# BIOCHEMICAL MECHANISMS OF BACTERIAL UTILIZATION OF DISSOLVED AND PARTICULATE ORGANIC MATTER IN THE UPPER OCEAN

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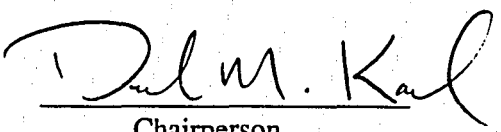

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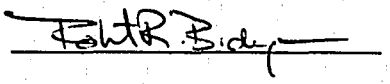

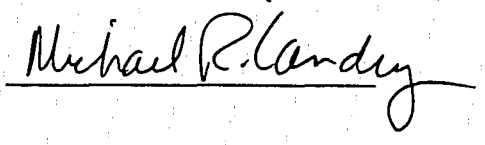

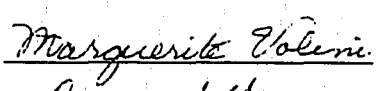

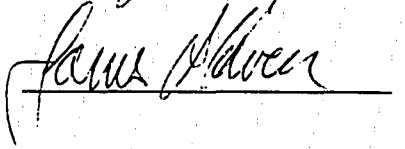

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## ABSTRACT

Dissolved and particulate organic matter (DOM, POM) in the ocean is one of the largest pools of reduced carbon on earth; it is about the same size as the atmospheric carbon pool. The chemical composition of this pool and the mechanisms by which it is produced and consumed by organisms are poorly understood. The decomposition process does not necessarily occur in Redfield ratio, and therefore uncouples the carbon and nitrogen cycles. Understanding the decomposition process is critical to understanding the oceanic carbon cycle and quantifying carbon transport by advection and sedimentation.

Enzymatic hydrolysis is a crucial first step in bacterial utilization of polymeric DOM. Bacterial ectoenzymes were studied in the subtropical Pacific, in the equatorial Pacific and in Antarctica. Leucine aminopeptidase and α- and β-glucosidase appear to be ubiquitous in seawater and marine particles. These enzymes exhibit similar $K_m$'s in different regions of the ocean, suggesting broad cross-habitat homology, but show adaptation to *in situ* temperature in each region. Glucosidases show greater specificity than peptidases. Variation in the relative activities of these enzymes indicates significant seasonal and geographic variation in rates of utilization of different components of DOM. β-glucosidase activity is much greater in the equatorial Pacific than in the other environments studied, and is low in relation to leucine aminopeptidase in Antarctica. Leucine aminopeptidase expression by Antarctic bacterioplankton is regulated by availability of particular amino acids, notably histidine, and may indicate widespread auxotrophy for these compounds.

Particle associated bacterial communities show assemblages of ectoenzymes and temperature responses of those enzymes distinct from those of free-living bacteria;

consistent patterns are observed among the various types of particles. Zooplankton fecal pellets appear to be a minor component of sinking particle flux in the subtropical Pacific. Acidic mucopolysaccharides are abundant in sinking particles, and rates of hydrolysis of these compounds are low. Hydrolytic enzymes and exopolysaccharides of bacteria provide a mechanism for decoupling the carbon and nitrogen cycles in the decomposition process which can produce a net flux of carbon across the thermocline in a region expected to be a net sink for atmospheric $CO_2$.

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## LIST OF ABBREVIATIONS

*Organic Matter Components and Characteristics*

AMPS: acidic mucopolysaccharides
DOM/DOC/DON: dissolved organic matter/carbon/nitrogen
DMCHO: dissolved monosaccharides (sugars)
DPCHO: dissolved polysaccharides
DFAA: dissolved free amino acids
DCAA: dissolved combined amino acids (proteins/peptides)
HMW: high molecular weight
LMW: low molecular weight
PDOM: polymeric dissolved organic matter
POM/POC/PON: particulate organic matter/carbon/nitrogen

*Enzymes and cell structures*

AGase: α-glucosidase
BGase: β-glucosidase
EPS: exopolysaccharide
LAPase: leucine aminopeptidase

*Reagents and Methods*

4MUF: 4-methyumbelliferone
BNAPH: β-naphthylamine
DAPI: 4'6-diamidino-2-phenylindole
HTCO: high-temperature catalytic oxidation
LLBN: L-leucyl-β-naphthylamine
MBTH: 3-methyl-2-benzothiazolinone hydrazone hydrochloride
MUAG: 4-methyumbelliferyl-α-D-glucoside
MUBG: 4-methyumbelliferyl-β-D-glucoside
MUBGal: 4-methyumbelliferyl-β-D-galactoside
MUBGluA: 4-methyumbelliferyl-β-D-glucuronic acid
MUBXyl: 4-methyumbelliferyl-β-D-xyloside
MUNAGA: 4-methyumbelliferyl-N-acetyl-β-D-glucosaminide
PIT: Particle Interceptor Trap

*Study sites and research projects*

(Station) ALOHA: A Long-term Oligotrophic Habitat Assessment
EQPAC 92: 1992 EQuatorial PACific process study (US-JGOFS)
HOT: Hawaii Ocean Time-series
JGOFS: Joint Global Ocean Flux Study
LTER: Long-Term Ecological Research
RACER: Research on Antarctic Coastal Ecosystem Rates
TOGA-TAO: Tropical Ocean Global Atmosphere - Tropical Atmosphere Ocean

*Hydrographic Features and Regions*

ACC: Antarctic Circumpolar Current
WAP: Western Antarctic Peninsula

# CHAPTER 1
# INTRODUCTION

## 1.1 BACTERIA AND ORGANIC MATTER IN THE OCEAN

Bacteria in seawater live among a complex mixture of substances including both nutrients and toxins. Understanding of both the physiology of natural bacterioplankton and the composition of the dissolved organic matter (DOM) that they rely upon for nutrition is very limited. It is a long-standing tenet of marine microbiology that most bacteria in seawater are gram-negative (ZoBell, 1946). Although the Archaea (Archaebacteria) do not readily fit the gram-negative/positive dichotomy (Beveridge and Graham, 1991), molecular phylogenetic analysis has verified that most bacteria in open-ocean surface waters are gram-negative Bacteria (Eubacteria) (Giovannoni et al., 1990a,b).

Gram-negative bacteria have an additional membrane (the outer membrane) outside the primary (cell or plasma) membrane; the space between these (the periplasmic space) is filled with a variety of macromolecules (the periplasm) including hydrolytic enzymes (Inouye, 1979; Graham et al., 1991; Whitfield and Valvano, 1993). The enzymes and other macromolecules that make up the periplasm may be embedded in the outer surface of the cell membrane and both inner and outer surfaces of the outer membrane. Along with exopolysaccharides external to the outer membrane, these are the means by which the cell interacts with the medium in which it lives (Beveridge and Graham, 1991; Whitfield and Valvano, 1993).

Gram-negative bacteria maintain enzymes on the cell surface, in the periplasm or embedded in the membranes, for hydrolysis of substrates too large to be transported

intact across the cell membrane. Gram-positive bacteria, in contrast, are generally believed to secrete their extracellular hydrolytic enzymes directly into the medium (Inouye, 1979). While this characteristic has made certain gram-positive bacteria extremely useful for industrial purposes (Priest, 1987), it may explain why gram-negative bacteria seem to adapt more readily to life in a very dilute medium.

Water is in general a nutritionally dilute environment in comparison to many other bacterial habitats, such as other organisms and their decaying remains. The open ocean is particularly so when compared to coastal, estuarine and most fresh waters. These generally receive substantial inputs of allochthonous organic matter of mostly vascular plant origin. Macrophyte algae are frequently present, and rates of phytoplankton production are generally greater than further offshore. It is important in the context of this thesis to note that the Antarctic continent is an important exception in this respect. Although marine primary production is elevated in nearshore waters as about the other continents, inputs of terrestrially derived organic matter are almost completely absent.

The observation that bacterial growth in seawater can consume a large fraction (30-60%) of contemporaneous primary production (Williams, 1981; Azam et al., 1983; Fuhrman, 1992) has stimulated great interest in the interaction of heterotrophic bacteria with nonliving organic matter in the ocean. Understanding this interaction, however, is still greatly hampered by the inability to chemically characterize much of the dissolved organic matter (DOM) and to culture most of the bacteria in seawater. Significant progress has been made in recent years, through such methods as high-temperature catalytic oxidation (Sugimura and Suzuki, 1988), $^{14}C$ accelerator mass spectrometry (Druffel et al., 1992), $^{13}C$ nuclear magnetic resonance spectroscopy (Benner et al., 1992), molecular phylogenetic analysis (Giovannoni et al., 1990a,b; DeLong et al.,

1993), and, not least, fluorogenic substrate analogues for ectoenzymatic activity (Chróst, 1991). Other important progress has come by applying fairly conventional methods to problems whose importance was only recently recognized. These include the modified MBTH method for dissolved carbohydrates (Pakulski and Benner, 1992; 1994), identification of acidic mucopolysaccharide particles by Alcian Blue staining (Alldredge et al., 1993), and transmission electron microscopy of organic colloids (Wells and Goldberg, 1991).

Bacterial biomass forms a greater fraction of total plankton biomass in the open ocean than in more eutrophic waters (Christian and Karl, 1994). Reasons for this are uncertain, but have important implications regarding the pathways through the foodweb by which organic carbon reaches the bacteria. Because small ($< 2$ μm) cells are the dominant primary producers in oceanic ecosystems, protozoa form the bulk of primary and secondary consumers, and biomass and production of metazoan zooplankton is a small fraction of primary production relative to other ecosystems. The hypothesis that most of the organic matter flux to the bacteria is from the excreta of animals (Jumars et al, 1989) is untenable for open ocean ecosystems. The protozoa, however, are also a potentially significant source of DOM to the bacteria (Antia et al, 1981; Andersson et al, 1985; Nagata and Kirchman, 1992; Radek and Hausmann, 1994) and in oceanic ecosystems the net flow of carbon and energy may be from protozoa to bacteria (Hagström et al., 1988).

The total dissolved organic carbon (DOC) pool in seawater is many times greater than the plankton biomass. A significant fraction of this consists of long-lived, biologically refractory substances. Open ocean surface waters contain a substantial pool of DOC that has been considered both biologically labile and chemically refractory. This pool was generally not observed with ultraviolet-oxidation or wet-chemical-

oxidation methods and was "discovered" only with the advent of high-temperature catalytic oxidation (HTCO) methods (Sugimura and Suzuki, 1988; Hedges and Lee, 1993). The presence of this pool in surface waters and its absence from deep water led to the hypothesis that it was more biologically labile than the DOC observed with the older methods, which shows little variation with depth and therefore has a mean lifetime on the order of the mixing time of the ocean (~1000 y). However, DOC measured by non-HTCO methods does show *some* elevation in surface waters (Druffel et al., 1992). The most biologically labile substances (simple sugars and amino acids) are oxidizable by these methods, but form a small fraction of the total DOC. So the non-HTCO DOC contains both very long- and short-lived substances, while the HTCO-DOC appears to have a shorter mean residence time but contains many substances that may be much longer-lived than the compounds most readily utilized by bacteria. A substantial fraction of this appears to be polysaccharide (Benner et al., 1992; Pakulski and Benner, 1994), but of what kind and what importance as bacterial substrates is uncertain. The relatively constant concentration of this polysaccharide pool in hydrographically and ecologically dissimilar oceanic regions (Pakulski and Benner, 1994) suggests that a substantial fraction of it is long-lived.

Proteins and polysaccharides are a central theme of this thesis. It deals with proteins and carbohydrates as bacterial substrates, and it deals with the proteins (enzymes) and carbohydrates that bacteria synthesize on their cell surfaces to obtain nutrients from the medium. While polysaccharides are substantially more abundant in the ocean, proteins and peptides may play a greater role in regulating the rate of growth of bacteria. Many bacterial species require particular amino acids for growth, as well as minor cell constituents such as vitamins and nucleotides (Guirard and Snell, 1962). Microbiologists have long used the enterobacterium *Escherichia coli* as a model

organism (Neidhardt, 1987), but its ability to grow solely on simple carbon compounds such as glucose and inorganic forms of nitrogen, phosphorus and sulfur may be rare among bacteria in nature (Gottschalk, 1986). While natural bacterioplankton can utilize inorganic forms of these elements (Cuhel et al, 1982; Björkman and Karl, 1994; Kirchman, 1994), whether they possess the ability to synthesize all or most of their cell constituents *de novo* is much more uncertain.

This issue is of great importance for understanding the regulation of bacterial biomass and production in plankton communities, and in other natural microbial communities such as those in soils and sediments. It is central to the concept of heterotrophy as it applies to osmotrophs, and to conceptual models of nutrient limitation. The Liebig-Brandt-Blackman concept of a single limiting nutrient can not necessarily be extrapolated from photoautotrophs to osmoheterotrophs. Limitation of biomass - the concept originally articulated by Liebig (1843) - is conceivable, although in nature populations are more likely to be limited by grazing. But the concept of limitation of growth rate by the least abundant nutrient or element that has been so successfully applied to phytoplankton cultures (Droop, 1983) does not apply to heterotrophic bacteria.

The marine bacterioplankton are a mixed community, both physiologically and taxonomically diverse, and substantially uncharacterized. Yet there are characteristics that appear ubiquitous: as in all of biology there is great diversity but little real novelty. Uptake of organic monomers like glucose or leucine, and the activities of enzymes such as leucine aminopeptidase and β-glucosidase, are observed wherever someone has cared to look: in seawater, in organic aggregates, in surface sediments, in the brine channels of sea ice. Certainly not all of the bacteria express these characteristics, but wherever there is water and oxygen, the basic heterotrophic metabolism asserts itself.

Despite this unity there is little known about the kinds of compounds that form the bulk of the "diet" of natural bacterioplankton, about the rates at which the various fractions of DOM are consumed, about auxotrophic requirements for compounds that can not be readily synthesized, about how the rate of growth is regulated by the environment, and finally, about the pathways by which nonliving organic matter reaches the bacteria from its photosynthetic origin.

The hydrolytic enzymes leucine aminopeptidase (EC 3.4.1.1) and α- and β-glucosidase (EC 3.2.1.20 and 3.2.1.21) are ubiquitous in the ocean and constitute an important mechanism by which bacteria obtain organic nutrients present in polymeric form (proteins or peptides, and carbohydrates) in the medium. By studying these enzymes I have attempted to address some of the broader questions about marine bacteria: What kinds of substrates are utilized by bacteria? How does utilization of particular substrates vary in time and space? At what rate are the major pools utilized? How is growth rate regulated by the mixture of available substrates? What can be inferred about the origin of the DOM that is utilized?

## 1.2 ECTOENZYMES

It has long been known that bacteria in seawater must have enzymes outside the cell to hydrolyze polymeric substrates which they can not assimilate directly. Such enzymes had long been studied, but indirectly, in cultured isolates (Kriss et al., 1963; Prescott and Wilkes, 1966; Merkel and Sipos 1971). Perry (1972) attempted to assay alkaline phosphatase directly in open ocean waters using 3-o-methylfluorescein phosphate, but the sensitivity was inadequate to give realistic estimates of *in situ* activity. Maeda and Taga (1973) developed an assay for deoxyribonuclease activity

based on generation of deoxyribose, which they used mostly in sediments but also in the surface waters of Tokyo Bay. Joint and Morris (1982, p. 66) wrote in an early review:

"Bacteria are incapable of ingesting particulate organic matter; they have no mechanism of phagocytosis or pinocytosis and all organic matter must be soluble before it can be transported across the cell membrane. Of course, bacteria do utilize particulate organic matter but these complex organic molecules must be acted upon by extracellular enzymes to produce soluble organic compounds."

At the time there were few published studies of such enzymes in natural waters, and no simple and reproducible method for studying them. The following year Hoppe (1983) and Somville and Billen (1983) published the first studies of bacterial extracellular enzymes *in situ* using fluorigenic substrate analogues derived from 4-methylumbelliferone and β-naphthylamine, more sensitive successors to the fluorogen used by Perry (1972). These early studies, however, and most of those that followed, were conducted in eutrophic coastal and fresh waters (summarized in Chróst, 1991a). Rosso and Azam (1987) determined aminopeptidase activities in mesotrophic shelf waters (Santa Monica Basin). More recently, activities in open ocean waters have been determined (Hoppe, 1991; Hoppe et al., 1993; Christian and Karl, 1995a,b).

Early investigations of leucine aminopeptidase and α- and β-glucosidase in coastal seawater suggested that the proteolytic enzyme had a much higher $K_m$ than the glucosidases (Hoppe, 1983; Somville and Billen, 1983; Somville, 1984). Later studies in eutrophic European lakes showed β-glucosidase with much higher $K_m$ than these initial observations (Chróst, 1989). Size fractionation studies showed that the enzymes

were largely associated with the bacterial size fraction (Hoppe, 1983; Somville and Billen, 1983; Vives-Rego et al., 1985). The enzymes were therefore believed to be bound to cell surfaces and not free in seawater, i.e., periplasmic enzymes or ectoenzymes. This characterization has recently been challenged, however, and in some cases a substantial fraction of the enzymatic activity may pass a 0.2 µm membrane filter. This is believed to be a consequence of microzooplankton grazing (Karner et al., 1994) but the mechanism is not well understood.

Chróst (1991b) argued against use of the term "exoenzyme" to signify extracellular enzymes, as it conflicts with the other meaning of this term. An exohydrolase catalyzes hydrolysis of polymers at their ends (removing single residues); an endohydrolase catalyzes hydrolysis at other points. By this definition, then, the enzymes studied using fluorigenic substrate analogues are exohydrolases, as in most cases only a single sugar or amino acid residue is bound to the fluorogen molecule. There have been few studies of endohydrolases in the ocean. Merkel and Sipos (1971) characterized an endopeptidase from a marine *Vibrio* sp. Hollibaugh and Azam (1983) estimated rates of degradation of dissolved protein using $^{14}C$ and $^{125}I$ labeled proteins. Most of the studies published since 1983 have been of exohydrolases only, but competitive inhibition of hydrolysis of substrate analogues by polymers (Martinez and Azam, 1993a) suggests that this is in fact the rate limiting step in bacterial utilization of polymeric organic matter. Chróst (1991b) further argued that the term "ectoenzyme" should be used as he believed that the evidence for a periplasmic location for these enzymes was strong (see also Martinez and Azam, 1993b). Notwithstanding recent reports that a substantial fraction of enzymatic activity in seawater occurs free in solution, I will use refer to enzyme activities determined in seawater as "ectoenzymes" which signifies "ecto- and otherwise extracellular exohydrolases."

## 1.3 EXOPOLYSACCHARIDES

Many bacteria synthesize polysaccharides exterior to the cell wall and the outer membrane, which frequently contain a large percentage of acidic or anionic sugars (Costerton et al., 1981; Whitfield and Valvano, 1993). The functions of exopolysaccharide (EPS) for free-living bacteria are diverse and poorly understood (Dudman, 1977). In seawater these may include adhesion to surfaces, scavenging of dissolved substances and catalysis of oxidation-reduction reactions of metal ions. EPS may also help to prevent viral infection (Lindberg, 1990), and otherwise mediate interactions with other organisms.

Exopolysaccharides are important for understanding the ecology of marine bacteria and ocean biogeochemistry for several reasons. Firstly, they mediate adhesion to particles and therefore catalyze the transformation of particulate organic matter (POM) to DOM (Azam and Cho, 1987). Secondly, they form a carbon-rich and potentially refractory fraction of sinking POM, and therefore affect the C/N ratio of sinking particles and the flux of carbon across the pycnocline (Martin et al., 1987; Karl et al., 1988). Finally, EPS itself is a potentially significant source of DOM, and one that may be long-lived and refractory.

## 1.4 STUDY AREAS: BACTERIAL PROCESSES IN WARM AND COLD OCEANS

The three study areas were the North Pacific near Hawaii (principally Station ALOHA), the equatorial Pacific (principally the South Equatorial Current) and the

Antarctic Peninsula region of the Southern Ocean. These three study areas present stark contrasts, ranging from a low latitude, oligotrophic convergent gyre environment, to an equatorial, open-ocean upwelling environment, to a high-latitude, coastal upwelling environment. Water temperatures ranged from the freezing point of sea water (-1.68 °C) to nearly 30 °C. However, all three of these study areas experience little seasonal variation in water temperature.

The Antarctic Peninsula and the subtropical North Pacific present an extreme contrast with respect to nutrients. In the subtropical gyre, concentrations of organic nitrogen and phosphorus are much greater than those of the inorganic pools, which are consistently less than 0.1 μM (Karl et al., 1993). In the waters off the Antarctic Peninsula inorganic nutrient concentrations are high ($[NO_3^-]$>20μM) except during occasional intense phytoplankton blooms, whereas organic nitrogen concentrations are among the lowest observed in the ocean (Karl et al., 1995a). The equatorial current system is intermediate in this respect, with higher inorganic nutrient concentrations and lower organic nutrient concentrations than in the subtropical gyre.

These three environments are all oligotrophic relative to temperate coastal oceans, estuaries and most fresh waters. In each case total plankton biomass (and bacterial biomass) is low relative to these systems, with the exception of the brief, intense blooms observed in the coastal waters of the Antarctic Peninsula. In coastal Antarctic waters, an extreme temporal uncoupling of photosynthesis and respiration results in rapid accumulation of biomass, but not necessarily in production of dissolved organic matter available to the heterotrophic bacteria (Karl et al., 1995a).

Primary production per unit biomass is high in the subtropical and equatorial oceans, as are rates of nutrient recycling. The dominance of picophytoplankton at low latitudes and their absence in Antarctica has important consequences for the dynamics

of microbial food webs and production of organic matter. The microbial loop concept as articulated by Azam et al. (1983), with microzooplankton as grazers primarily of heterotrophic bacteria, is valid only when the primary producers are nanoplankton or larger. When microzooplankton graze extensively on phytoplankton they may be an important source of DOM for the heterotrophic bacteria as well as consumers of them, and a net energy transfer from microzooplankton to bacteria becomes thermodynamically possible (Hagström et al., 1988).

Dinitrogen fixation by *Trichodesmium* spp. plays an important role in biogeochemical cycling at Station ALOHA, and shifts the ecosystem from nitrogen to phosphorus limitation during periods of calm weather (Letelier, 1994; Karl et al., 1995b). Dinitrogen fixation tends to increase during late summer and early autumn, when the trade winds are weakest (Wyrtki and Myers, 1976) and in some years this ecosystem state is observed for much of the year (Karl et al., 1995b). During these periods the ecosystem most resembles the classical oligotrophic ocean model, with high rates of primary production but little new or export production (i.e., rapid recycling of N and P within the euphotic zone). However, a regular seasonal increase in particulate export is observed during the autumn trade wind minimum, and this is hypothesized to result from input of "new" nitrogen by dinitrogen fixation (Karl et al., 1995c). Another significant recent development is the observation that a large fraction of export production at Station ALOHA consists of diatoms (Karl et al., unpublished data). Whether periodic diatom production is forced by cross-thermocline mixing as hypothesized by Goldman (1993) and is therefore contemporaneous with periods of instability (which is correlated on an interannual basis with decreased primary production and increased export production) is not clear. The seasonal distribution of

the flux of diatoms to the deep ocean suggests that at least some of this flux is driven by dinitrogen fixation (i.e., peak flux occurs in summer).

