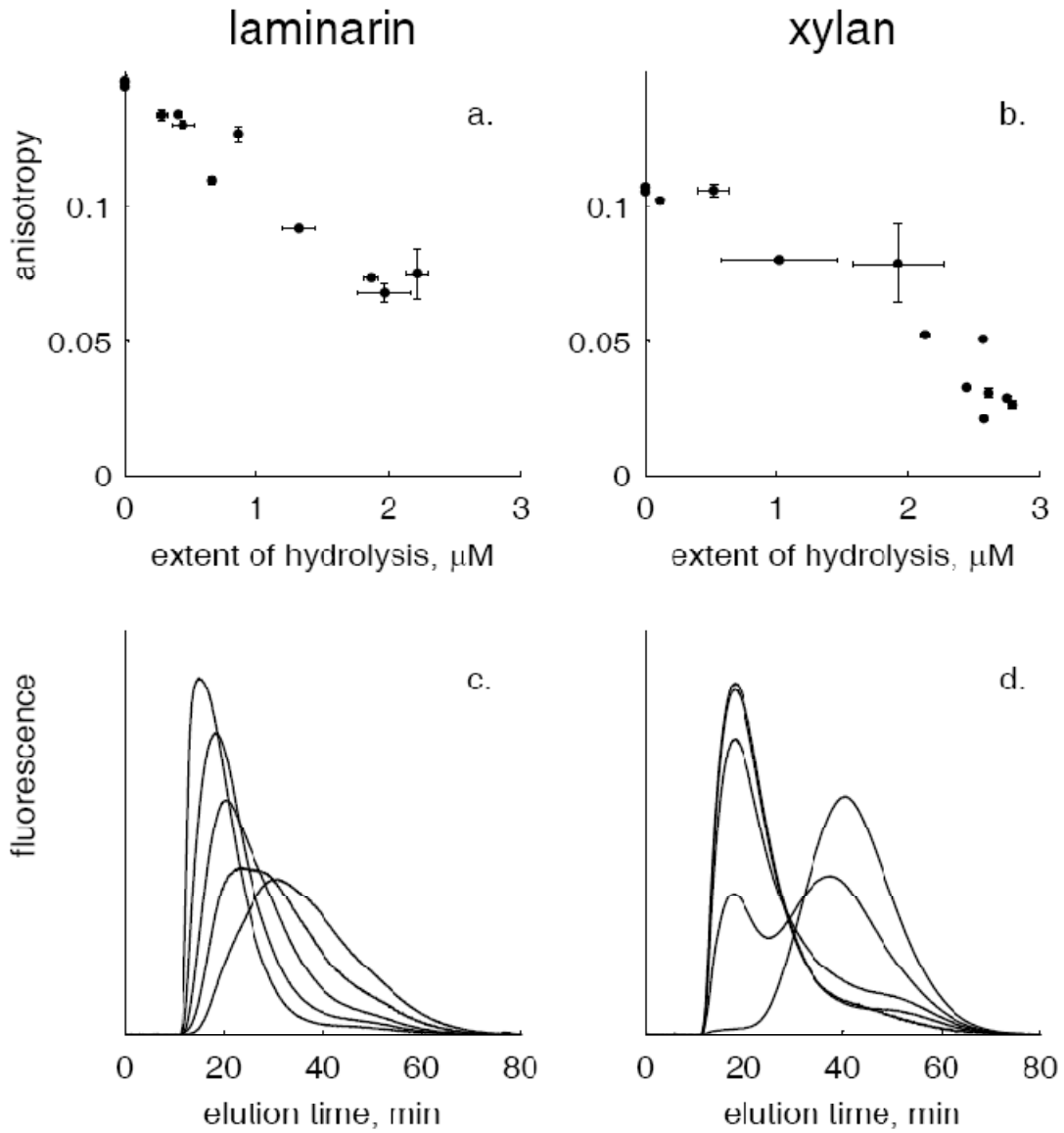
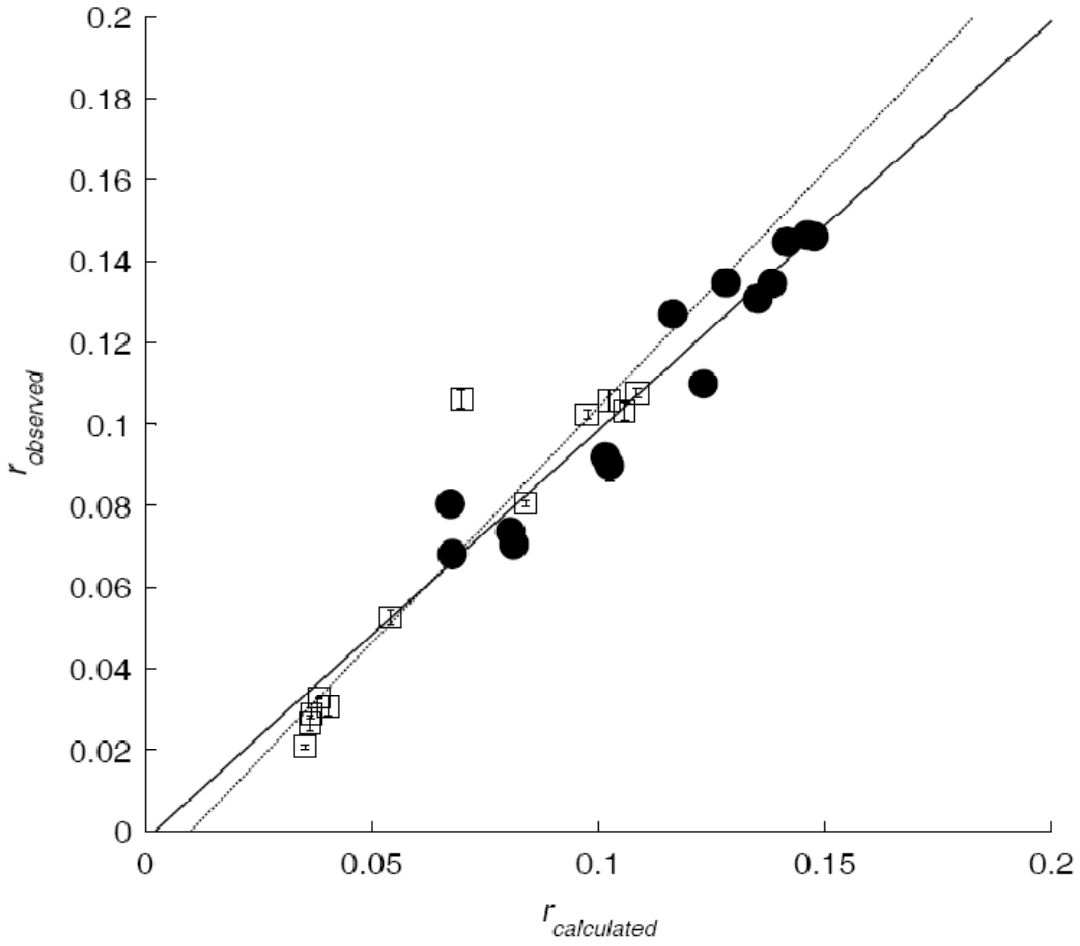