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# CHAPTER 2

# BACTERIAL ECTOENZYMES IN THE OCEAN: AMINOPEPTIDASES AND GLUCOSIDASES

## 2.1 INTRODUCTION

The method of using fluorigenic substrate analogues as tracers for ectoenzymatic activity in natural waters was introduced by Hoppe (1983) and Somville and Billen (1983); a useful summary is given by Hoppe (1993). A significant departure from the methods used by other investigators was the use of mercuric chloride as a preservative and as a control for background fluorescence and abiotic hydrolysis. A paper presenting the rationale and justification for this modification has been published (Christian and Karl, 1995); section 2.3.3 is drawn from this paper. Sections 2.3.1 through 2.3.3 deal largely with the methodology; sections 2.3.4 through 2.3.6 summarize results concerning the biochemistry of the enzymes.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Enzymes

The substrate analogues used were derivatives of 4-methylumbelliferone (4MUF) and β-naphthylamine (BNAPH) principally L-leucyl-β-naphthylamine (LLBN), 4-methylumbelliferyl-α-glucoside (MUAG), and 4-methylumbelliferyl-β-glucoside (MUBG). Several other 4MUF derivatives were used in certain experiments (4-methylumbelliferyl-β-galactoside, 4-methylumbelliferyl-β-xyloside,

4-methylumbelliferyl-β-glucuronic acid, and 4-methylumbelliferyl-β-N-acetylglucosamine) and are described as 4MUF-X, where X is the common abbreviation for the attached sugar moiety.

The enzymes hydrolyzing LLBN, MUAG and MUBG are referred to as leucine aminopeptidase (EC 3.4.1.1), α-glucosidase (EC 3.2.1.20), and β-glucosidase (EC 3.2.1.21), and abbreviated as LAPase, AGase, and BGase, respectively. However, it should not be assumed that the enzymes of natural seawater bacteria are identical to those described in the historical enzymological literature (e.g., Delange and Smith 1971) or that *in vivo* activities can be attributed to a single enzyme. Rath and Herndl (1994) found two distinct isozymes of β-glucosidase in samples taken from macroparticulate matter in the Adriatic Sea. Although diversity was low in this microenvironment, their results suggest that seawater samples should not be considered analogous to samples of individual enzymes *in vitro*.

### 2.2.2 Study areas

Experiments were carried out in the Pacific subtropical gyre (Station ALOHA), in the equatorial Pacific, and in the Antarctic Peninsula region of the Southern Ocean. The equatorial study area was visited only once, in March-April 1992 (RV Thomas G. Thompson cruise TT008). Cruises to Station ALOHA (HOT cruises) are referred to as HOT-## beginning with HOT 1 in October 1988. Cruise designations and dates in Antarctica are as follows: RACER 3 (December 1991 - January 1992), RACER 4 (July-August 1992), PD92-09 (November 1992), NBP93-02 (March-May 1993), PD94-01 (January-February 1994), and PD94-12 (December 1994).

### 2.2.3 External fluorescence standards

4MUF and BNAPH were obtained from Sigma Chemical Co. Stock solutions were made up in distilled water to a final concentration of 100 µM. 4MUF (~20 mg) was first dissolved in 2 ml methyl cellosolve (2-methoxyethanol, Sigma Chemical), then diluted to 1 l with distilled, deionized water (ddw). BNAPH (~15 mg) was dissolved directly in 1 l ddw by heating in a glass bottle with magnetic stirring (BNAPH should first be crushed to a fine powder to accelerate dissolution). The liquid is brought to boiling, then the heat is reduced to just less than 100 °C and the bottle kept loosely capped to minimize evaporation. BNAPH can also be dissolved in methyl cellosolve, but this approach was used on only one cruise.

These stock solutions were serially diluted to concentrations of 10, 1, and 0.1 µM in ddw and 100, 200, and 500 µl aliquots of these solutions were added to 6 ml of seawater. Fluorescence was determined in a Perkin-Elmer LS-5 spectrofluorometer with quartz or polymethacrylate (Sigma Chemical) cuvettes. Polymethacrylate has transmission properties similar to quartz for wavelengths greater than ~300 nm.

### 2.2.4 Ectoenzyme assays

Stock solutions of the fluorescent substrate analogues were prepared in much the same way, with methyl cellosolve used in 1:500 ratio for 4MUF derivatives. LLBN is water soluble at room temperature and does not require methyl cellosolve. The fluorescent substrate analogue (MUBG or LLBN) was added to 6 ml of seawater and incubated for 12-24 h. All incubations were conducted in the dark. Samples not analyzed immediately following the incubation were poisoned with 0.1 ml of a

saturated solution of mercuric chloride (final concentration ~4 mM) to stop the reaction, and stored frozen if the analysis was not conducted within 24 h (cf. Christian and Karl 1995). Mercuric chloride precipitates excess LLBN which was removed by filtration (0.2 μm) or centrifugation prior to fluorescence determination.

Fluorescence was measured in a Perkin-Elmer LS-5 or LS-5B spectrofluorometer. Fluorescence was corrected for nonenzymatic hydrolysis and background fluorescence of the substrate analogue by subtracting the fluorescence of control samples with mercuric chloride added at time zero. Activity in nmol $l^{-1}$ $h^{-1}$ was calculated as $(F-F_0)\cdot A/t$, where $F_0$ is the control fluorescence, A is the concentration in nM given by 1 fluorescence unit and t is the incubation time in hours.

Fluorescence of 4-methylumbelliferone (4MUF) was measured at 447 nm with excitation at ~360 nm. Because the pH of surface seawater is generally lower in Antarctica than at our other two study sites, the excitation spectrum of 4MUF is shifted towards shorter wavelengths and its overlap of the excitation spectrum of MUBG is increased. Background fluorescence is decreased and the sensitivity of the method improved if the fluorescence is determined at an excitation wavelength greater than the maximum for free 4MUF fluorescence. Excitation and emission wavelengths for β-naphthylamine (BNAPH) were 337 and 411 nm respectively. Bandwidths were 10 nm in all cases.

### 2.2.5 Data analysis

The relationship of enzymatic activity to substrate concentration is described by the Henri-Michaelis-Menten (HMM) equation

$$V = \frac{V_{max} S}{K_m + S} \qquad (2.1)$$

where V is the activity at substrate concentration S, $V_{max}$ is the activity at saturating substrate concentration, and $K_m$ is the substrate concentration at which $V=V_{max}/2$. The exact expression for $K_m$ (in terms of rate constants) depends on the reaction mechanism, which is not known. Under certain conditons $K_m$ approximates the equilibrium constant for the binding of the substrate to the enzyme ($K_s$).

Equation 2.1 can be linearized by several different transformations to permit the values of $K_m$ and $V_{max}$ to be estimated either graphically or by a standard Model I linear regression. The three commonly used transformations are

$$V = V_{max} - K_m (V/S) \qquad (2.2)$$

$$S/V = (K_m/V_{max}) + (1/V_{max}) S \qquad (2.3)$$

$$1/V = (1/V_{max}) + (K_m/V_{max}) (1/S) \qquad (2.4)$$

The last (equation 2.4) is the Lineweaver-Burk, or "double reciprocal" plot. These linear transformations give poor estimates of $K_m$ and $V_{max}$, because in the transformation of the data errors in the smallest values become disproportionately important (Dowd and Riggs, 1965; Atkins and Nimmo, 1975). Direct fitting of equation 2.1 by iterative nonlinear least-squares techniques such as Gauss-Newton is preferable because the sum of squares of the residuals of the raw data is minimized rather than that of the transformed data (Johnson and Faunt, 1992). With digital computers now universally accessible there is little reason to rely on linear

transformations to estimate $K_m$ and $V_{max}$, although these techniques are still useful for rapid visualization and for graphical representation of inhibition.

Models were fit using a Gauss-Newton algorithm based on Tarantola (1987) and Johnson and Faunt (1992). If equation 2.1 is expressed as d = g(m) where d=V and $m=[V_{max}\ K_m]^t$, the sum of the squares of the residuals d-g(m) can be minimized by iteratively applying the equation

$$\sum_{j=1}^{k} \frac{\partial g_i}{\partial m_j}(n_j - m_j) = d_i - g_i(m) \qquad (2.5)$$

where $\partial g_i / \partial m_j$ is the derivative of g with respect to $m_j$ evaluated at the *i*th value of the independent variable (S in equation 2.1) and $g_i(m)$ is the *i*th value of g(m), to derive a new value of m (here called n) nearer to the optimal value. This equation can be written in matrix form as

$$G\,(n-m) = d - g(m) \qquad (2.6)$$

where G is a matrix of the partial derivatives of g such that $G_{ij} = \partial g_i / \partial m_j$. In order to solve this equation for n it is necessary to invert G, which is achieved by creating the Hessian matrix $G^tG$, which is square and invertable. Equation 2.6 can then be written as

$$n = m + (G^tG)^{-1}\,G^t\,(d-g(m)) \qquad (2.7)$$

At each iteration m is set equal to n until convergence is achieved. Given the four assumptions outlined by Johnson and Faunt (1992), this algorithm will converge upon the maximum likelihood value of m (which is equivalent to that which minimizes the sums of squares of the residuals if the assumptions are met). The Hessian matrix is equal to the variance-covariance matrix for m, from which the *a posteriori* variances and correlation coefficients for the model parameters are derived.

I have used the simplest case with no data weighting (i.e., all data points weighted equally). As with all nonlinear curve-fitting routines, this is somewhat sensitive to "first guess" values, and the initial value of m must be reasonable for convergence to occur. In most cases values derived from equation 2.4 using model I linear regression were used, although in a few cases these failed to converge and a visual search of the parameter space was required.

If a second, competing substrate is added to the incubation vessel, equation 2.1 is expanded to

$$V = \frac{V_{max} S}{K_m(1 + I/K_i) + S} \qquad (2.8)$$

where I is the concentration of the second substrate and $K_i$ is the equilibrium constant for its binding to the enzyme. Equation 2.8 can be easily solved for $K_m$, $K_i$, and $V_{max}$ using equations 2.5-2.7, but arriving at a first-guess value was more difficult than with the simpler form. In general values of $K_m$ and $V_{max}$ derived for the "no inhibitor" treatment were adequate but sometimes several trials were needed to find an appropriate value of $K_i$.

## 2.3 RESULTS

### 2.3.1 Precision

Duplicate or triplicate subsamples from a single water sample (i.e., Niskin® or GoFlo® bottle) show varying degrees of reproducibility in the three study areas and among the different assays. This variance is the aggregate of analytical variance, sampling variance at the finest level (6 ml subsamples from a single 125-500 ml sampling bottle) and incubation effects. The actual variance of the activities in a given environment is necessarily greater if it is assumed to incorporate the observed variance into the larger scale spatial and temporal variability. Temporal (which includes a spatial component) variability can be estimated for Station ALOHA and for the equatorial Pacific where repeated hydrocasts were taken at the same location (see Chapter 3).

Precision for the different assays is summarized in Table 2.1. In each environment the spatial and temporal distribution of the samples is slightly different. At Station ALOHA the samples were collected on various cruises spanning several years, at a single location. On EQPAC 92 the data are from a single cruise, taken at a single location over a period of about 20 days. On cruises in Antarctica samples were collected at various locations, and statistics are given only for individual cruises. Measures of precision necessarily differ between cases where duplicate and triplicate subsamples were collected. Where triplicates were taken precision is given as the coefficient of variation (CV) given by

$$CV = \frac{s}{\bar{x}} \cdot 100\% \qquad (2.9)$$

where s and $\bar{x}$ are the standard deviation and the mean. Where only two subsamples were taken the mean percent difference (MPD) is given by

$$MPD = \frac{|x_1 - x_2|}{\bar{x}} \cdot 100\% \qquad (2.10)$$

### 2.3.2 Linearity

Because ectoenzyme activities in oligotrophic ocean waters tend to be low, it was necessary to incubate samples for extended periods, in most cases 12-24 h. It is therefore necessary to establish the linearity of the change in fluorescence over time, to determine whether activities integrated over the course of the incubation are representative of initial velocities and whether bottle effects are significant. In general the rate of change of fluorescence over time is constant (i.e., the time course of fluorescence is linear) over periods considerably longer than the incubation periods normally employed (up to 54 h, see Figure 2.1). In one case (in Gerlache Strait on RACER 3) the time course for BGase was strongly nonlinear: the mean activity was significantly greater over 40.5 h than over 16.5 h. The conditions observed in this profile were somewhat atypical for this environment, and nonlinear responses were not observed at all depths. The nonlinear response was greatest in the anomalous activity peak observed at approximately 50 m. This is an atypical feature for this environment, and repeated sampling at the same location showed it to be a transient one (Christian and Karl, 1992; see Chapter 3).

Table 2.1. Precision of duplicate or triplicate subsamples incubated for ectoenzyme activity. CV = coefficient of variation (equation 2.9); MPD = mean percent difference (equation 2.10). Alternate solution for equatorial LAPase has 3 outliers removed (see Chapter 3).

| Area or Cruise | Enzyme | Statistic | Mean | Min | Max | N |
|---|---|---|---|---|---|---|
| HOT | BGase | CV | 29.4 | 2.1 | 129.2 | 24 |
| HOT | LAPase | MPD | 15.0 | 1.0 | 75.8 | 28 |
| EQPAC92 | BGase | MPD | 9.8 | 0.0 | 55.7 | 30 |
| EQPAC92 | LAPase | MPD | 28.9 | 0.6 | 174.1 | 30 |
| EQPAC92 | LAPase | MPD | 13.4 | 0.6 | 44.4 | 27 |
| PD9209 | LAPase | MPD | 5.9 | 0.0 | 13.1 | 17 |
| NBP9302 | BGase | CV | 8.7 | 2.9 | 18.6 | 5 |
| NBP9302 | LAPase | MPD | 9.9 | 0.0 | 50.0 | 15 |

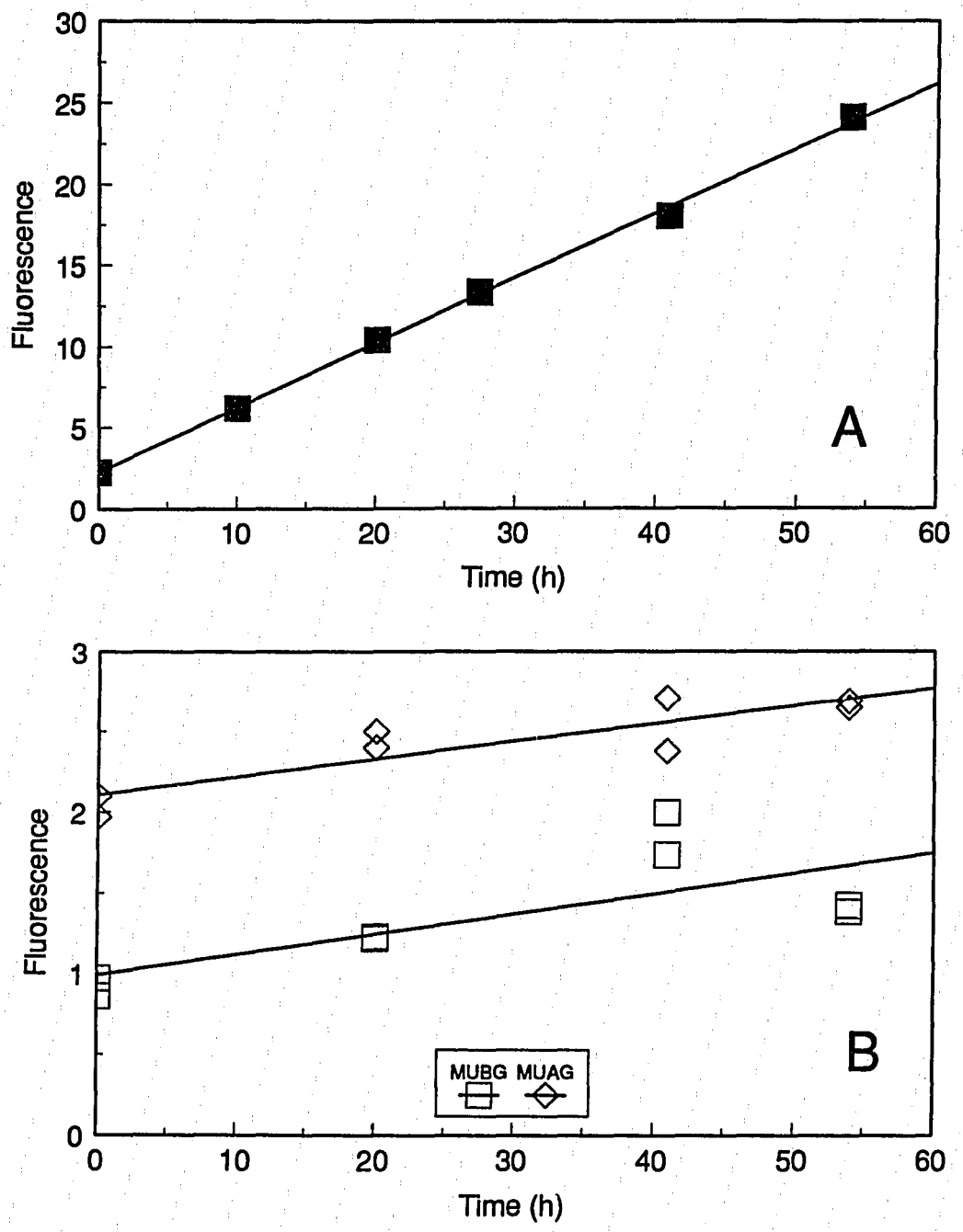


Figure 2.1. Time course of fluorescence evolved from hydrolysis of (a) L-leucyl-β-naphthylamine and (b) 4MUF-α-glucoside and 4MUF-β-glucoside.

### 2.3.3 Preservation

In order to establish the viability of preserving samples with mercuric chloride, the effect of mercuric ion on fluorescence of 4MUF and BNAPH in seawater was determined. The fluorescence yield of 4MUF in seawater poisoned with 4 mM $HgCl_2$ was approximately 5% less than in unpoisoned seawater, over a wide concentration range (Figure 2.2a). Reduction in fluorescence was greater for BNAPH (~30%) but the percent loss was constant over a concentration range of at least three orders of magnitude (Figure 2.2b). Standard curves can be fitted to a straight line, with $r^2$ always >0.99 and usually >0.999. In oligotrophic waters the "turbidity effect" due to particulate matter is minimal; filtering may be necessary in eutrophic waters.

To determine the effect of the ionic strength of the menstruum on the reduction of fluorescence yield by $Hg^{2+}$, I prepared solutions of NaCl, $MgCl_2$, and $NaHCO_3$ at concentrations approximately equivalent to their molarities in seawater at 35 ‰ (585, 50, and 2 mM, respectively). NaCl and $MgCl_2$ solutions also contained 2 mM $NaHCO_3$ to buffer against pH changes. Figure 2.3a shows the relative fluorescence yield of 4MUF in these three solutions and in seawater, with and without added $HgCl_2$. The percent reduction in fluorescence yield was highly variable. It appears that either $Na^+$ or $Mg^{2+}$ will prevent the inhibition of fluorescence by $Hg^{2+}$, to an extent that depends on the concentration of the other cation, i.e., on the ionic strength of the solution. Figure 2.3b shows the relative fluorescence yield of BNAPH in the same solutions. While the reduction of fluorescence yield due to $Hg^{2+}$ was significantly greater than for 4MUF in solutions of high ionic strength such as seawater, dependence on the ionic strength of the solution was weak and the loss of fluorescence was less than for 4MUF in solutions of low ionic strength. The bars in Figure 2.3 represent slopes of standard

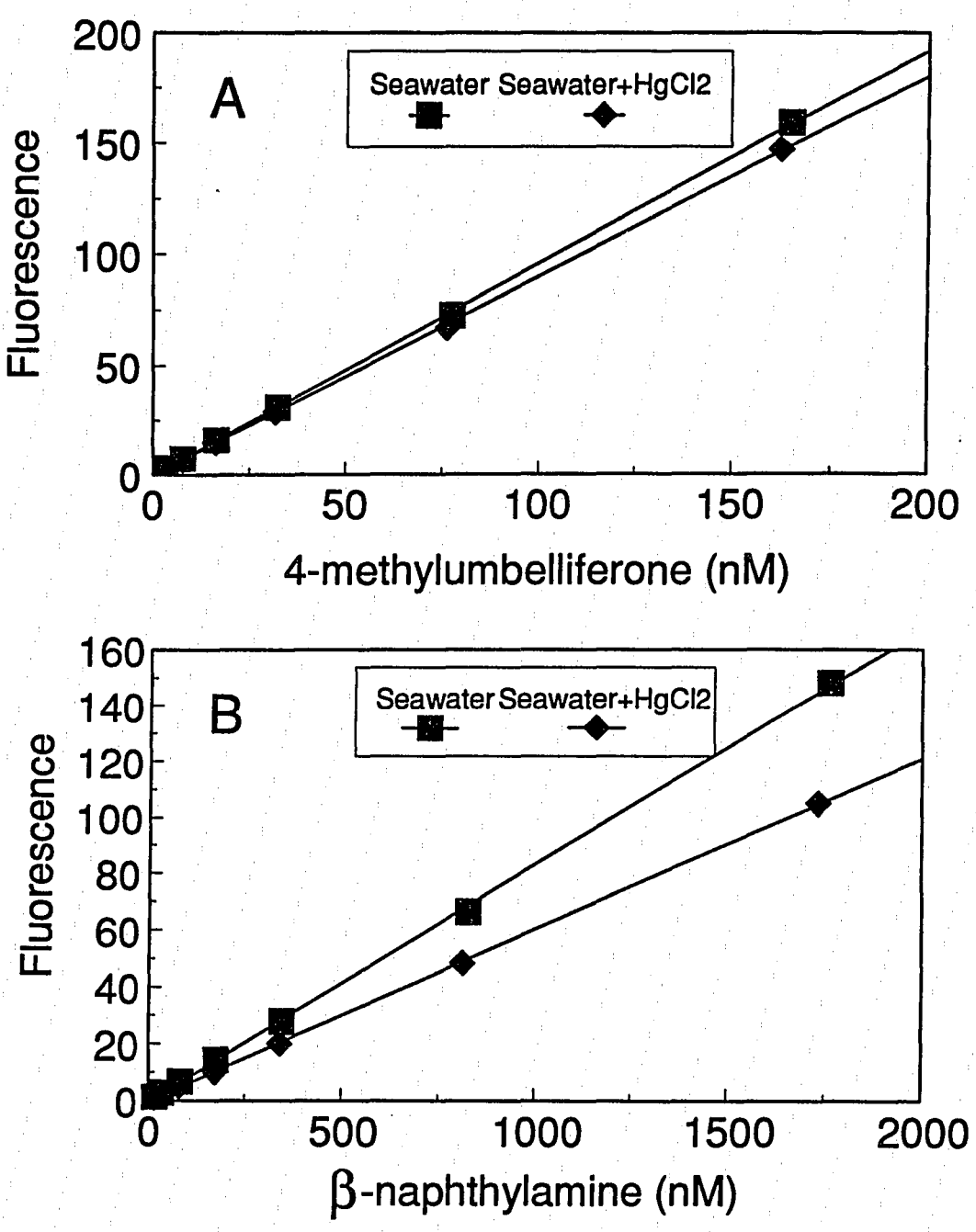


Figure 2.2. Fluorescence (arbitrary units) versus concentration for (a) 4-methylumbelliferone and (b) β-naphthylamine in subtropical North Pacific surface seawater with and without 4 mM $HgCl_2$.

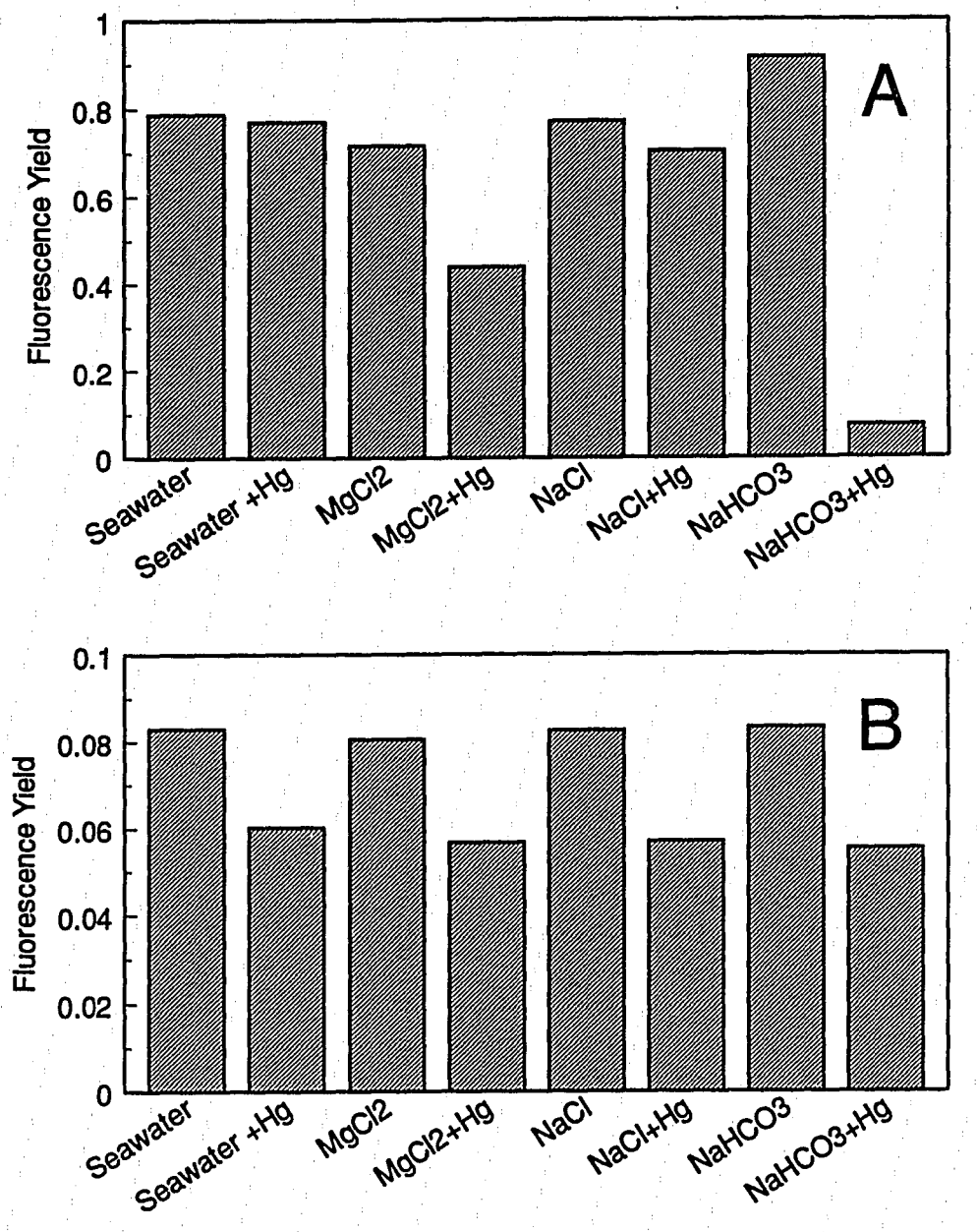


Figure 2.3. Effect of $HgCl_2$ on fluorescence of (a) 4-methylumbelliferone and (b) β-naphthylamine in solutions of NaCl, $MgCl_2$ and $NaHCO_3$ at concentrations equivalent to seawater with S‰ = 35.0 (585 mM NaCl + 2 mM $NaHCO_3$, 50 mM $MgCl_2$ + 2mM $NaHCO_3$, 2 mM $NaHCO_3$). Fluorescence yield is least-squares linear regression of fluorescence (arbitrary units) on concentration with zero-intercept model (each curve is three points at decadally increasing concentration; $r^2$>0.9999 in all cases).

curves with three points at decadally increasing concentrations; $r^2$ in all cases was >0.9999.

Mercuric chloride does not affect the excitation or emission spectra for either 4MUF or BNAPH in seawater. Figure 2.4 illustrates the excitation (at constant emission wavelength), and emission (at constant excitation wavelength) spectra. Each curve was normalized to a constant maximum value, by a linear correction based on the percent offset at the fluorescence peak, to facilitate comparison of spectra.

To test the stability of fluorescence over time, I prepared 10-15 identical standards in subtropical North Pacific surface seawater with 4 mM $HgCl_2$. After thawing, the apparent concentration of each was determined as the relative fluorescence divided by the slope of the standard curve (fluorescence/concentration) for freshly prepared standards. The stability of fluorescence over time in samples stored frozen at -20 °C is illustrated in Figure 2.5.

For 4MUF there was no reduction in apparent concentration over 18 months (Figure 2.5a). The calculated slope of the Model I linear regression is positive (0.003 nmol $l^{-1}$ $d^{-1}$) but not significantly different from 0 (P~0.30). The correlation coefficient (r) for this relationship is 0.36, which is not significant at the 0.05 level. For BNAPH there was a significant reduction in fluorescence over time (Figure 2.5b). The correlation coefficient in this case is 0.941, which is significant at the 0.001 level. The relationship is best fit by a nonlinear model; it appears that the rate of loss of fluorescence yield asymptotically approaches zero (i.e., the apparent concentration drops to a constant value). In this experiment the asymptote falls at ~60% of the initial concentration. Because this experiment was conducted with multiple samples at a single concentration, it is possible that the percent loss is a function of the initial concentration. However, a subsequent experiment showed that the linearity of

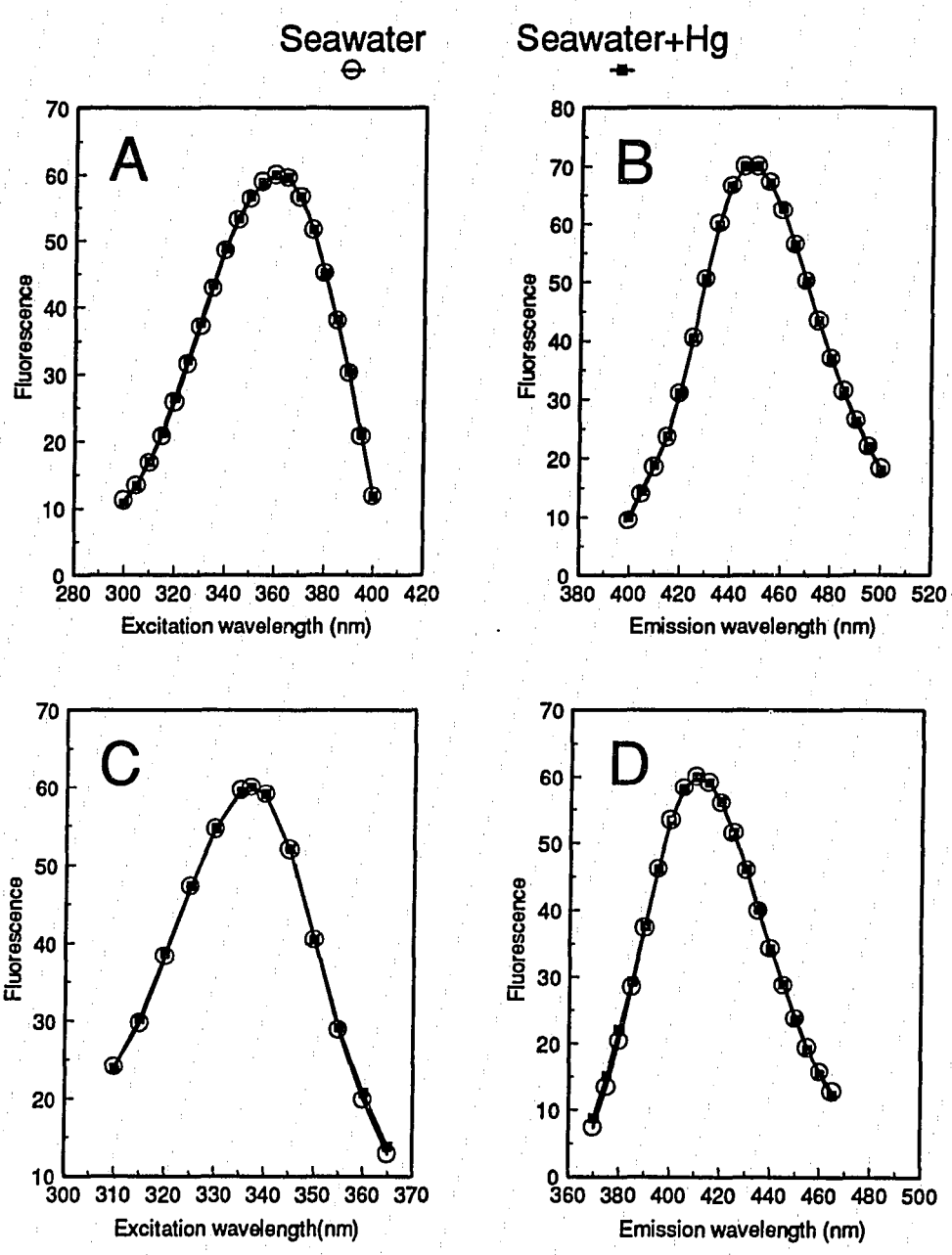


Figure 2.4. Fluorescence spectra (arbitrary units) for 4-methylumbelliferone and β-naphthylamine in subtropical North Pacific surface seawater with and without 4 mM $HgCl_2$: (a) excitation spectrum for 4MUF with emission at 447 nm (b) emission spectrum for 4MUF with excitation at 360 nm (c) excitation spectrum for BNAPH with emission at 411 nm (d) emission spectrum for BNAPH with excitation at 337 nm. Spectra are normalized to constant peak fluorescence (linear correction).

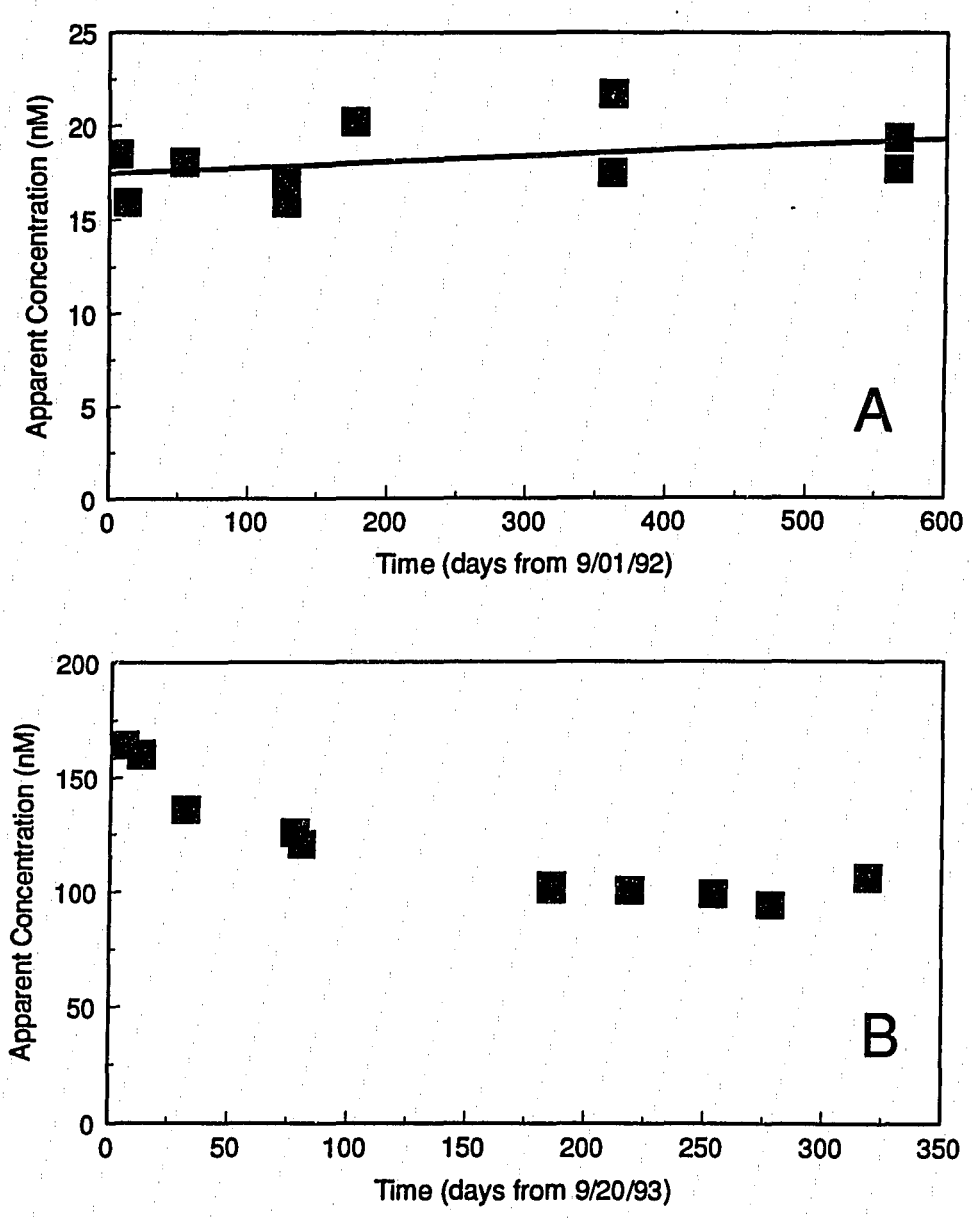


Figure 2.5. Stability over time of fluorescence of (a) 4-methylumbelliferone (initial concentration = 16 nM), with Model I linear regression, (b) β-naphthylamine (initial concentration = 160 nM).

preserved "standard curves" is maintained for at least 136 days and standards of different initial concentrations do not "converge" to similar fluorescence (Figure 2.6).

Poisoned or heat-killed seawater controls are needed to correct for both autofluorescence and autohydrolysis of the substrate analogues. The substrate analogues used in ectoenzymatic analysis fluoresce strongly, but at different wavelengths than the free fluorogens. At the peak excitation and emission wavelengths for 4MUF the autofluorescence of substrate analogues like 4MUF-β-glucoside is minimal. There is some overlap of the excitation and emission spectra for the free fluorogens and the unhydrolyzed substrate analogues, so it is likely that at least some of the fluorescence observed in poisoned controls is due to the latter. However, the tendency for the fluorescence in killed controls prepared from a single stock solution to increase over time suggests that some fraction of the fluorescence in poisoned controls is due to autohydrolysis of the substrate analogue, or hydrolysis by abiotic processes (Figure 2.7). The rate of hydrolysis is low enough, however, that a single stock solution can be used for several weeks.

Hydrolysis of LLBN to BNAPH appears to be catalyzed by $Hg^{2+}$ (data not shown). The rate of increase of fluorescence in killed controls following poisoning is greater than what would be expected by abiotic hydrolysis alone. It is therefore desirable that killed controls be frozen immediately rather than incubated alongside the live samples. This prevents the killed controls from controlling for abiotic hydrolysis in the live samples during the incubation period, but this error is likely to be small (Hoppe, 1993). Autohydrolysis of substrate analogues of this type appears to be an equilibrium process, that is, it occurs more or less instantaneously upon dilution and the percent dissociating decreases with increasing concentration. This results in a fluorescence-concentration relationship resembling the saturation kinetics of enzymes. Therefore,

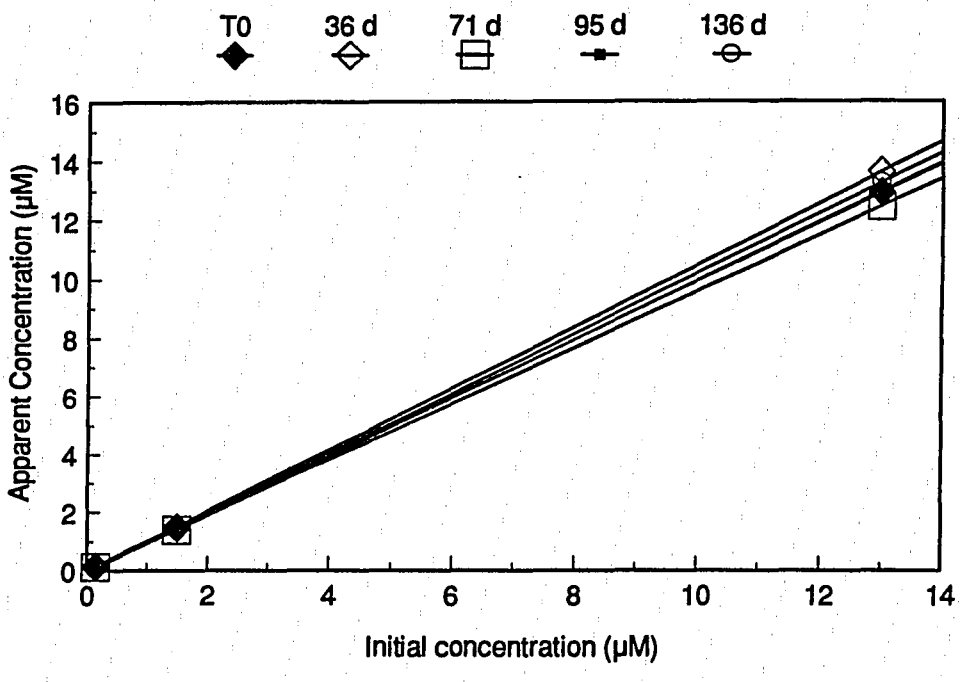


Figure 2.6. Stability over time of β-naphthylamine fluorescence over a wide range of initial concentrations, in replicate standards prepared in seawater with 4 mM $HgCl_2$ and stored frozen at -20 °C. Apparent concentration is the fluorescence divided by the ratio of fluorescence to concentration (least-squares linear regression of fluorescence on concentration with zero-intercept model) for freshly prepared standards.

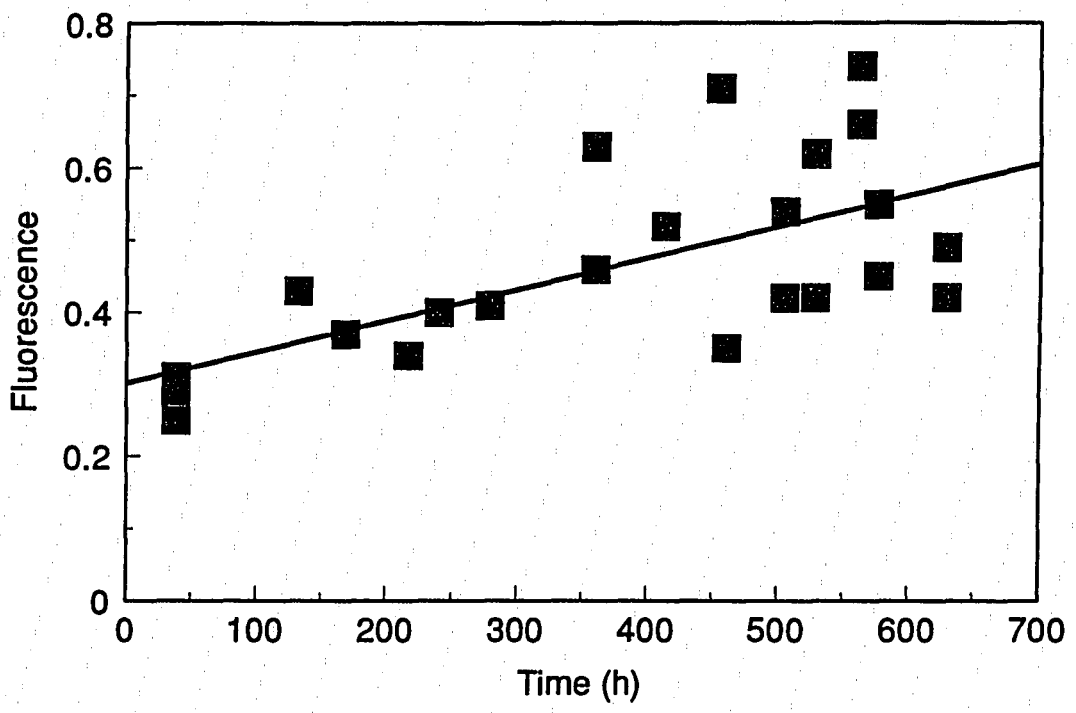


Figure 2.7. Fluorescence (arbitrary units) of $Hg^{2+}$-killed controls for β-glucosidase assay (1.6 µM 4-MUF-β-glucoside, 4 mM $HgCl_2$) collected on RACER 4 (July-August 1992). Linear regression statistics: a=0.30, b=4.3E-04, $r^2$=0.44, n=24. Time in hours from 00:00 on 07/15/92.

when working with 4MUF derivatives with high background fluorescence (e.g., 4MUF-phosphate, 4-methylumbelliferyl-β-N-acetylglucosamine), one must exercise caution when interpreting fluorescence as evidence of enzymatic activity.

In addition to $Hg^{2+}$, other metal cations such as $Cu^{2+}$ and $Ni^{2+}$ will inhibit bacterial ectoenzymes such as BGase (Figure 2.8). $Ag^{+}$ is a poor choice because its solubility in seawater is too low. The solubilities of $Cu^{2+}$ and $Ni^{2+}$ are also less than the 4 mM routinely used for $Hg^{2+}$, but these ions strongly inhibit BGase at their saturation concentration. $Ni^{2+}$ has previously been shown to strongly inhibit marine bacterial activity at 5 mM but not at 1 mM (Gonye and Jones, 1973).

### 2.3.4 Temperature and concentration response

The responses of LAPase, BGase and AGase to temperature and concentration of the substrate analogue have been studied in Antarctica, at Station ALOHA, and in the Equatorial Pacific. The responses of LAPase and BGase at saturating substrate concentration to temperature presents extremely interesting cross-habitat comparisons which will be dealt with at length in Chapter 3.