FIGURE 5-2: MEASURED ANISOTROPY VS. CALCULATED ANISOTROPY.

Fl-laminarin (filled circles); fl-xylan (unfilled squares). Error bars are +/- one standard deviation for replicate samples; these are generally smaller than the data points.



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Chapter 6 Dissertation Conclusions

This work demonstrates the existence of substantial regional variation over small spatial scales in microbial community function at the level of processing of individual complex macromolecules (Chapter 3). It explores some of the potential reasons for this variation using two approaches: by probing some potential reasons for the inability of certain communities to access putatively labile compounds (Chapter 4) and by exploring active lifetimes of extracellular enzymes, an important but ill-constrained factor that determines the conditions under which extracellular enzyme production can be a viable strategy for carbon acquisition by marine heterotrophs. Finally, it investigates a novel analytical method to measure polysaccharide hydrolysis rates in environmental samples (Chapter 5), which may useful in future investigations of this sort.

The fields of marine organic biogeochemistry and marine microbial ecology have historically suffered from similar handicaps: we have been long unable to characterize the subjects at the center of the field. As described in the introduction, current analytical techniques to characterize DOC and POC fall short of offering full characterization. On the microbial ecology side, the situation is superficially better: construction of clone libraries based on the 16s genes present in clone libraries is now routine, and newer methods (e.g. V6 tag sequencing and metagenomic methods) can provide huge amounts of raw data about the nucleic acid sequences present in an environmental sample. However, these methods are semi-quantitative at best and typically fail to describe, for instance, the relationships among

genes within a single organism, or the circumstances under which genes identified are expressed. A large fraction of marine organic matter is chemically uncharacterizable (Hedges et al. 2000), while a large fraction of the genes present in seawater are of unknown function.