The responses of the enzymes to varying concentrations of the model substrates are more uncertain, particularly for AGase and BGase. Estimation of $K_m$ for AGase and BGase proved difficult due to the low activities present in the two study areas where the experiments were conducted (Station ALOHA and Antarctica). Several methods of obtaining a more concentrated sample were attempted, including aging of seawater (3/1/93), determination of activities associated with particles collected in sediment traps rather than with free-living bacterioplankton (3/2/93), and concentration of suspended particulate matter on a Whatman GF/F filter (4/1/93). While these

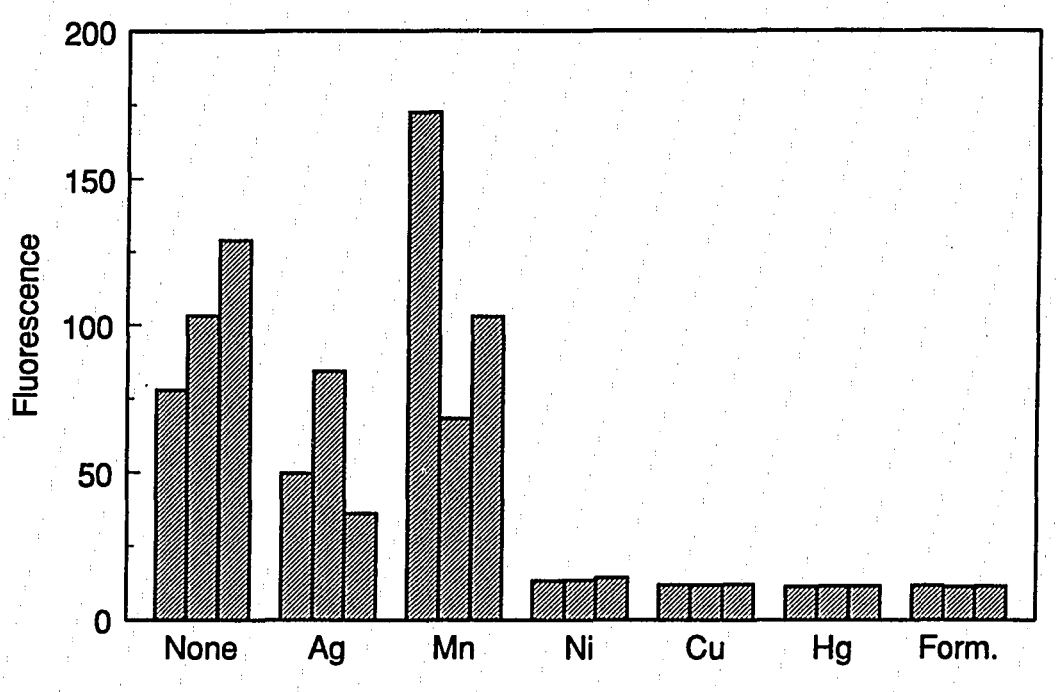


Figure 2.8. Fluorescence (arbitrary units) of subtropical North Pacific surface seawater samples inoculated with 1.6 µM 4MUF-β-glucoside and stored for 29 days at room temperature (~ 22° C) with various potentially inhibitory metal cations. No inhibitor and 1% formalin are included for comparative purposes. All metal ions (Me) were added at 4 mM as $MeCl_2$, except Ag as $Ag_2SO_4$. There are three replicates for each treatment.

approaches may give somewhat artificial values, all of the values obtained were less than 2 μM, and most fell within a much smaller range of 0.01-0.1 μM (Table 2.2). In several experiments an increase in $K_m$ with increasing temperature was observed, but in one case (late autumn in Antarctica) both AGase and BGase showed similar or lower values at 10 °C than at 0 °C. Values for BGase are within the general range observed by Somville (1984), rather than the much greater values observed by Chróst (1989), and suggest broad cross-habitat homology of this enzyme.

$K_m$ for LAPase in Antarctica generally increased with increasing temperature, although there was a fair amount of variance in the relationship (Figure 2.9). A significant amount of this variance is likely to be environmental rather than analytical (temperature response varied among regions and seasons). $K_m$ for Antarctica was in the range of about 50-300 μM over all temperatures assayed (-1.7 to +20 °C) and 100-160 μM at *in situ* temperature (-1.7 to +2 °C). A minimum at 4-6 °C was observed several times, and consistently enough to suggest that it was not purely stochastic variability. $K_m$ for LAPase at Station ALOHA is in the same range. It is clear that $K_m$ for LLBN exceeds that for MUBG and MUAG by several orders of magnitude.

### 2.3.5 Specificity

On the fall 1993 cruise in Antarctica (NBP-9302) an extensive effort was made to determine the specificity of the enzyme or enzymes hydrolyzing LLBN by competitive inhibition experiments with various dipeptides. All of the dipeptides used showed some evidence of competitive inhibition. There was a fair amount of variance and the kinetic interpretation is somewhat speculative. However it is reasonable to

Table 2.2. Estimates of $V_{max}$ (nmol $l^{-1}$ $d^{-1}$) and $K_m$ (μM) for α- and β-glucosidase using 3 linear transformations of the Henri-Michaelis-Menten equation and a nonlinear least-squares direct fit. n=5 in all cases except on 3/2/95 (n=8). RSS = residual sum of squares.

| AGase | | | Eq. 2.2 | Eq. 2.3 | Eq. 2.4 | NLLS |
|---|---|---|---|---|---|---|
| Antarctica | | | | | | |
| 5/7/93 | 0°C | $V_{max}$ | 0.34 | 0.53 | 0.31 | 0.38 |
| | | $K_m$ | 0.013 | 0.30 | 0.012 | 0.048 |
| | | $r^2$ | 0.03 | 0.76 | 0.03 | |
| | | RSS | 5.26E-02 | 6.81E-02 | 5.65E-02 | 4.94E-02 |
| | 10°C | $V_{max}$ | 0.79 | 0.77 | 0.79 | 0.79 |
| | | $K_m$ | 0.013 | 0.006 | 0.013 | 0.014 |
| | | $r^2$ | 0.68 | 1.00 | 0.74 | |
| | | RSS | 3.34E-03 | 5.98E-03 | 3.35E-03 | 3.34E-03 |
| 12/21/94 | 0°C | $V_{max}$ | 0.36 | 0.25 | 0.35 | 0.38 |
| | | $K_m$ | 0.057 | -0.071 | 0.064 | 0.071 |
| | | $r^2$ | 0.17 | 0.92 | 0.45 | |
| | | RSS | 3.96E-02 | 3.93E+00 | 4.23E-02 | 3.90E-01 |
| BGase | | | | | | |
| Station ALOHA | | | | | | |
| 3/1/93 | 20°C | $V_{max}$ | 0.99 | 1.02 | 0.98 | 0.99 |
| | | $K_m$ | 0.042 | 0.093 | 0.042 | 0.043 |
| | | $r^2$ | 0.36 | 0.98 | 0.44 | |
| | | RSS | 4.05E-02 | 6.81E-02 | 4.09E-02 | 4.05E-02 |
| 3/2/93 | 5°C | $V_{max}$ | 1.20 | 1.58 | 1.06 | 1.38 |
| | | $K_m$ | 0.056 | 0.19 | 0.043 | 0.11 |
| | | $r^2$ | 0.74 | 0.98 | 0.89 | |
| | | RSS | 2.06E-01 | 1.88E-01 | 3.44E-01 | 1.28E-01 |
| | 20°C | $V_{max}$ | 53.3 | 53.9 | 51.4 | 53.9 |
| | | $K_m$ | 0.36 | 0.37 | 0.34 | 0.36 |
| | | $r^2$ | 0.95 | 0.99 | 1.00 | |
| | | RSS | 5.58E+01 | 5.58E+01 | 6.32E+01 | 5.53E+01 |

Table 2.2. (continued) Estimates of $V_{max}$ and $K_m$ for α- and β-glucosidase.

Antarctica

| | | | | | | |
|---|---|---|---|---|---|---|
| 4/14/93 | 0°C | $V_{max}$ | 0.15 | 0.17 | 0.15 | 0.19 |
| | | $K_m$ | 0.48 | 0.67 | 0.51 | 0.82 |
| | | $r^2$ | 0.83 | 0.93 | 0.99 | |
| | | RSS | 4.93E-04 | 3.48E-04 | 4.48E-04 | 3.21E-04 |
| | 20°C | $V_{max}$ | -3.28 | -86.41 | -9.29 | 26.84 |
| | | $K_m$ | -1.04 | -11.91 | -1.59 | 1.95 |
| | | $r^2$ | 0.28 | 0.05 | 0.98 | |
| | | RSS | 4.31E+02 | 2.04E+01 | 4.92E+05 | 1.02E+01 |
| 5/7/93 | 0°C | $V_{max}$ | 0.39 | 0.49 | 0.38 | 0.41 |
| | | $K_m$ | 0.039 | 0.16 | 0.034 | 0.059 |
| | | $r^2$ | 0.42 | 0.98 | 0.50 | |
| | | RSS | 1.24E-02 | 1.99E-02 | 1.35E-02 | 1.14E-02 |
| | 10°C | $V_{max}$ | 1.14 | 1.36 | 1.13 | 1.20 |
| | | $K_m$ | 0.028 | 0.11 | 0.028 | 0.043 |
| | | $r^2$ | 0.40 | 0.99 | 0.44 | |
| | | RSS | 7.27E-02 | 1.26E-01 | 7.46E-02 | 6.62E-02 |
| 12/21/94 | 0°C | $V_{max}$ | 0.96 | 1.26 | 0.75 | 1.35 |
| | | $K_m$ | 0.39 | 0.67 | 0.27 | 0.79 |
| | | $r^2$ | 0.68 | 0.93 | 0.91 | |
| | | RSS | 3.48E-02 | 1.15E-02 | 9.55E-02 | 1.05E-02 |

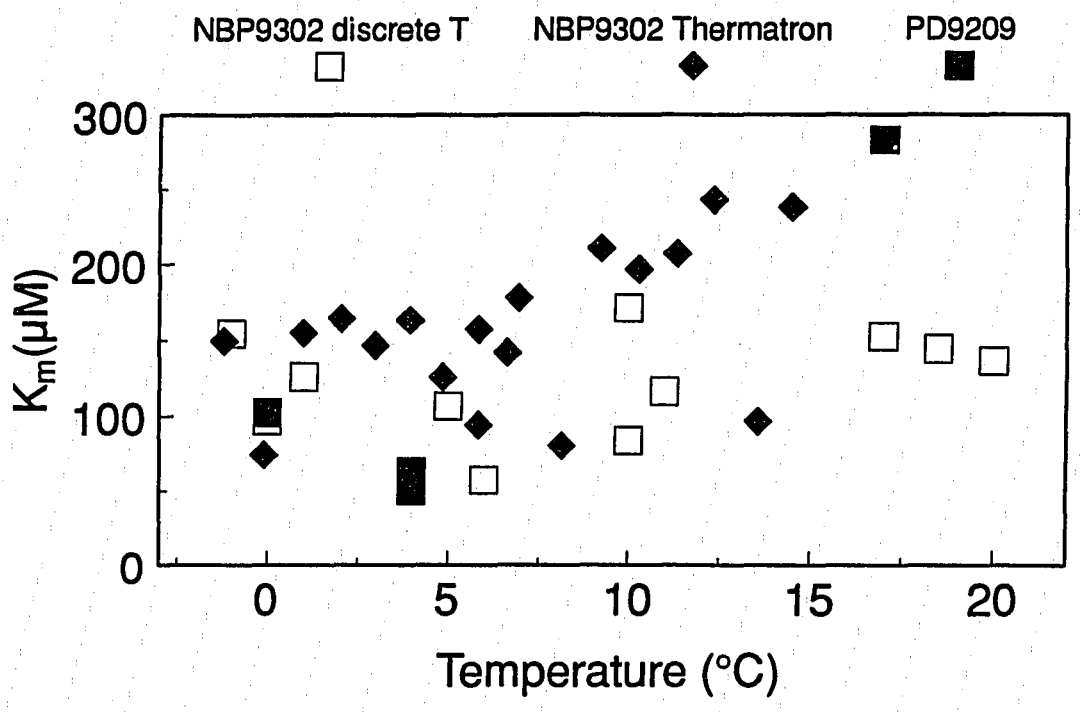


Figure 2.9. Relationship of $K_m$ for hydrolysis of L-leucyl-β-naphthylamine in Antarctic seawater to incubation temperature. Three data sets are shown: cruise designations are explained in section 2.2. The thermatron is described in section 3.2.

characterize the enzyme in question as a nonspecific aminopeptidase, as hydrolysis of LLBN was not more strongly inhibited by L-Leu-L-Leu than by other dipeptides.

Double-reciprocal (Lineweaver-Burk) plots of these experiments roughly followed the pattern expected for competitive inhibition (Figure 2.10). The plot for glutamic acid conforms well to the classic competitive inhibition pattern, but this was the "best" example out of some 14 experiments conducted in this fashion. Most of the others follow the same general pattern but with too much variance to distinguish from uncompetitive or noncompetitive inhibition. The values of $K_m$ and $V_{max}$ calculated by nonlinear least-squares are suggestive of competitive inhibition, with $K_m$ increasing with increasing concentration of the competing dipeptide (Table 2.3). However, the *a posteriori* variances of the model parameters were generally large, and in some cases $V_{max}$ also increased.

Fitting the expanded HMM equation (equation 2.8) resulted in consistent values of $K_m$ and $V_{max}$ on 4/16/93 and 4/21/93 (Table 2.3), and these values are also consistent with those estimated for the simpler form of the equation on samples containing LLBN only. The estimated values of $V_{max}$, $K_m$, and $K_i$ predicted the measured activities fairly well (Figure 2.11). On 4/27/93 none of the rates observed on the addition of competing dipeptides, with the exception of Glu-Glu, conformed to the competitive inhibition model. For the other dipeptides the nonlinear least-squares algorithm failed to converge. The reason for this, on inspection of the raw data, is that the activities observed in the presence of potentially competing substrates are higher than those observed in seawater alone. This may be simply sampling and analytical variance, but it may also be a result of the conditions in which the water was collected, i.e., basically winter conditions, with low activities. It is possible that the added

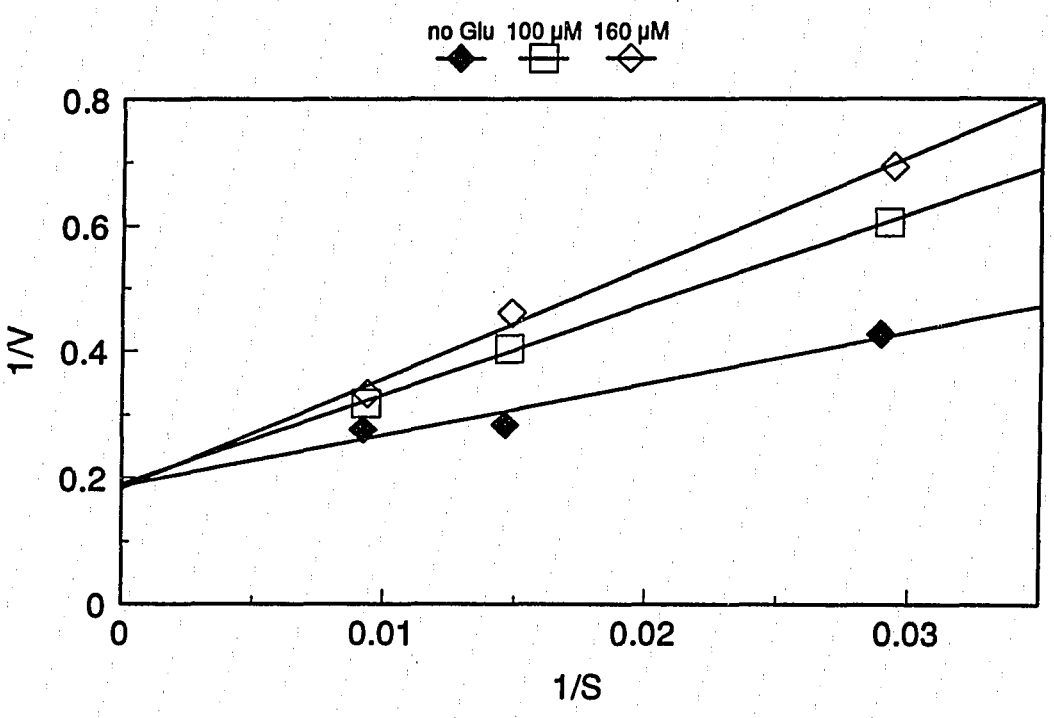


Figure 2.10. Example double reciprocal (Lineweaver-Burk) plot showing competitive inhibition of L-leucyl-β-naphthylamine hydrolysis by L-Glu-L-Glu. Substrate concentrations (S) are in µM and activities (V) in nmol $l^{-1}$ $h^{-1}$. Lines are model I linear regression.

Table 2.3. Estimates of $V_{max}$ (nmol $l^{-1}$ $h^{-1}$) and $K_m$ (μM) for hydrolysis of L-leucyl-β-naphthylamine in the presence of potentially competing dipeptides of various amino acids. Values for single concentrations were derived using nonlinear least-squares direct fit of the standard Henri-Michaelis-Menten equation (n=3). Values in last row of each group show values of $V_{max}$, $K_m$, and $K_i$ determined from expanded Henri-Michaelis-Menten equation (equation 2.8) with n=12 on 4/16/93 and n=9 on 4/21/93 and 4/27/93. Standard deviations of model parameter estimates are given in the column following each parameter.

| | | $V_{max}$ | (s) | $K_m$ | (s) | $K_i$ | (s) |
|---|---|---|---|---|---|---|---|
| 4/16/93 | | | | | | | |
| LLBN only | | 13.3 | (2.6) | 67 | (36) | | |
| L-Leu | 50 μM | 16.0 | (3.9) | 111 | (60) | | |
| | 100 μM | 15.7 | (3.8) | 107 | (57) | | |
| | 230 μM | 14.2 | (4.1) | 110 | (68) | | |
| | Eq. 2.8 | 14.7 | (1.7) | 84 | (26) | 599 | (534) |
| D-Leu | 50 μM | 15.1 | (3.4) | 94 | (50) | | |
| | 100 μM | 14.1 | (3.3) | 91 | (51) | | |
| | 230 μM | 14.2 | (3.2) | 83 | (45) | | |
| | Eq. 2.8 | 14.1 | (1.5) | 81 | (24) | 4241 | (2067) |
| Gly | 50 μM | 15.4 | (3.2) | 89 | (45) | | |
| | 100 μM | 15.4 | (3.9) | 110 | (61) | | |
| | 230 μM | 27.0 | (22.4) | 420 | (489) | | |
| | Eq. 2.8 | 14.9 | (1.8) | 78 | (26) | 229 | (117) |
| | Eq. 2.8* | 14.5 | (1.7) | 77 | (24) | 411 | (329) |
| 4/21/93 | | | | | | | |
| LLBN only | | 15.1 | (3.1) | 87 | (44) | | |
| Met | 225 μM | 24.7 | (7.6) | 196 | (105) | | |
| | 415 μM | 17.4 | (6.2) | 156 | (103) | | |
| | Eq. 2.8 | 18.1 | (2.7) | 117 | (40) | 1632 | (1667) |
| Ser | 225 μM | 18.4 | (5.3) | 145 | (82) | | |
| | 415 μM | 14.0 | (3.4) | 85 | (48) | | |
| | Eq. 2.8 | 15.6 | (2.1) | 94 | (33) | 2633 | (4531) |
| Ala | 225 μM | 13.7 | (2.6) | 64 | (33) | | |
| | 415 μM | 23.6 | (8.0) | 191 | (112) | | |
| | Eq. 2.8** | 14.8 | (2.1) | 79 | (31) | 3426 | (8756) |

Table 2.3. (continued) Estimates of model parameters for hydrolysis of L-leucyl-β-naphthylamine in the presence of potentially competing dipeptides.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Glu | 225 µM | 7.0 | (3.7) | 99 | (117) | | |
| | 415 µM | did not converge | | | | | |
| | Eq. 2.8 | 14.7 | (2.9) | 81 | (41) | 45.4 | (18.4) |
| 4/27/93 | | | | | | | |
| 0 °C | LLBN only | 5.0 | (3.2) | 35 | (66) | | |
| L-Leu | 100 µM | 9.4 | (9.5) | 106 | (188) | | |
| | 160 µM | 17.1 | (35.4) | 262 | (717) | | |
| | Eq. 2.8 | did not converge | | | | | |
| Glu | 100 µM | 5.4 | (6.8) | 79 | (192) | | |
| | 160 µM | 6.4 | (11.6) | 121 | (363) | | |
| | Eq. 2.8 | 5.2 | (2.8) | 40 | (60) | 134 | (193) |
| Met | 100 µM | 6.9 | (5.4) | 64 | (108) | | |
| | 160 µM | 16.4 | (45.0) | 299 | (1053) | | |
| | Eq. 2.8 | 6.9 | (3.6) | 71 | (79) | 3242 | (34549) |
| Gly | 100 µM | 6.2 | (4.0) | 46 | (74) | | |
| | 160 µM | 10.7 | (15.5) | 152 | (337) | | |
| | Eq. 2.8 | did not converge | | | | | |
| 10 °C | LLBN only | 7.9 | (2.7) | 28 | (32) | | |
| Gly(10°C) | 100 µM | 14.3 | (6.9) | 81 | (77) | | |
| | 160 µM | 13.9 | (7.2) | 84 | (83) | | |
| | Eq. 2.8 | did not converge | | | | | |

* 1 outlier removed (alternate solution)
** 1 outlier removed (necessary for convergence)

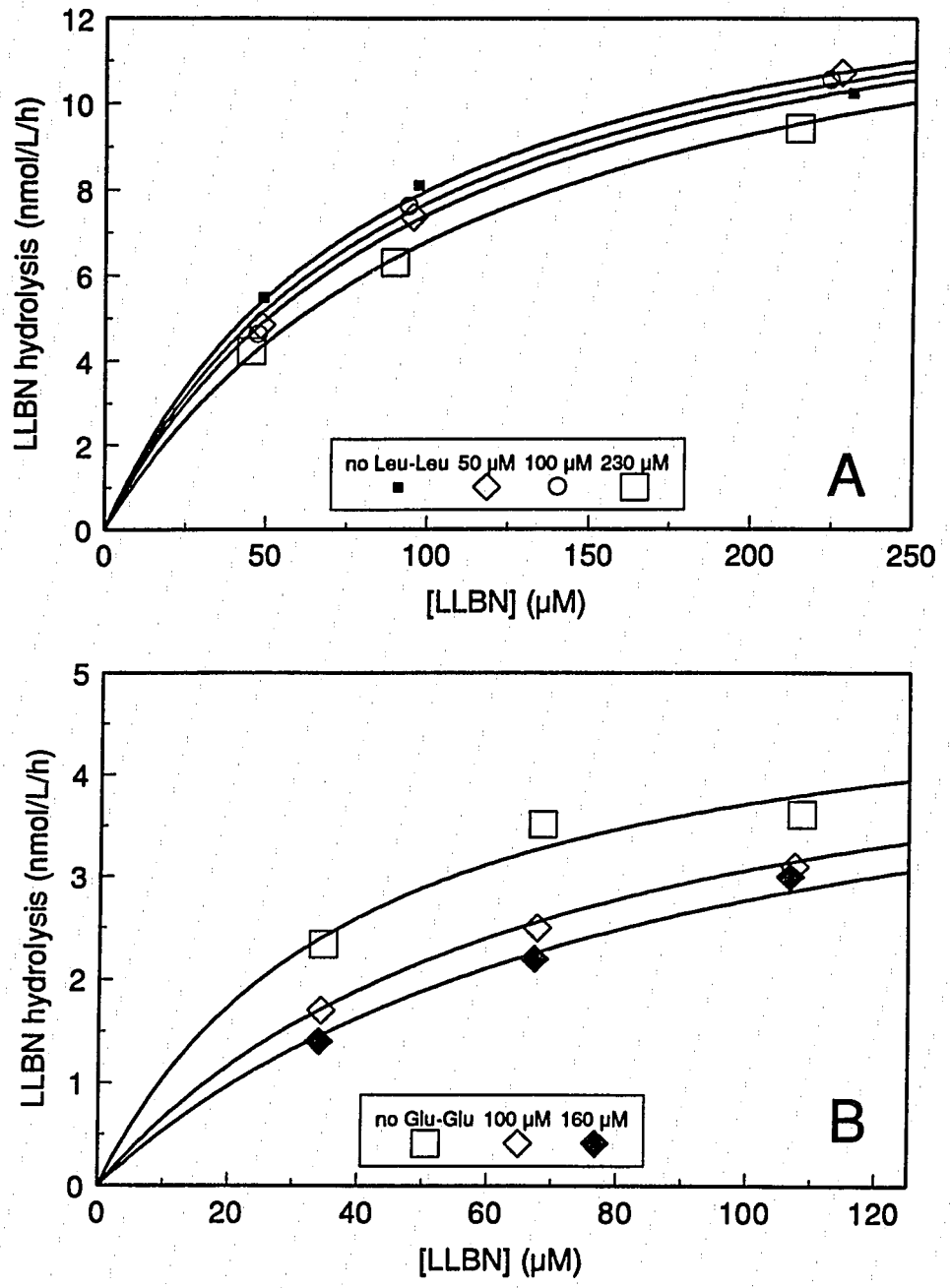


Figure 2.11. Rate of LLBN hydrolysis in the presence of dipeptides of (a) L-leucine and (b) L-glutamic acid (symbols), shown relative to values predicted by expanded Henri-Michaelis-Menten equation (equation 2.8) with model parameters estimated by nonlinear least-squares (curves).

dipeptides induced activities that had been repressed as the bacterioplankton entered a dormant stage with the onset of winter.

Relative affinities for the various amino acids are uncertain. Several experiments were conducted in non-kinetic fashion (i.e., with a single concentration each of LLBN and the competing dipeptide) to attempt to establish a rank order (Figure 2.12), but the differences observed in these experiments were too small to draw firm conclusions. The order does not seem to follow a simple gradient of hydrophilicity/hydrophobicity. However, the enzyme appears to have greatest affinity for glutamic acid, with hydrophobic amino acids including leucine showing lower affinity (this was not observed in the first kinetic experiment). It is possible that the enzyme has greatest affinity for hydrophilic amino acids, some of which are among the amino acids most abundant in seawater. If so, *in situ* activity may be systematically underestimated by using an analogue based on a hydrophobic amino acid (leucine). However, differences in affinity appear relatively small.

An experiment was conducted at Station ALOHA (HOT 50, October 1993) to determine the specificities of enzymes hydrolyzing various 4MUF-glycosides relative to cellobiose (β-D-Glu-β-D-Glu) (Figure 2.13). Percent inhibition by cellobiose was greatest for β-glucosidase and β-galactosidase, followed by α-glucosidase and β-xylosidase (note different scales used), and least for β-glucuronidase and N-acetyl-β-glucosaminidase (chitobiase). The high percent inhibition of β-galactosidase suggests that this may not be a distinct enzyme but rather that the enzyme or enzymes hydrolyzing MUBG also hydrolyze 4MUF-β-Gal. The enzyme(s) hydrolyzing 4MUF-β-Xyl appear to be distinct, but activity of this enzyme was low. The high percent inhibition of α-glucosidase is somewhat surprising given reports of broad specificity β-hexosidases that are nonetheless highly specific for β-anomers (Plant et al., 1988), but

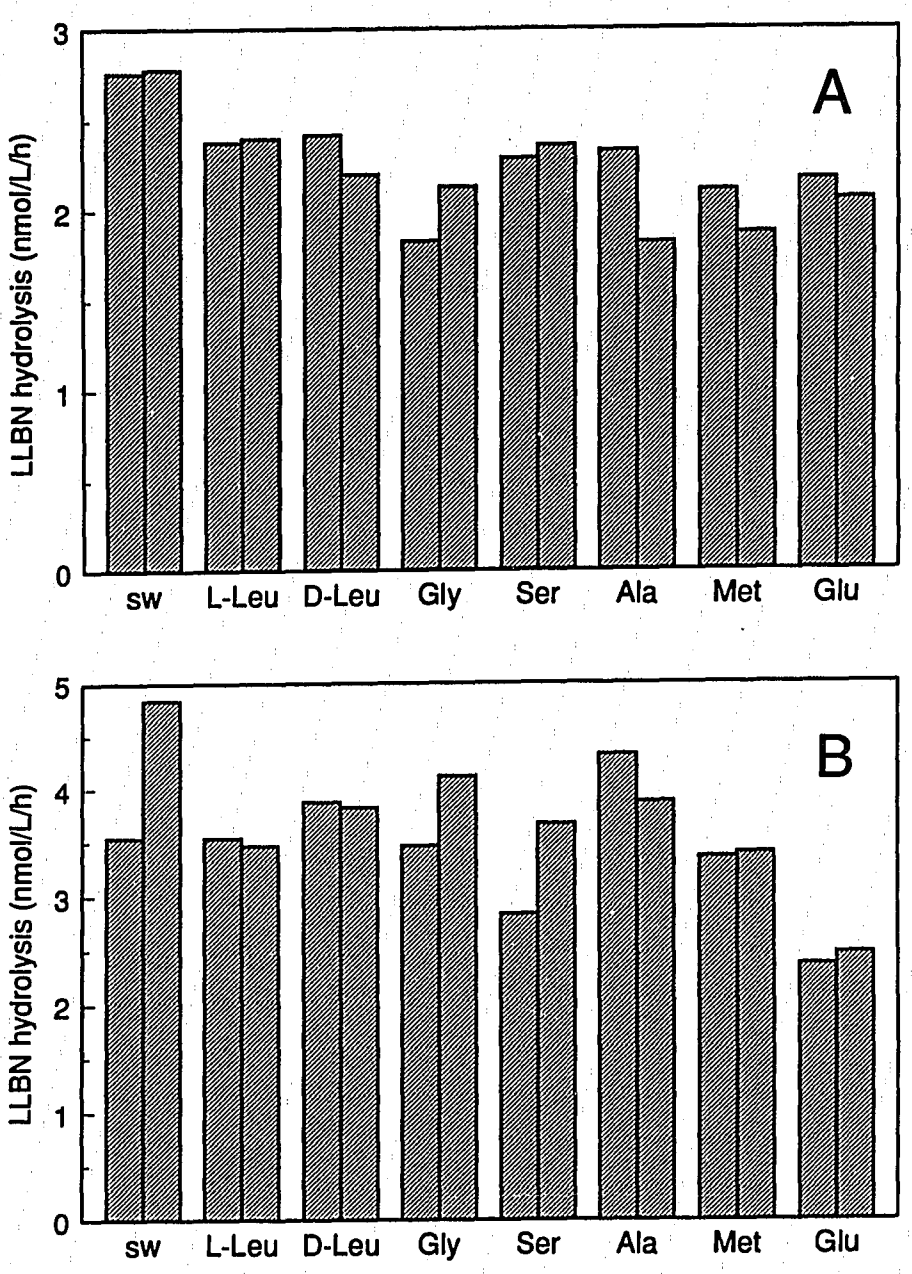


Figure 2.12. Rate of LLBN hydrolysis in the presence of potentially competing dipeptides of various amino acids. In (a) dipeptide concentrations are 80 µM and L-leucyl-β-naphthylamine concentration is 35 µM. In (b) these concentrations are 148 µM and 49 µM respectively. All amino acids are L-forms except where specified; there are 2 replicates for each treatment.

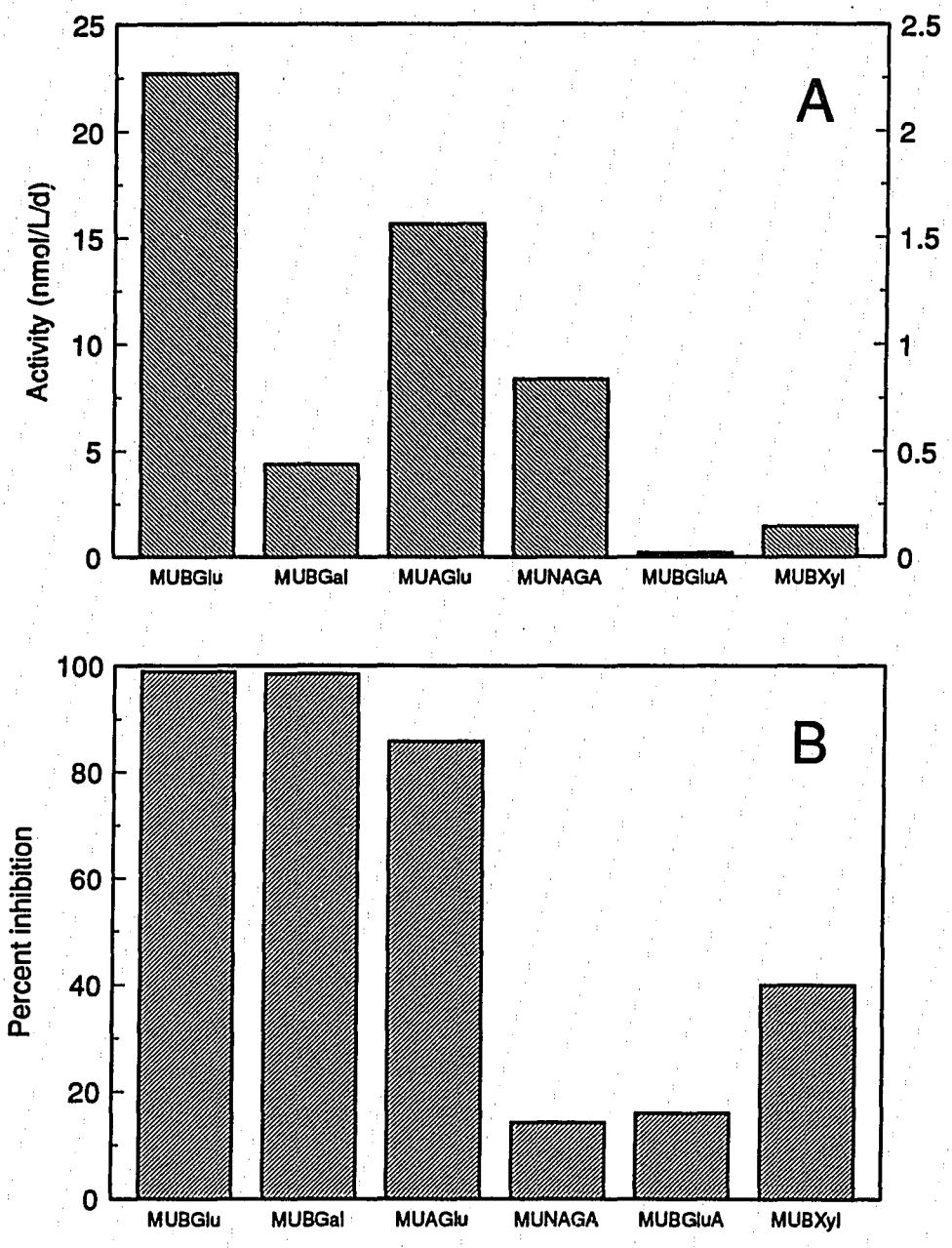


Figure 2.13. Competitive inhibition of hydrolysis of 4MUF-glycosides by cellobiose. Left hand scale for MUBGlu and MUBGal; others on right hand scale. (a) Activities *in situ* (substrate concentration = 1.6 μM) (b) percent inhibition relative to activities shown in (a) when 100 μM cellobiose added in addition to substrate analogue.

apparently nonspecific α/β glucosidases were also observed in Antarctic waters in December 1994 (see Chapter 3). Of the six enzyme activities measured in this experiment, β-glucosidase was by far the highest. β-galactosidase was next but lower by a factor of about five. Therefore, if this is in fact a single enzyme, its affinity for glucose is much greater than for galactose. Similar experiments in Antarctic waters, but using only MUAG and MUBG, gave inconsistent results regarding specificity for α and β anomers.

### 2.3.6 Inhibition by trace (metal) elements

BGase activity in seawater showed little inhibition by $Hg^{2+}$ or $Ni^{2+}$ at concentrations of 10 nM to100 μM, and by $Cu^{2+}$ only at the high end of this range (Figure 2.14). By contrast, $Cu^{2+}$ can inhibit the growth of phytoplankton at concentrations as low as 3-5 nM (Fitzwater et al., 1982). Interestingly, at 10 nM the activity was greater in the presence of each of these 3 cations than in unamended seawater controls. Although the amended treatments were not replicated, each point falls well outside the range of three replicate controls.

## 2.4 DISCUSSION

Although I have for convenience referred to the enzymes measured by names of familiar enzymes likely to hydrolyze the model substrates employed, the true identities of the enzymes remain obscure. Both glucosidases and peptidases appear to have relatively broad specificity, but not exactly matching the affinities reported for similar enzymes in the literature. The ionic cofactors of these enzymes are also uncertain.

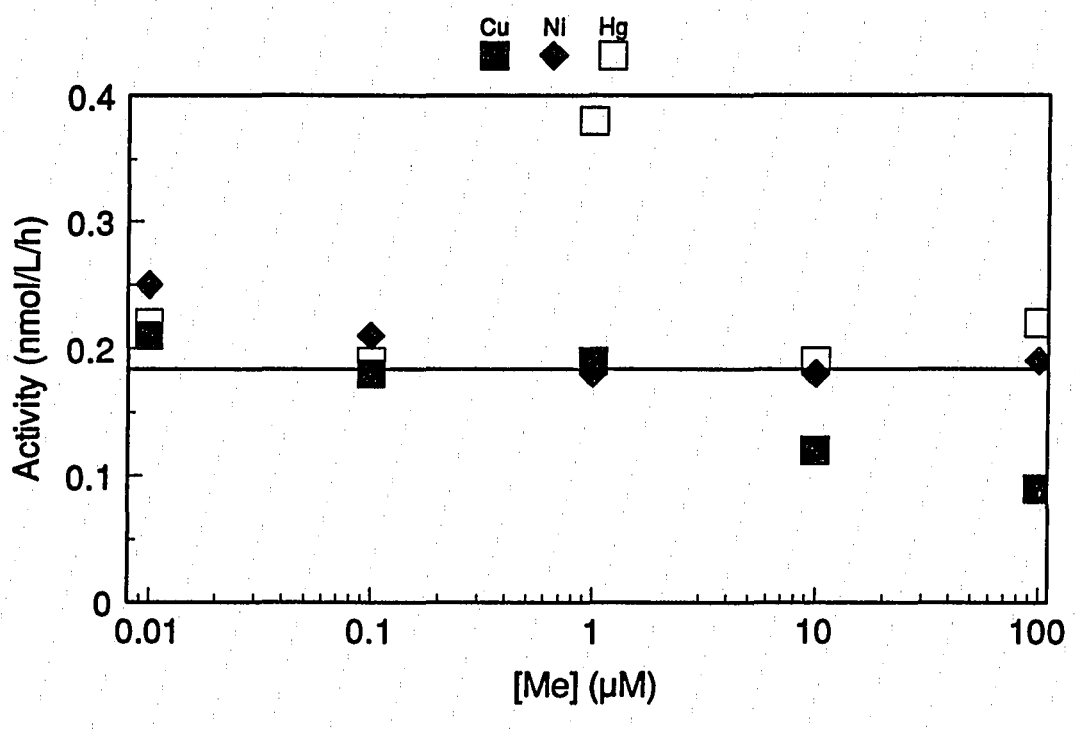


Figure 2.14. β-glucosidase activity in seawater in presence of $Cu^{2+}$, $Ni^{2+}$, and $Hg^{2+}$ ions, over a range of concentrations from 10 nM to 100 µM. Horizontal line shows activity in unamended seawater control (mean of 3 replicates). 4MUF-β-glucoside added at 1.6 µM.

I have referred to the enzyme hydrolyzing LLBN as "leucine aminopeptidase," implying an enzyme that most rapidly cleaves leucine residues but with relatively broad specificity (Delange and Smith, 1971). Broad specificity enzymes would seem evolutionarily advantageous in this context (ectoenzymes whose function is to get nutrients from the medium). The competitive inhibition experiments described above suggest that the LAPase in Antarctic seawater may have greatest affinity for glutamic acid. Aminopeptidases A have greatest affinity for acidic residues (Delange and Smith, 1971). These experiments are not conclusive, however. Competitive inhibition of LLBN hydrolysis by these dipeptides demonstrates only that they bind to the active site. It is only assumed that they are hydrolyzed.

Several N-terminal aminopeptidases have been described from bacterial sources. *Escherichia coli* has at least five, of which three have broad specificity (Miller, 1987). An aminopeptidase from *E. coli* was found to be extremely similar to bovine lens leucine aminopeptidase (blLAP), with overall 31% sequence identity and 52% sequence identity between the C-terminal domains, which in blLAP encodes the active site (Kim and Lipscomb, 1994). This was hypothesized to be aminopeptidase I, isolated by Vogt (1970). These enzymes are also very similar to one produced by a marine bacterium, *Aeromonas proteolytica* (Prescott and Wilkes, 1966; Prescott et al., 1971). The sequence similarity of aminopeptidases in bacteria and mammals suggests that this enzyme is evolutionarily ancient, so similar enzymes may be found in natural aquatic bacteria. That such an enzyme is broadly distributed among prokaryotes is suggested by the similar $K_m$ values observed in widely separated regions of the ocean (Section 2.3.4; Somville and Billen, 1983; Fontigny et al, 1987) and the presence of a very similar

enzyme in cultures of the cyanobacterium *Synechococcus* sp. (Martinez and Azam, 1993).

Fontigny et al. (1987) suggested that metalloproteases dominated LAPase in seawater because activity was strongly inhibited by EDTA. Leucine aminopeptidase was initially reported to be activated by Mn or Mg (Delange and Smith, 1971). It has since been demonstrated that it is principally a Zn enzyme, although variants may also be activated by Mn or Co (Kim and Lipscomb, 1994). A variety of Zn proteases have been identified from both gram-positive and gram-negative bacteria (Häse and Finkelstein, 1993). Aminopeptidase A is a Zn enzyme (Wang and Cooper, 1993). Of the bioactive trace elements, Zn and Co have among the lowest concentrations in open ocean surface water (Donat and Bruland, 1995), so Mn could play an important role in activating this enzyme in the ocean. Although activation may be less strong at comparable concentrations, concentrations of Mn in open ocean surface waters are much higher than those of Zn or Co. This does not apply in Antarctic oceanic waters, however, as input of aeolian-source metals including Mn is small and concentrations of nutrient-type elements like Zn relatively high (Duce and Tindale, 1991; Duce et al., 1991; Martin et al., 1990). Neritic Antarctic waters are enriched in both types of elements (Martin et al., 1990).

β-glucosidases are broadly distributed among diverse kinds of organisms, and basic structural similarities among those studied to date suggest that this enzyme is also evolutionarily very ancient (Esen, 1993). β-glucosidases described from thermophilic Archaea have broad specificity for β-D-glycosides (Plant et al., 1988; Nucci et al., 1993). It can reasonably be hypothesized that this represents the ancestral condition, and that the more specific forms found in, for example, higher plants (Conn, 1993) and

cellulolytic bacteria (Ware et al., 1990) have evolved in response to the particular requirements of these organisms.

β-glucosidases and related enzymes have been studied in fresh water where the rate of degradation of cellulose is of interest (Münster, 1991). The presence of β-glucosidase activity in open ocean water far from any source of terrestrially-derived organic matter indicates that planktonic organisms produce β-linked polysaccharides, and that these are used by planktonic bacteria unlikely to be closely related to obligately cellulolytic forms.

The β-glucosidases of the thermophilic bacteria have broad specificity for β-D-glycosides but are nonetheless highly specific for β anomers (Plant et al., 1988; Nucci et al., 1993). Somville (1984) found strong specificity for α and β anomers in a pond near Brussels. Only α-D-glucopyranosides competitively inhibited hydrolysis of MUAG, and only β-D-glucopyranosides competitively inhibited hydrolysis of MUBG. However, α-D-glucopyranosides noncompetitively inhibited hydrolysis of MUBG and β-D-glucopyranosides noncompetitively inhibited hydrolysis of MUAG. This is not inconsistent with my observations regarding inhibition of MUAG hydrolysis by cellobiose, which neither distinguished between competitive and noncompetitive inhibition nor determined whether the cellobiose was actually hydrolyzed by the enzymes hydrolyzing MUAG. However, experiments in Antarctica in December 1994 provided evidence of a glucosidase that is not specific for α or β anomers (see Chapter 3).

Somville (1984) reported $K_m$'s for both α and β glucosidase of ~0.01 μM, which was consistent in marine, fresh and estuarine waters of varying trophic status. This value is consistent with values in Antarctica and the subtropical Pacific, both in the water column and associated with sinking particles, suggesting broad cross-habitat

homology of these enzymes. Chróst (1989) observed much greater values (70-140 µM) of $K_m$ for β-glucosidase in the Plußsee (northern Germany). Given the proximity, and presumably ecological similarity, of this lake to Somville's pond in Belgium, these values are difficult to interpret. While Somville's results and mine suggest that this enzyme has similar characteristics in diverse and widely separated bodies of water, it is not clear why the enzymes in a pond in Belgium should be similar to those in remote regions of the ocean and different by four orders of magnitude from a lake a few hundred kilometers away. Münster (1991) reported intermediate values (~10 µM) in Lake Mekkojarvi (Finland).

β-glucosidases are not generally thought to have a trace element cofactor; they may be activated by $Ca^{2+}$ (Gottschalk, 1986). The high concentrations of $Cu^{2+}$, $Ni^{2+}$, and $Hg^{2+}$ required for inhibition in seawater suggest that, if this enzyme has an ionic cofactor, it is likely to be a major ion such as $Ca^{2+}$, or is very tightly bound to the enzyme. However, LAPase as well may require high concentrations of metal ions for significant inhibition in seawater. Tubbing and Admiraal (1991) reported inhibition of LAPase by $Cu^{2+}$ in the Rhine River, at concentrations of 0.1-1 µM. Vives-Rego et al. (1986) found that $Cu^{2+}$ was a weak inhibitor of LAPase in coastal seawater in comparison to $Ni^{2+}$ or $Zn^{2+}$, and these ions required concentrations of several hundred µM to achieve 50% reduction in activity.