6.1 Role of this work

I attempted to circumvent these problems by examining the functioning of microbial communities with respect to degradation of model compounds. (I used a suite of polysaccharides, but any other set of structurally-characterized, environmentally realistic model macromolecules might have been equally informative.) The work from the Gulf of Mexico demonstrated variation in microbial community function over short spatial scales, and over depth gradients. We know that microbial community composition in surface water may vary over the length scales examined here, and that thermoclines often divide distinct microbial communities (Morris et al. 2005). (Amplified Ribosomal Intergenic Spacer Analysis [ARISA] performed after this dissertation was defended by Kai Ziervogel and Jennifer Biddle on the water samples investigated in Chapter 3 confirm that similar patterns held true in the samples described here, as well.) It is also well-established that microbial community function can vary in a gross sense over spatial scales. For instance, N_2 fixation varies spatially in the tropical North Atlantic due to inputs of nutrients from the Amazon River plume. However, this sort of variation can be understood without opening the 'black box' of microbial community composition. In this example, N_2 fixation can generally be predicted from nutrient concentrations, without knowing the identity of the microbes present.

Variations in community function at the fine scale measured here cannot be predicted from any environmental parameters of which we are aware.

6.2 Implications for the future

One obvious focus of future research, therefore, is to explain the causes of the variations in community function that were observed here. One place to start is by examining the nature of the structures that are usually hydrolyzed (e.g. laminarin) versus those that are not (e.g. pullulan). Chapter 4 attempted to probe the reasons for the failure of Svalbard communities to hydrolyze pullulan, but was unable to identify a single, simple reason that pullulan was not consistently hydrolyzed in those samples. The data were consistent with the absence of genes for pullulanase in the community, and that may be the most parsimonious explanation. However, as explained in Chapter 4, it is not possible to prove the absence of a gene for a specific function in the environment, and even if it were, such proof would raise the question of why the gene is absent.

Elizabeth Kujawinski has suggested that it will be more productive to understand the microbial processing of organic matter if biochemical pathway, rather than the microbial species, is taken to be the relevant level of organization. Functional genes clearly correlate well with 16s genes at the level of basic metabolic functions because horizontal transfer of entire metabolic systems is thought to be rare. The same is not necessarily true for extracellular polysaccharide hydrolases. Polysaccharide hydrolytic gene systems would seem to be much more modular, in that a new polysaccharide hydrolase would potentially be useful to an organism as long as the organism already had the ability to use some of the component monomers. For that reason, polysaccharide hydrolases would seem to be highly

susceptible to horizontal transmission, indicating that a species-based approach is unlikely to be fruitful.

However, an approach based on recognizing biochemical systems is tremendously challenging. Molecular examination of the genes present in samples may be feasible for well-conserved genetic systems for which we can be reasonable certain that we can identify all of the relevant genes (many genetic systems related to nitrogen cycling may currently fit this bill, for instance). For the initial steps of the degradation of complex organic matter, however, a molecular approach is not yet practical, because the number of molecules subject to degradation is huge, their structures are extremely diverse, and the structures of enzymes needed to degrade them are equally numerous and diverse. The approach employed here is a first step towards identifying biochemical systems present or absent in the environment.

Other geochemically-based approaches might be possible. For instance, Bunte and Simon (1999) measured concentrations and uptake rates of a suite of monosaccharides during a timeseries in lake water. They did not calculate actual uptake rates for specific monosaccharides, however. Changes in ratios of those uptake rates might have provided insight into potential seasonal changes in microbial community composition. It would also be interesting to examine degradation of other types of macromolecules. For instance, the existence of relatively broad-spectrum proteases might hydrolysis of proteins in seawater is much less selective than polysaccharides.

Further description of the relationship between the presence of biochemical systems and rates of biogeochemical processes will be an intermediate step towards what our field's goal should be, which is the ability to make accurate predictions of biogeochemical activity and microbial community composition (which, again, may not be best defined using 16s

genes). To gain this predictive understanding, we need to understand marine microbes, as Farooq Azam has put it, from a microbe's-eye view (Azam and Malfatti 2007). Each chapter in this work represents a step in that direction. Chapter 2 (enzyme lifetimes) provides data that could be useful in models that seek to provide a microbe's eye view of extracellular enzyme production. The evidence in Chapter 3 of variation in microbial community function suggests that the microbial world looks different in different parts of the ocean, and microbes respond accordingly. Chapter 4 tries to understand the reasons a microbial community would fail to access a labile substrate, and Chapter 5 presents a new means with which to obtain that microbes' eye view. These results advance marine biogeochemistry by enhancing our understanding of the selectivity with which marine organic matter is degraded, and advance marine microbial ecology by helping to understand the potential breadth of ecological niches that marine microbes may occupy.

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