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# CHAPTER 3

# SPATIAL AND TEMPORAL PATTERNS OF ECTOENZYME ACTIVITY

## 3.1 INTRODUCTION

Fluorogenic tracers of ectoenzymatic activity in marine and fresh waters have now been in use for more than a decade (Hoppe, 1983; Somville and Billen, 1983). Because of its utility in determining rates and patterns of bacterial utilization of dissolved organic matter (DOM), and the relative ease of measurement, this is becoming one of the cornerstone methods of aquatic microbial ecology (Hoppe, 1991, 1993). Most studies of ectoenzymes to date have been conducted in freshwater, estuarine and nearshore marine environments (Chróst, 1991), i.e., in relatively eutrophic environments, usually with significant inputs of allochthonous organic matter. The overwhelming majority have been in temperate zones. While some data have been collected from oceanic (Hoppe, 1991), polar (Billen, 1991), and tropical (Hoppe et al., 1988) environments, these studies reported the activity of only a single enzyme (leucine aminopeptidase).

Most studies of bacteria in seawater have focused on estimating biomass and rates of biomass production. Information regarding the substrates utilized *in situ* is extremely limited. Measurements of ectoenzymatic activity can complement these other studies in several important ways. First, they can be used to estimate the rates of utilization of different components of DOM and thereby to constrain estimates of biomass production and growth efficiency. Second, the enzymes that are expressed by bacteria in a given environment can give clues as to patterns of substrate utilization in that environment that are not apparent from uptake rates of macromolecular precursors

like thymidine or leucine. Finally, the temperature responses of the enzymes in a given environment can also indicate phenotypic differences among bacterial communities.

I have measured the activities and temperature responses of leucine aminopeptidase (LAPase), α-glucosidase (AGase) and β-glucosidase (BGase) in three oceanic provinces from which few data regarding ectoenzymatic activities of natural bacterioplankton communities have been published: the subtropical gyre of the North Pacific, the Pacific equatorial current system, and the high latitude, permanently cold waters of the Southern Ocean. The existence of a latitudinal gradient in the relative activities of proteases and polysaccharases of marine bacteria was first observed by Kriss et al. (1963), who used less quantitative selective enrichment methods. Using fluorogenic substrate analogues, I have determined that the relative activities of these enzymes show very pronounced differences among latitudinal and climatic zones, and that these differences follow a latitudinal pattern that is at least qualitatively similar to that observed with the old methods. Much of this material, in particular the cross-habitat comparisons described in Section 3.3.1, is drawn from a forthcoming paper (Christian and Karl, 1995a).

In contrast to the equatorial and subtropical study sites, which involved repeat measurements at a single location, experiments in Antarctica have been conducted over a broad area and in all seasons in this highly seasonal environment. Temporal variability is an important consideration when comparing results from Antarctica to the other study sites, which experience little seasonality, so some aspects of seasonal variation are described in Section 3.3.1. Covering a broad area has made it possible to discern spatial patterns within the Antarctic study area, as well as temporal variations on time scales both longer and shorter than seasonal, that have important implications for the ecology and biogeochemistry of the region. These are described in detail in Section

3.3.2. Parts of this section are drawn from papers published in the *Antarctic Journal of the United States* (Christian and Karl, 1992; 1993; 1994).

## 3.2 MATERIALS AND METHODS

### 3.2.1 Study areas

Samples were collected from oceanic (Pacific subtropical gyre and equatorial Pacific) and polar (Antarctic Peninsula region of the Southern Ocean) environments (Figure 3.1). In the subtropical Pacific, experiments were conducted at the US-JGOFS time-series Station ALOHA (22°45' N, 158°00' W; cf. Karl and Winn, 1991), and in the equatorial Pacific, in the US-JGOFS study area at 0°, 140° W, in the South Equatorial Current (Murray et al., 1994). In the Southern Ocean, field studies were conducted in the Palmer Peninsula Long-Term Ecological Research (LTER) study area (Quetin and Ross, 1992; Waters and Smith, 1992). The equatorial data were collected on a single cruise in March-April 1992 (RV Thomas G. Thompson cruise TT008). Station ALOHA and the Antarctic study area were visited repeatedly from 1991 through 1994.

Antarctic data are from several cruises. RACER 3 and 4 (December-January 1991-92 and July-August 1992, respectively) were conducted primarily in Gerlache Strait; NBP93-02 (March-May 1993) and PD94-01 (January-February 1994) covered a broader area, both inside and outside the coastal island chain along the Antarctic Peninsula. PD94-12 (December 1994) included coastal (Paradise Harbor) and shelf (near Victor Hugo Island) stations, and a profile in ice-covered Crystal Sound. Some of the data on temperature responses for Antarctic LAPase were collected on PD92-09 (November 1992). The Palmer LTER grid (Figure 3.2) is described in detail by Waters

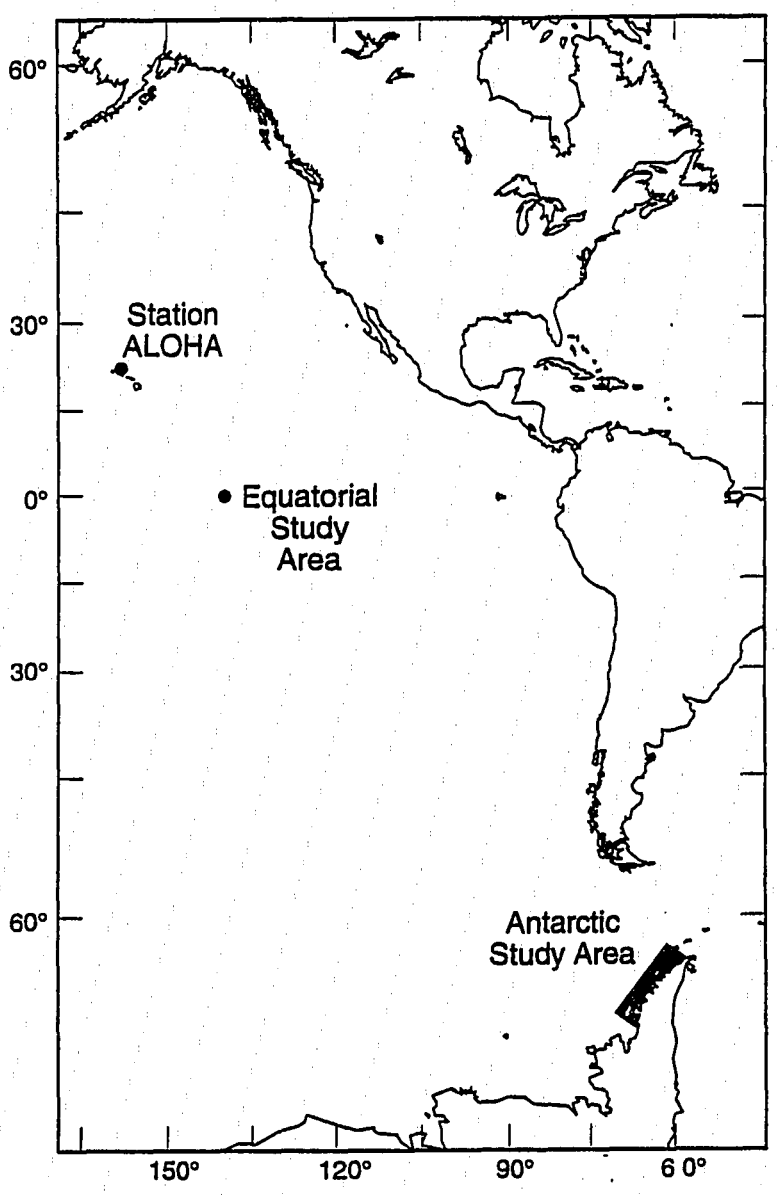


Figure 3.1. Locations of study sites.

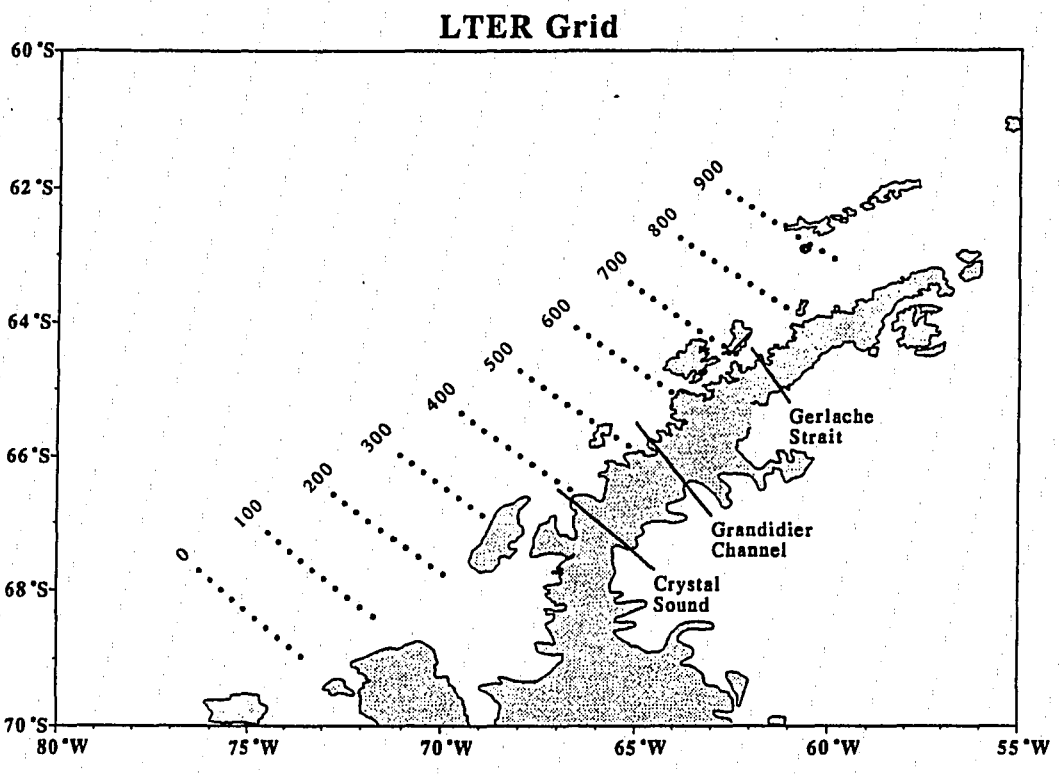


Figure 3.2. Palmer LTER study area with gridlines.

and Smith (1992). The grid consists of 10 parallel lines 200 km long and 100 km apart running approximately perpendicular to the coastline, numbered 000 through 900 from south to north. Along each line stations are numbered xxx.yyy, where xxx is the line number and yyy is the distance in kilometers from the inshore end of the line. To illustrate, station 900.200 is at the northwestern corner of the grid, and the center of the grid lies between stations 500.100 and 400.100.

### 3.2.2 Ectoenzyme assays

The methods used here are those described in Chapter 2. The fluorescent substrate analogues used were 4-methylumbelliferyl-α-glucoside (MUAG), 4-methylumbelliferyl-β-glucoside (MUBG), and L-leucyl-β-naphthylamine (LLBN). These were added to 6 ml of seawater and incubated for 12-24 h. On RACER 4 (winter cruise in Antarctica) incubation time was increased to 48 h. Samples not analyzed immediately were poisoned with 0.1 ml of a saturated solution of mercuric chloride (final concentration ~4 mM) to stop the reaction and frozen until analyzed (Christian and Karl, 1995b). Mercuric chloride precipitates excess LLBN which was removed by filtration (0.2 μm) or centrifugation prior to fluorescence determination. All incubations were conducted in the dark at approximately *in situ* temperature. Incubation temperature was 28°C at the equator and 0°C in Antarctica. At Station ALOHA incubation temperature was approximately *in situ* temperature, but varied slightly on different cruises; the data presented here are normalized to 25°C.

All routine determinations were conducted at saturating substrate concentration (1.6 μM MUAG or MUBG, 1 mM LLBN) to facilitate comparison of total enzyme among samples. These should therefore be considered potential activities rather than

estimates of activity *in situ*. This potential activity is described as $V_{sat} = k_2E_o \sim V_{max}$, where $k_2$ is the rate constant for the catalytic (hydrolysis) step, and $E_0$ is the total enzyme present in the sample.

BNAPH experiences some loss of fluorescence with long-term storage (Christian and Karl, 1995b). Because samples were stored for varying lengths of time there is some additional variance associated with this effect. Because of uncertainty about the exact rate of loss of fluorescence, I have not attempted to correct these values to their presumed initial concentration. BNAPH activities from RACER 3 and 4 are probably underestimated by 10-20%, and in the equatorial Pacific by about 30%.

### 3.2.3 Sampling and replication

Water was collected in Niskin® or GoFlo® bottles, dispensed directly into polycarbonate or high-density polyethylene bottles, and subsampled by pipette into the incubation containers (generally scintillation vials for BGase and 15 ml polypropylene centrifuge tubes for LAPase). Where replicates were taken the value presented is the mean of the subsamples taken from a given Niskin® bottle. At Station ALOHA, LAPase activities are the mean of two replicates and BGase activities the mean of three. In the equatorial Pacific, activities are the mean of duplicate subsamples. In Antarctica, in most cases a single subsample was taken from each depth: variance among duplicate or triplicate subsamples was comparable to, or less than, in the other two study sites (see Chapter 2). In the temperature response experiments all subsamples are shown.

In the equatorial Pacific, LAPase activity in 3 of 30 samples was much greater in one of two replicate subsamples than in the other, and the lower value was consistent

with the rest of the data. These anomalies are probably due to the presence of particles rich in microbial activity (see Chapter 5). Alternative estimates of the mean activity that do not include these three points are given in Table 3.1.

Samples of sea ice rich in biomass ("brown ice") were collected from ice recently disrupted and fragmented by the ship's motion, using a Zodiac® placed on the ice by the ship's crane. Microbial communities from the underside of undisturbed ice were collected by SCUBA divers using a spring-loaded syringe device with a collection volume of ~0.5 l.

### 3.2.4 Temperature control

The activities of these enzymes at various temperatures were determined in several experiments. These were carried out in temperature-controlled water baths or in a set of flow-through deck incubators constructed for the HOT program. To determine the response to finer gradations of temperature, a device known as the "thermatron" was used, which consists of an aluminum block approximately 80x40x7 cm with 36 holes (9x4) each containing a 50 ml beaker of seawater in which samples can be placed (Figure 3.3). At either end of the block water flows through an opening approximately 0.5 cm in diameter spanning the width of the block. Water was circulated through these openings from two temperature-controlled water baths whose temperatures differed by ~15 °C, creating a gradient along the block of about 8°C in approximately 1°C intervals between samples. The block was housed in an insulated box constructed of 1.27 cm polyvinylchloride sheeting with styrofoam lining ~8 cm thick. When the temperature of the water bath at the cold end of the gradient was <0 °C, ethylene glycol was added to prevent freezing.

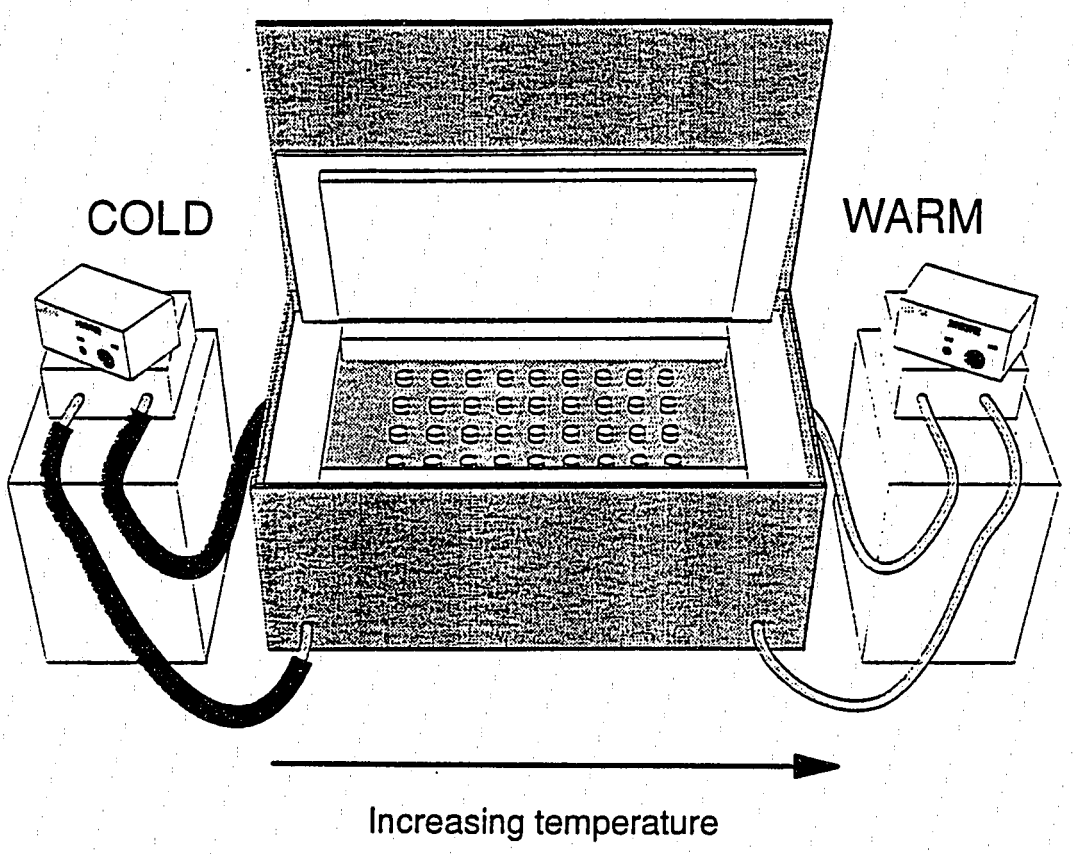


Figure 3.3. Schematic of the thermatron.

### 3.2.5 Incorporation of $^3$H-leucine

To determine the rate of incorporation of monomeric amino acids, water samples (25 ml) were incubated with L-[2,3,4,5] or [4,5]-$^3$H-leucine. Leucine was added at a final concentration of 5-10 nM with a specific activity of ~50 Ci/mmol. Following incubation (~6 h, in the dark) samples were filtered (Whatman GF/F) and frozen for transport back to Hawaii. The protein, RNA, and DNA components were then separated by differential acid-base hydrolysis (Karl, 1981). Values presented here are incorporation into protein only; this generally represents > 85% of assimilation in surface samples.

## 3.3 RESULTS

### 3.3.1 Activity ratios and temperature responses: cross-habitat comparison

#### *3.3.1.1 Activity ratios*

The ratio of LAPase to BGase ($V_{sat}/V_{sat}$) varied significantly and systematically among the different study areas. LAP/BG at the equator was much less than in the other two regions. BGase at the equator was 3-4 orders of magnitude greater than at Station ALOHA; LAPase was similar in these two study areas (Figure 3.4 and Table 3.1). LAP/BG at Station ALOHA was generally less than in Antarctica (Table 3.1). The difference in LAP/BG among the three study areas was in all cases highly

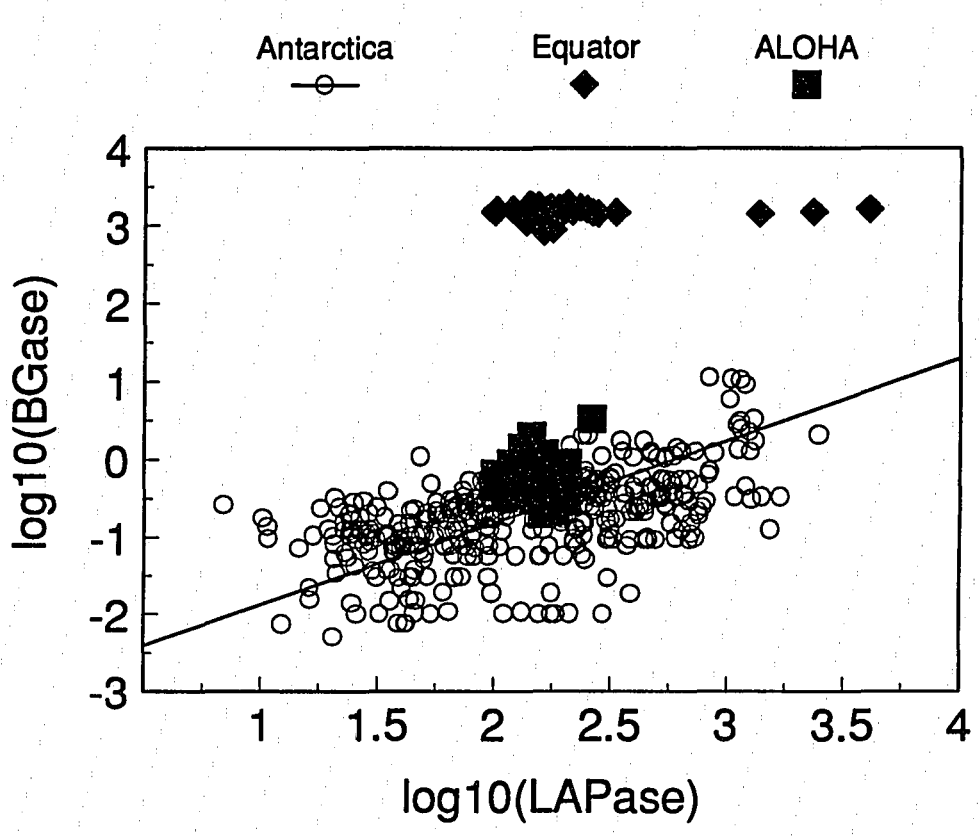


Figure 3.4. LAPase vs. BGase ($V_{sat}$, logarithms (base 10) of rates in nmol $l^{-1}$ $d^{-1}$) at *in situ* temperature in the three study areas. Samples are from ≤20 m in Gerlache Strait (RACER 3 and 4) and ≤80 m elsewhere. The regression equation (Model II, geometric mean method) for the Antarctic data is $\log_{10}(BGase)=-3.01+1.066\log_{10}(LAPase)$ ($r^2=0.311$, $P<0.001$, $n=398$).

Table 3.1. Mean values of $V_{sat}$ (nmol $l^{-1}$ $d^{-1}$) for LAPase and BGase for the three study areas and for five cruises in Antarctica. LAP/BG ratios are the ratio of the mean values for each environment or cruise. Values for Crystal Sound and Paradise Harbor are from December 1994. All data from depths ≤80 m, in Gerlache Strait and Paradise Harbor ≤20 m.

| Study Area | n | LAPase | BGase | LAP/BG |
|---|---|---|---|---|
| Equator | | | | |
| all data | 30 | 420 | 1519 | 0.276 |
| outliers removed | 30 | 192 | 1519 | 0.127 |
| ALOHA | 28 | 160 | 0.75 | 213 |
| Antarctica | | | | |
| All cruises | 427 | 273 | 0.45 | 604 |
| Dec. 1991 | 90 | 648 | 1.18 | 548 |
| Jul.-Aug. 1992 | 80 | 37 | 0.11 | 339 |
| Mar.-May. 1993 | 105 | 137 | 0.32 | 434 |
| Jan.-Feb. 1994 | 123 | 228 | 0.22 | 1052 |
| December 1994 | | | | |
| all data | 29 | 451 | 0.64 | 708 |
| Crystal Sound | 6 | 143 | 0.32 | 440 |
| Paradise Harbor | 15 | 726 | 0.98 | 740 |

significant ($P < 0.001$; Wilcoxon two sample test (nonparametric), or one-way ANOVA on log-transformed data).

The potential activity ($V_{sat}$) of LAPase in the subtropical Pacific and in Antarctica was 2-3 orders of magnitude greater than that of BGase. Because of seasonality, both enzymes had a much greater range in Antarctica than in the other two regions. For each of four cruises in Antarctica there is a cluster of points with variable LAP/BG ratio (i.e., the points do not fall along a line), and the mean ratio varied among the four cruises (Figure 3.5 and Table 3.1). Overall there was a strong positive correlation ($P < 0.001$), but a large amount of variance ($r^2 = 0.311$). These data span a large range of activities, and correlations within a given area and season can be weak (see section 3.3.2). The regression equation (Model II, geometric mean method) for these four cruises is $\log_{10}(\text{BGase}) = -3.01 + 1.066\log_{10}(\text{LAPase})$ (Figure 3.4).

*3.3.1.2 Temperature responses*

The temperature responses of these enzymes in the three regions differed significantly, which may reflect important differences among bacterial communities (Figure 3.6). The temperature relationships at Station ALOHA were more similar to those in samples from Antarctica than to equatorial waters, although the surface water temperature at Station ALOHA (23-27°C) is very similar to that in the equatorial study area, which is within the same range at most times. More extreme temperatures are observed in association with El Niño events, and temperatures during March-April 1992 were among the warmest recorded in 12 years of continuous measurement (TOGA-TAO array; McPhaden, 1993). Water temperature in Antarctica is near 0°C at all times of the year. All regression lines have $r^2 > 0.95$ except for Antarctic LAPase

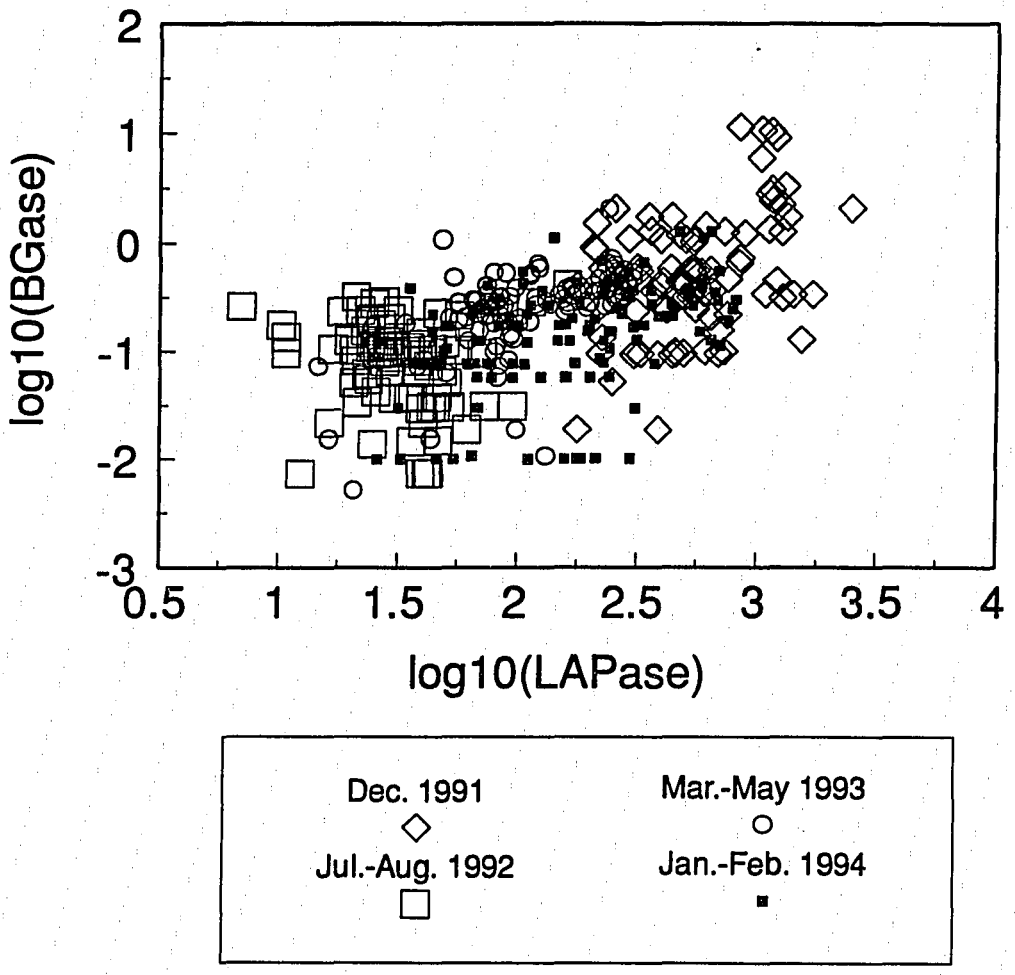


Figure 3.5. LAPase vs. BGase ($V_{sat}$, logarithms (base 10) of rates in nmol $l^{-1}$ $d^{-1}$) at *in situ* temperature on four cruises in Antarctica in various seasons: Dec. 1991 (RACER 3), Jul.-Aug. 1992 (RACER 4), Mar.-May 1993 (NBP93-02), and Jan.-Feb. 1994 (PD94-01).

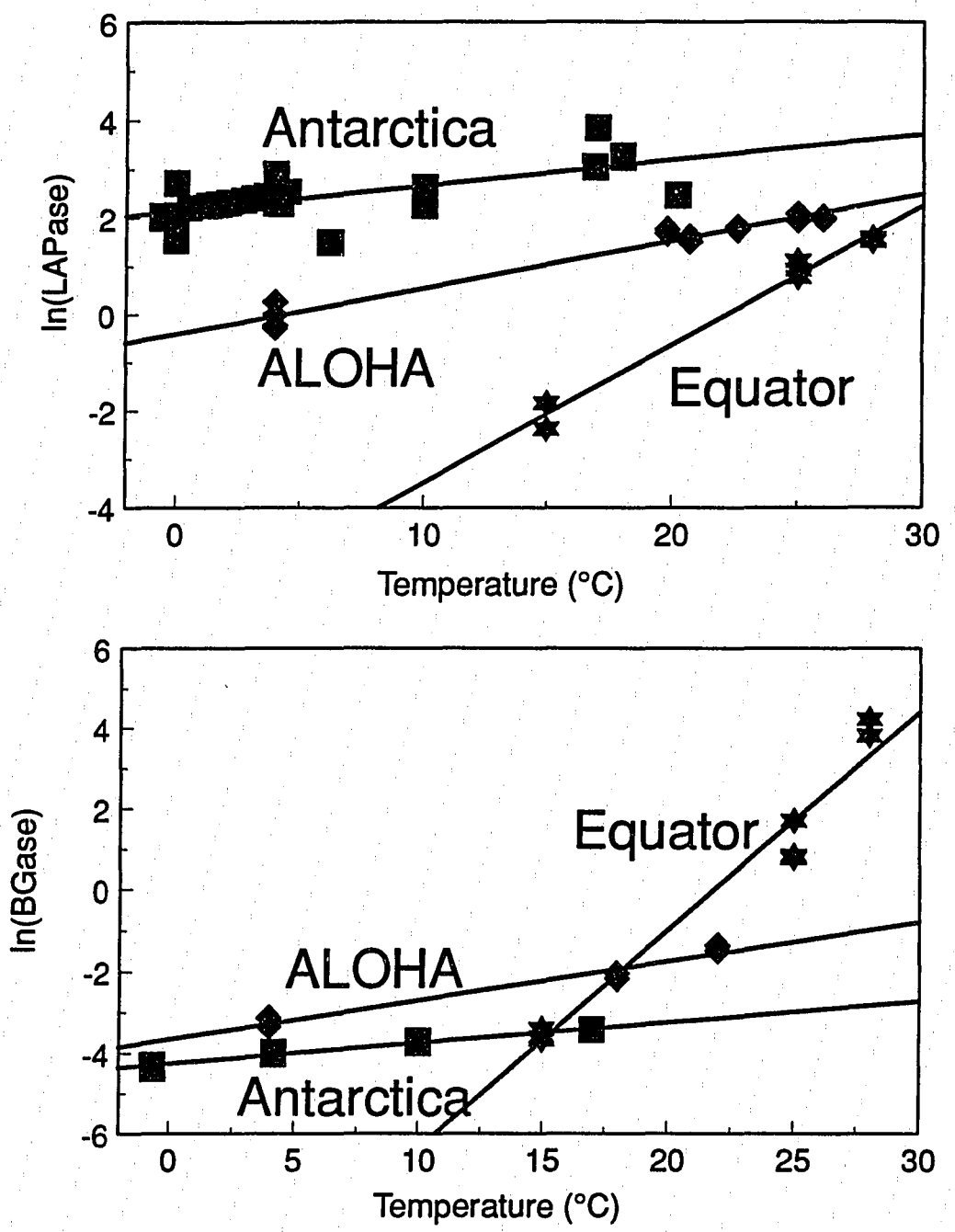


Figure 3.6. Temperature dependence of $V_{sat}$ for LAPase (top) and BGase (bottom), natural logarithms of rates in nmol $l^{-1}$ $d^{-1}$. Regression statistics are given in Table 3.2.

Table 3.2. Regression statistics for temperature dependence of ectoenzyme activity (model I linear regression of $\ln(V_{sat})$ on incubation temperature in degrees Celsius).

| Enzyme | Location | Intercept | Slope | $r^2$ |
|---|---|---|---|---|
| LAPase | Antarctica | 2.14 | 0.052 | 0.49 |
| LAPase | Station ALOHA | -0.40 | 0.096 | 0.97 |
| LAPase | Equator | -6.35 | 0.286 | 0.98 |
| BGase | Antarctica | -4.25 | 0.051 | 0.98 |
| BGase | Station ALOHA | -0.16 | 0.095 | 0.96 |
| BGase | Equator | -11.79 | 0.540 | 0.96 |

($r^2 = 0.49$) which combines several experiments with similar slopes but different intercepts (Table 3.2). The slopes of both semilog temperature relationships at Station ALOHA are greater than those in Antarctica by about a factor of 2 (Table 3.2). The coincidence of the slopes of the activities for these two enzymes is quite remarkable. It is also interesting that the slopes are so much greater in the equatorial Pacific than at Station ALOHA, given the small difference in water temperature.

$V_{sat}$ for LAPase in Antarctica did not have a temperature optimum over the range 0-20°C (maximum summer water temperature is 1-2°C). At low ($<K_m$) substrate concentrations (nearer to those actually present in the ocean), the reaction was only weakly dependent on temperature (Figure 3.7). This apparent temperature-independence may result because the rate-limiting step in this concentration range (substrate binding) is reversible. Because the rate constants for both forward and back reactions presumably increase with temperature, there is no increase in rate of the overall reaction. Only when substrate concentration increases and the second, nonreversible step (hydrolysis) becomes rate-limiting does temperature become a significant consideration.

### 3.3.2 Ectoenzyme activities in the Western Antarctic Peninsula region

#### *3.3.2.1 Spatial/temporal noncovariance of BGase and LAPase*

During the 1991-92 austral summer (RACER 3), two "fast grids" (30-40 stations sampled over ~72 h) of surface water samples for LAPase and BGase were taken in Gerlache Strait. Because these experiments were conducted at saturating substrate concentration, the results represent potential rather than actual activities. It

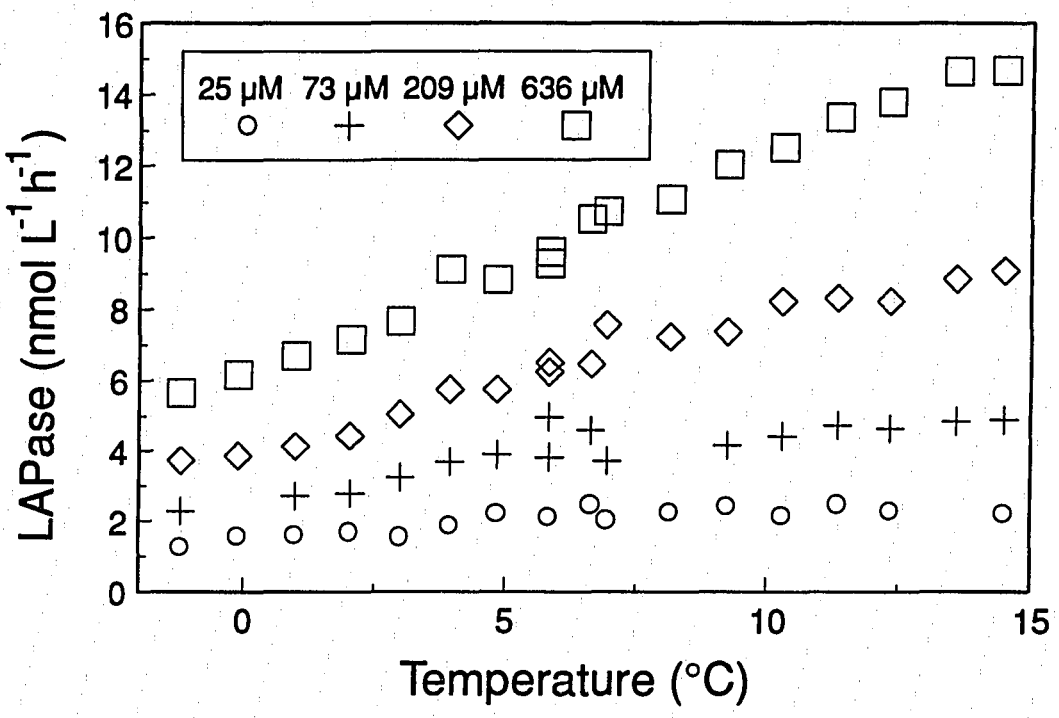


Figure 3.7. LAPase activity (nmol $l^{-1}$ $d^{-1}$) in Antarctic seawater at incrementally increasing temperature in the "thermatron" at four concentrations (25, 73, 209 and 636 µM) of LLBN. Composite of two experiments normalized to 636 µM rate in region of overlap.

has been suggested that such potential activity measurements are an index of bacterial biomass rather than growth or activity (Billen et al., 1990). This implies that the potential activities of the two enzymes should be strongly positively correlated. However, activities of these two enzymes were uncoupled in space or time, or both, during two "fast grids" of surface samples (Figure 3.8). On fast grid B (12/22-12/25) there was a significant positive correlation between the two, but a fair amount of variance about the mean relationship ($r^2$=0.269, P<0.005; if the three "outlier" points (see Figure 3.8a) are removed $r^2$=0.691). On fast grid C (12/27- 12/30) the two activities were not significantly correlated ($r^2$=0.095).

*3.3.2.2 Temporal variability at Station A*

The activities of these enzymes were extremely variable in time and space. Over four occupations of Station A (64° 11.7' S, 61° 19.5' W) at intervals of 5-7 days these enzyme activities were highly variable (Figure 3.9). On Dec. 19 a large peak in the activities of both enzymes was observed at 50 m. This peak was not present on Dec. 12. A trace of this peak remained on Dec. 25, but disappeared by Dec. 30, by which time an even larger peak appeared at less than 20 m. This pattern was similar for both enzymes, and the two activities were significantly correlated overall (P<0.001, $r^2$=0.489, n=32).

The Station A profiles show that the surface samples taken at the fast grid stations may miss much of the activity. This could alias the spatial pattern since the maximum activity sometimes occurs at or near the surface. Although there was no single sampling depth at which one could reasonably expect to observe the greatest activity, activities were generally greatest in the mixed layer (<20 m). The event

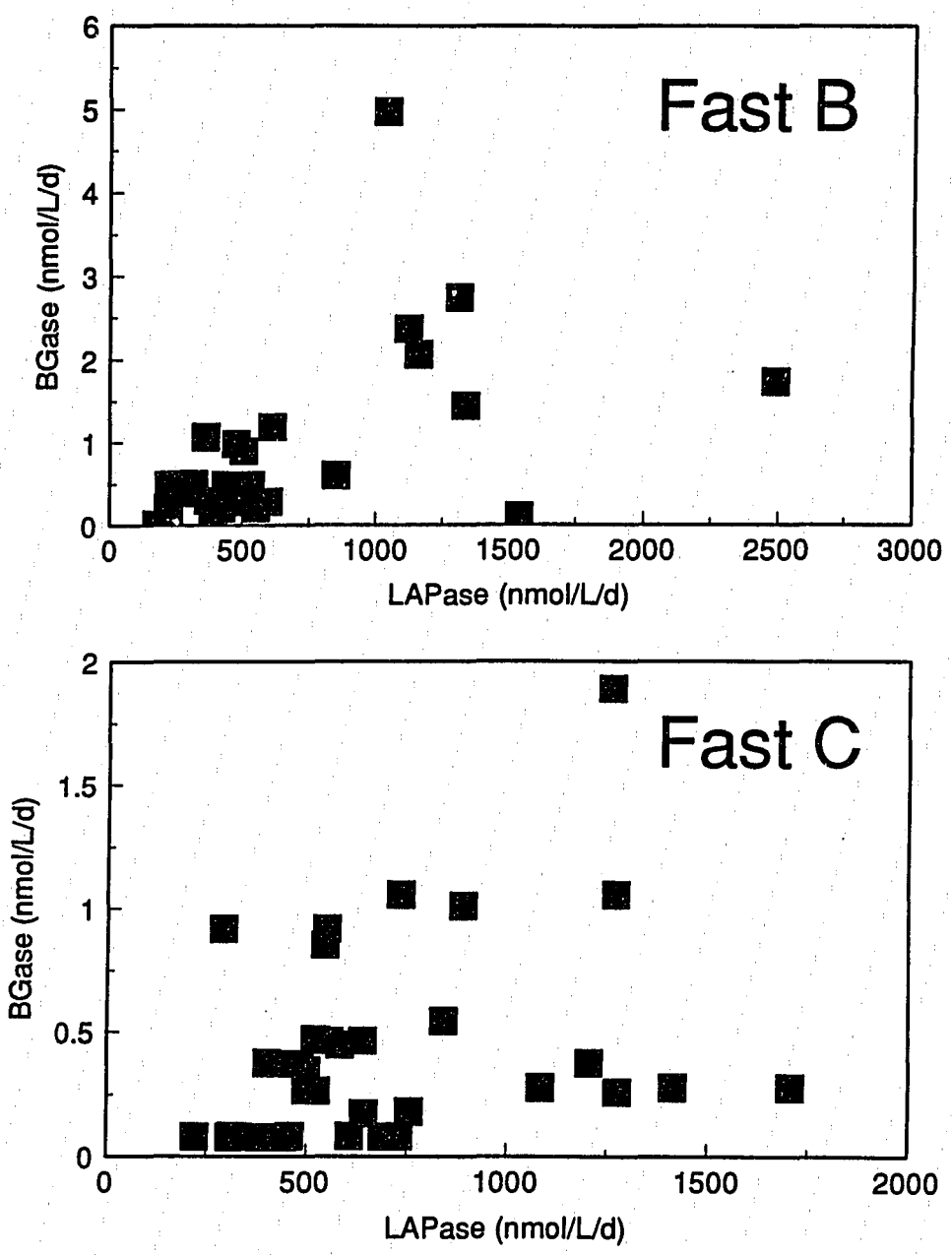


Figure 3.8. Relationship between BGase and LAPase activities (nmol $l^{-1}$ $d^{-1}$) on fast grid B (12/22/91 - 12/25/91) and fast grid C (12/27/91 - 12/30/91).

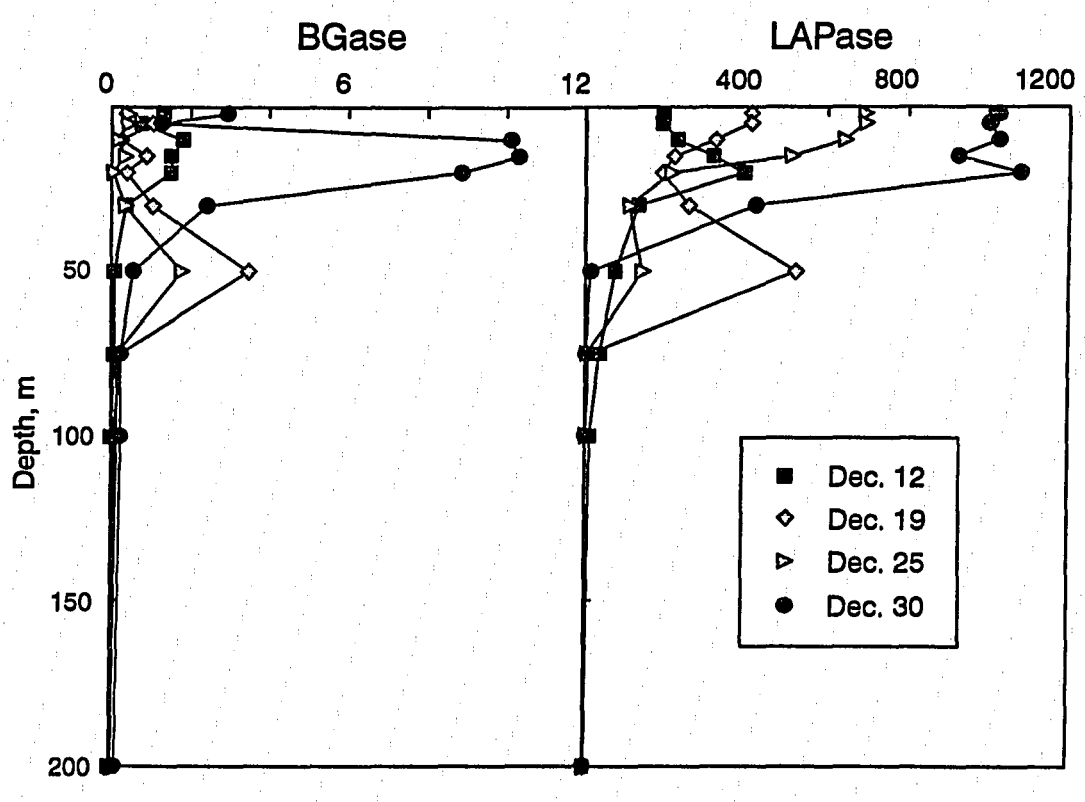


Figure 3.9. Depth profiles of BGase and LAPase activity (nmol $l^{-1}$ $d^{-1}$) at Station A (64° 11.7' S, 61° 19.5' W) in December 1991.

observed at Station A on Dec. 19-25 may have been caused by wind mixing of mixed layer water rich in living cells and organic matter below the euphotic zone where it was trapped when a new pycnocline formed. There were strong winds in Gerlache Strait during the week of Dec. 12-19. It might also have been caused by rapid sinking of phytoplankton (Alldredge and Gottschalk, 1989). Such events may be an important mechanism for removing primary production from the euphotic zone in Antarctic waters.

*3.3.2.3 Seasonal variation of LAP/BG ratio*

Results from four cruises in Antarctic waters suggest seasonal variation in the substrates utilized by bacterioplankton. The LAP/BG ratio was greatest in summer, and least in fall and winter, implying that glucose was more heavily utilized as the supply of dissolved proteins and peptides produced by spring-summer bloom processes decreased. LAP/BG on each of the summer cruises was significantly ($P < 0.001$, Wilcoxon or ANOVA as above) different from the fall and winter cruises. Differences between the two summer cruises and between the fall and winter cruises were not significant at $\alpha = 0.05$. The turnover time of this substrate pool would therefore be on the order of several months. This effect is more apparent in the more oligotrophic shelf waters surveyed during January-February 1994 (PD94-01) and March-May 1993 (NBP93-02) than in the RACER study area in Gerlache Strait. In the open shelf waters LAPase activities were about as great in autumn as in summer, while BGase activities were higher in autumn (Figure 3.5). A profile taken in Crystal Sound in December 1994 showed activity ratios similar to those observed on the winter cruise (Table 3.1); at this time Crystal Sound was still completely ice covered.

### *3.3.2.4 Absence of onshore-offshore gradient*

When the entire LTER grid was sampled in the autumn of 1993 (NBP93-02), onshore-offshore gradients were largely absent, and activities were generally as great in the offshore waters of the Antarctic Circumpolar Current (ACC) as in Bransfield Strait and near the coastal islands of the Palmer Archipelago. Activities in shelf waters were typically constant from the surface to a depth of 80-120 m, where they declined sharply. LAPase activity in the upper 80 m in autumn 1993 was not correlated with water depth, which ranged from less than 100 m to greater than 3000 m (Figure 3.10). Activities in the mixed layer in the most protected waters can be greater, however, particularly during phytoplankton blooms (Table 3.1).

### *3.3.2.5 Seasonal variation in geographic trends*

In autumn 1993, LAPase activity was relatively constant from the 900 to the 400 line and then declined towards the southern end of the grid (Figure 3.11). Because the cruise took place in the austral autumn and the southern stations were sampled last, it is difficult to distinguish between latitudinal and seasonal variation. Repeat visits to several stations near the center and at the northern end of the grid after the southern stations were sampled showed activities that had declined substantially from a month earlier. However, these activities were still higher than the activities at the southernmost stations.

The geographic trend in LAPase activity was very different the following summer (Jan.-Feb. 1994). The highest activities were observed at the inshore end of the

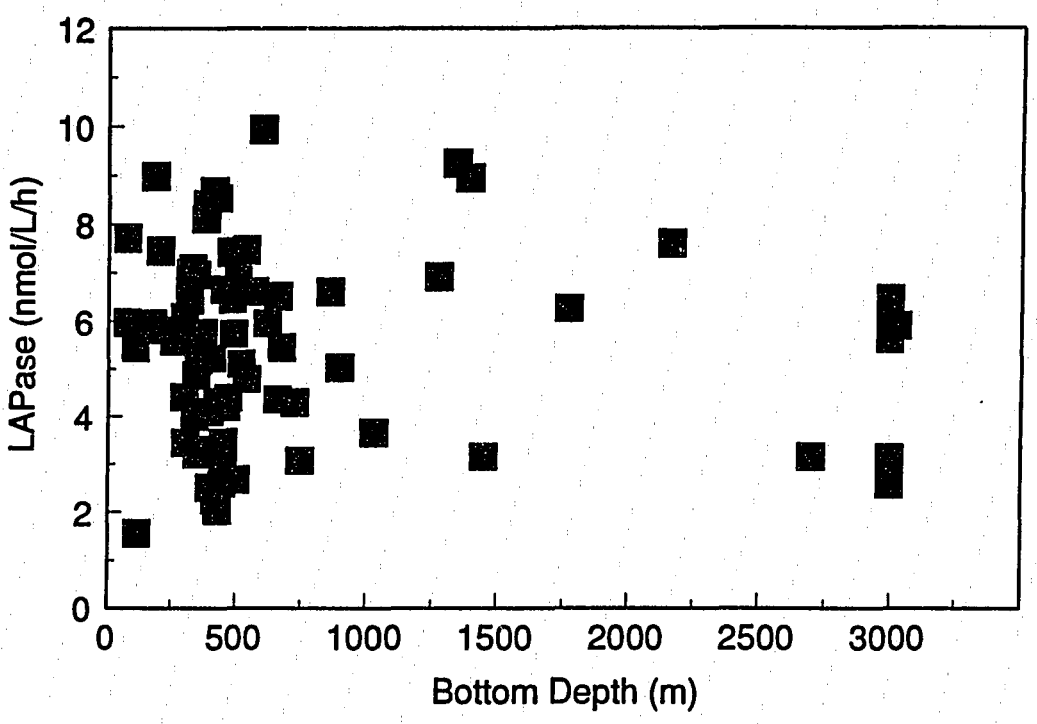


Figure 3.10. Relationship of depth-averaged mixed layer LAPase activity (nmol $l^{-1}$ $h^{-1}$) to water depth. Depths greater than 3000 m are listed as 3000 m.

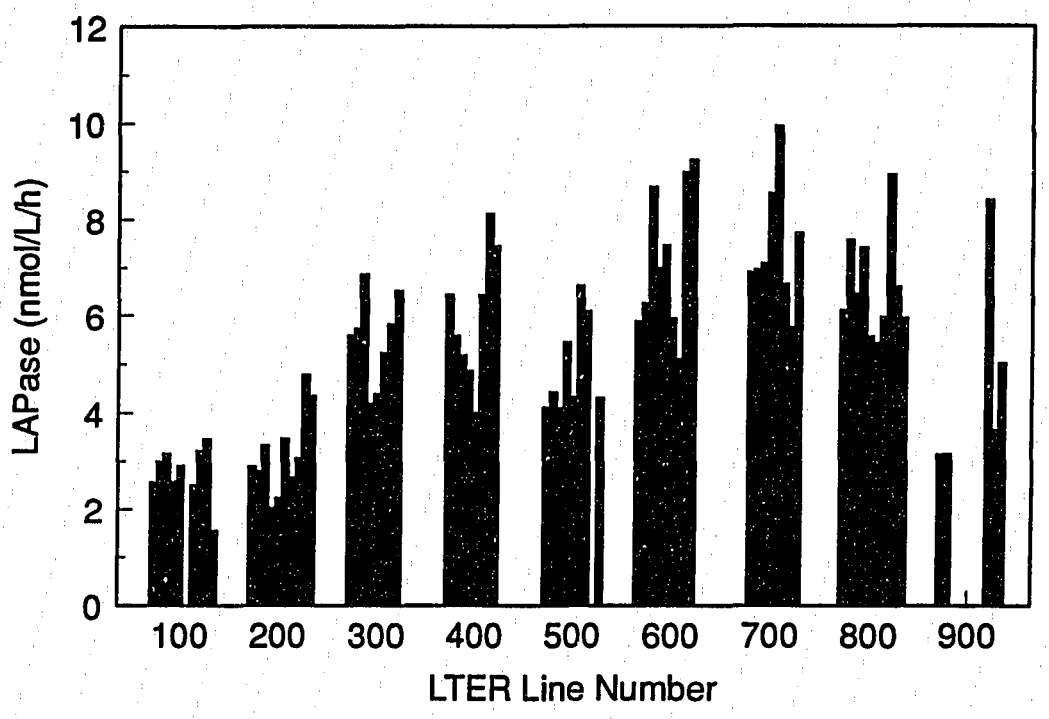


Figure 3.11 Depth-averaged LAPase activity (nmol $l^{-1}$ $d^{-1}$) on LTER lines 100 through 900. Bars in each histogram are individual stations, inshore to offshore from left to right.

300 line (Figure 3.12, station locations are given in Table 3.3). On the 400 line, depth-integrated LAPase was also elevated at the inshore stations, declining across the shelf. It is hypothesized that the north-south and onshore-offshore trends result from the recent presence of the annual pack ice, which extended to the inshore end of the 300 and 400 lines on this cruise but had long since disappeared further north.

*3.3.2.6 Effect of sea ice*

In January 1994 (PD94-01), fragments of sea ice rich in biomass ("brown ice") were encountered with increasing frequency, along a southward transect from Palmer Station to Crystal Sound through the Lemaire and Grandidier Channels. Depth-integrated LAPase gradually increased along this track. The southernmost stations (382.010 and 375.020) were completely ice-covered (but with fragmented, drifting ice, not land-fast ice). These had intermediate depth-integrated activities, lower than most of the (ice-free) stations of the 300 line. However, activities at or just below the surface were among the highest observed (Figure 3.12). Near-surface BGase activities were also greatest at the ice stations. AGase was also slightly elevated at station 375.020. BGase activity never approached the levels observed near the receding pack ice in Marguerite Bay in 1991-92 (up to 17 nmol $l^{-1}$ $d^{-1}$; see Karl et al., 1992 and Dore et al., 1992 for a description of the field experiment).

AG/BG ratios in the water column showed a relative decline from the northern to the southern end of the sampling area (Figure 3.13). ln(AG/BG) was significantly ($P<0.001$) negatively correlated with latitude, and with time from the start of the cruise, as the stations were occupied more or less from north to south (Table 3.3). The AG/BG ratio is plotted against BGase in Figure 3.13b. Although the apparent negative

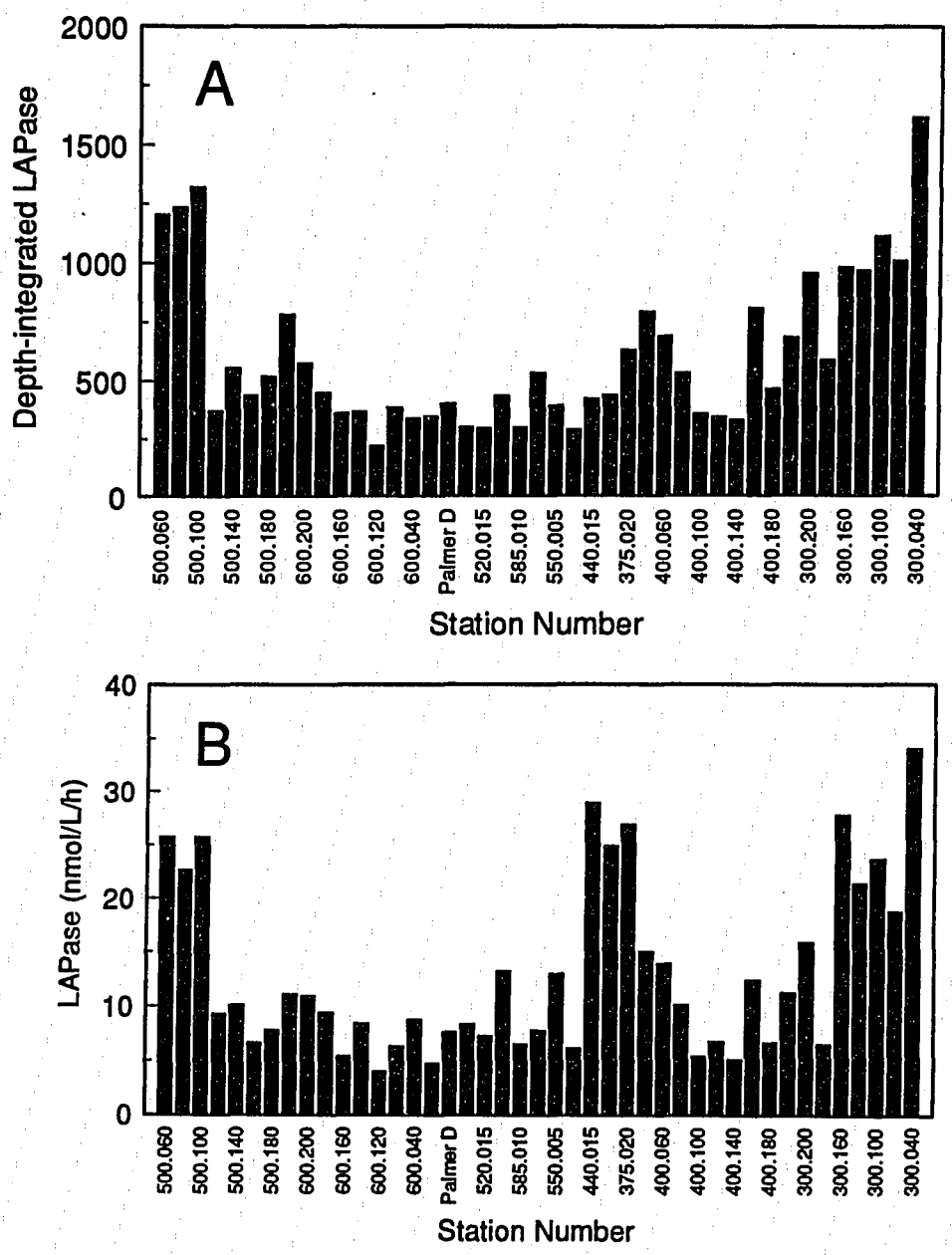


Figure 3.12. (a) LAPase activity integrated to 80 m (trapezoid rule), in μmol $m^{-2}$ $h^{-1}$. (b) LAPase activity (nmol $l^{-1}$ $h^{-1}$) at the shallowest depth sampled at each station (10 m at station 500.100, just below surface at all other stations), in nmol $l^{-1}$ $h^{-1}$. Stations shown in order occupied; only alternate stations named (see Table 3.3 for names of other stations).

Table 3.3. Stations occupied on PD94-01 with dates and positions.

| Station | Date | Day* | Latitude (S) | Longitude (W) |
|---|---|---|---|---|
| 500.060 | 1/12/94 | 0 | 65.48 | 66.15 |
| 500.080 | 1/12/94 | 0 | 65.36 | 66.46 |
| 500.100 | 1/13/94 | 1 | 65.23 | 66.78 |
| 500.120 | 1/13/94 | 1 | 65.11 | 67.09 |
| 500.140 | 1/14/94 | 2 | 64.99 | 67.39 |
| 500.160 | 1/14/94 | 2 | 64.86 | 67.69 |
| 500.180 | 1/14/94 | 2 | 64.74 | 68.00 |
| 500.200 | 1/14/94 | 2 | 64.61 | 68.29 |
| 600.200 | 1/15/94 | 3 | 63.97 | 66.86 |
| 600.180 | 1/15/94 | 3 | 64.09 | 66.56 |
| 600.160 | 1/15/94 | 3 | 64.21 | 66.26 |
| 600.140 | 1/16/94 | 4 | 64.33 | 65.96 |
| 600.120 | 1/16/94 | 4 | 64.45 | 65.65 |
| 600.100 | 1/17/94 | 5 | 64.58 | 65.34 |
| 600.080 | 1/17/94 | 5 | 64.70 | 65.03 |
| 600.040 | 1/17/94 | 5 | 64.93 | 64.40 |
| 600.060 | 1/17/94 | 5 | 64.81 | 64.72 |
| Palmer D | 1/24/94 | 12 | 64.81 | 64.05 |
| Palmer J | 1/24/94 | 12 | 64.77 | 64.13 |
| 620.015 | 1/25/94 | 13 | 64.94 | 63.72 |
| 602.017 | 1/25/94 | 13 | 65.06 | 64.00 |
| 585.010 | 1/26/94 | 14 | 65.21 | 64.13 |
| 575.010 | 1/26/94 | 14 | 65.28 | 64.27 |
| 550.005 | 1/27/94 | 15 | 65.48 | 64.54 |
| 510.000 | 1/27/94 | 15 | 65.78 | 65.04 |
| 440.015 | 1/28/94 | 16 | 66.15 | 66.32 |
| 382.010 | 1/29/94 | 17 | 66.56 | 67.13 |
| 375.020 | 1/29/94 | 17 | 66.54 | 67.40 |
| 400.040 | 1/31/94 | 19 | 66.25 | 67.34 |
| 400.060 | 1/31/94 | 19 | 66.13 | 67.66 |
| 400.080 | 1/31/94 | 19 | 66.00 | 67.97 |
| 400.100 | 2/1/94 | 20 | 65.88 | 68.28 |
| 400.120 | 2/1/94 | 20 | 65.75 | 68.59 |
| 400.140 | 2/1/94 | 20 | 65.62 | 68.90 |
| 400.160 | 2/2/94 | 21 | 65.50 | 69.20 |
| 400.180 | 2/2/94 | 21 | 65.37 | 69.50 |
| 400.200 | 2/2/94 | 21 | 65.24 | 69.80 |

Table 3.3. (continued) Stations occupied on PD94-01 with dates and positions.

| | | | | |
|---|---|---|---|---|
| 300.200 | 2/3/94 | 22 | 65.85 | 71.38 |
| 300.180 | 2/3/94 | 22 | 65.98 | 71.08 |
| 300.160 | 2/3/94 | 22 | 66.11 | 70.78 |
| 300.140 | 2/4/94 | 23 | 66.24 | 70.48 |
| 300.120 | 2/4/94 | 23 | 66.38 | 70.18 |
| 300.100 | 2/4/94 | 23 | 66.51 | 69.87 |
| 300.080 | 2/6/94 | 25 | 66.63 | 69.55 |
| 300.060 | 2/6/94 | 25 | 66.76 | 69.24 |
| 300.040 | 2/6/94 | 25 | 66.89 | 68.92 |

* Days from 1/12/94

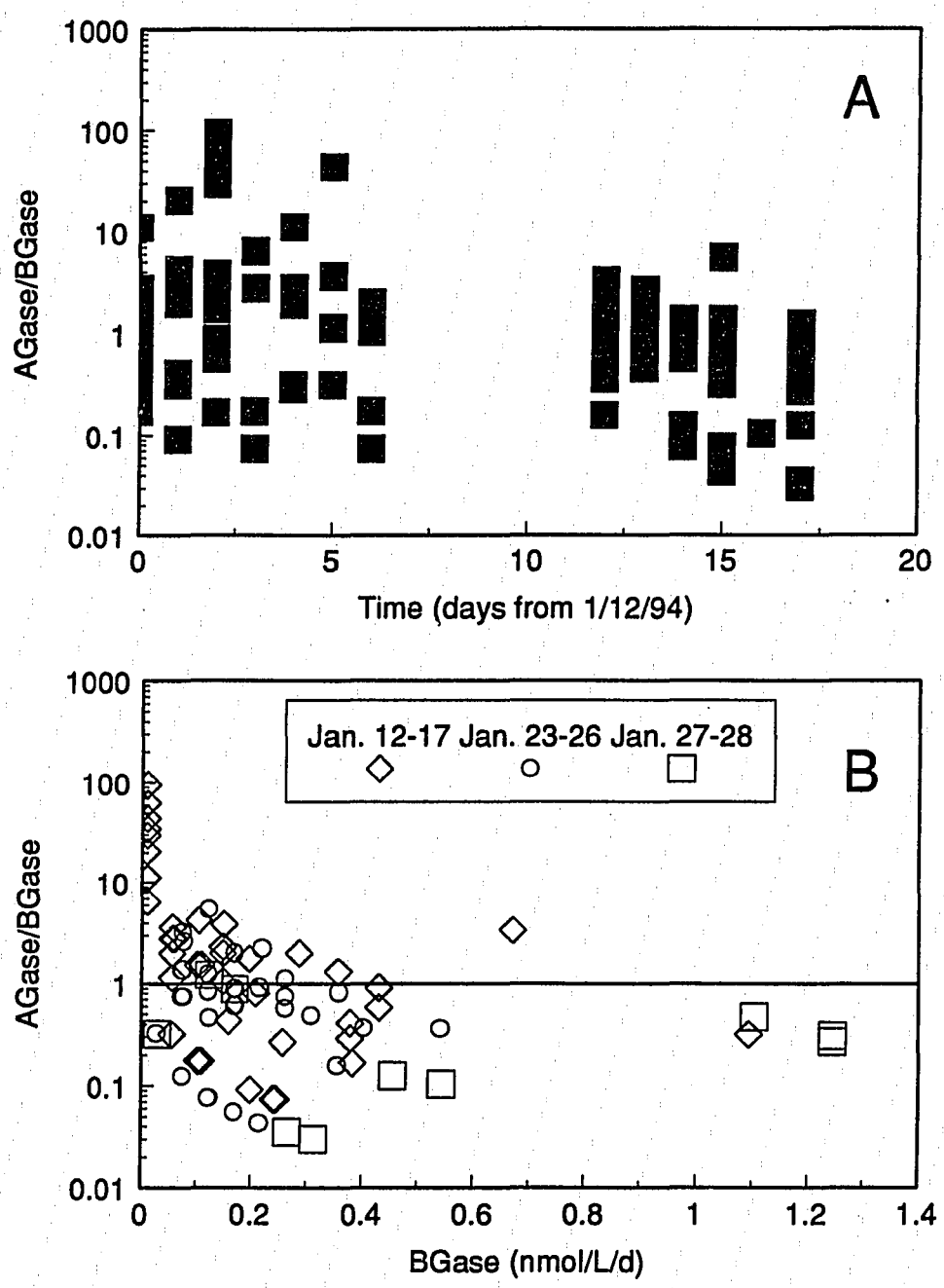


Figure 3.13. (a) AGase/BGase ratio at stations sampled from 1/12/94 through 1/29/94 (see Table 3.3 for names and locations of stations sampled each day). (b) AGase/BGase ratio relative to BGase (nmol $l^{-1}$ $d^{-1}$), at same stations shown in (a), for three time periods of sampling in different areas (see Table 3.2 for station locations).

correlation is not statistically meaningful as the variables are not independent, it is significant that the trend in the ratios follows the spatial and temporal order of sampling, i.e., high values of AG/BG were all at the northern end of the grid and low values mostly at the southern end (Figure 3.13b). This suggests the influence of ice melt on the composition of the bacterioplankton community, because BGase was dominant in the ice (AGase was undetectable). BGase activity near the surface was greatest near the ice edge, and the LAP/BG ratio was also significantly ($P<0.005$) negatively correlated with time from the start of the cruise. For depths less than 20 m, ln(AG/BG) was significantly ($P<0.05$) negatively correlated with LAPase.

It appears that waters with significant ice melt influence were enriched in BGase relative to both AGase and LAPase. LAP/BG ratios in brown ice are higher than in other enriched microenvironments such as fecal pellets (see Chapter 5), but lower than in the water column on this cruise. Water column LAP/BG ratios on this cruise were the highest encountered on five cruises in this region (Figure 3.5 and Table 3.1). Near-surface LAP/BG at the ice-covered stations was at the low end of the range, but a number of stations far from the ice exhibited comparable ratios.

*3.3.2.7 Nonspecific α/β-glucosidase observed in December 1994*

In December 1994 (PD94-12) the relationship between AGase and BGase was very different than that observed the previous year. Activities of these two enzymes were strongly positively correlated at all stations sampled, with a ratio near unity (Figure 3.14 and Table 3.4). The regression equation (Model II, geometric mean method) for all natural water samples on PD94-12 is BGase = -0.0067 + 0.984(AGase) ($r^2=0.922$, $P<0.001$, $n=49$). The slope is not significantly different from 1, and the

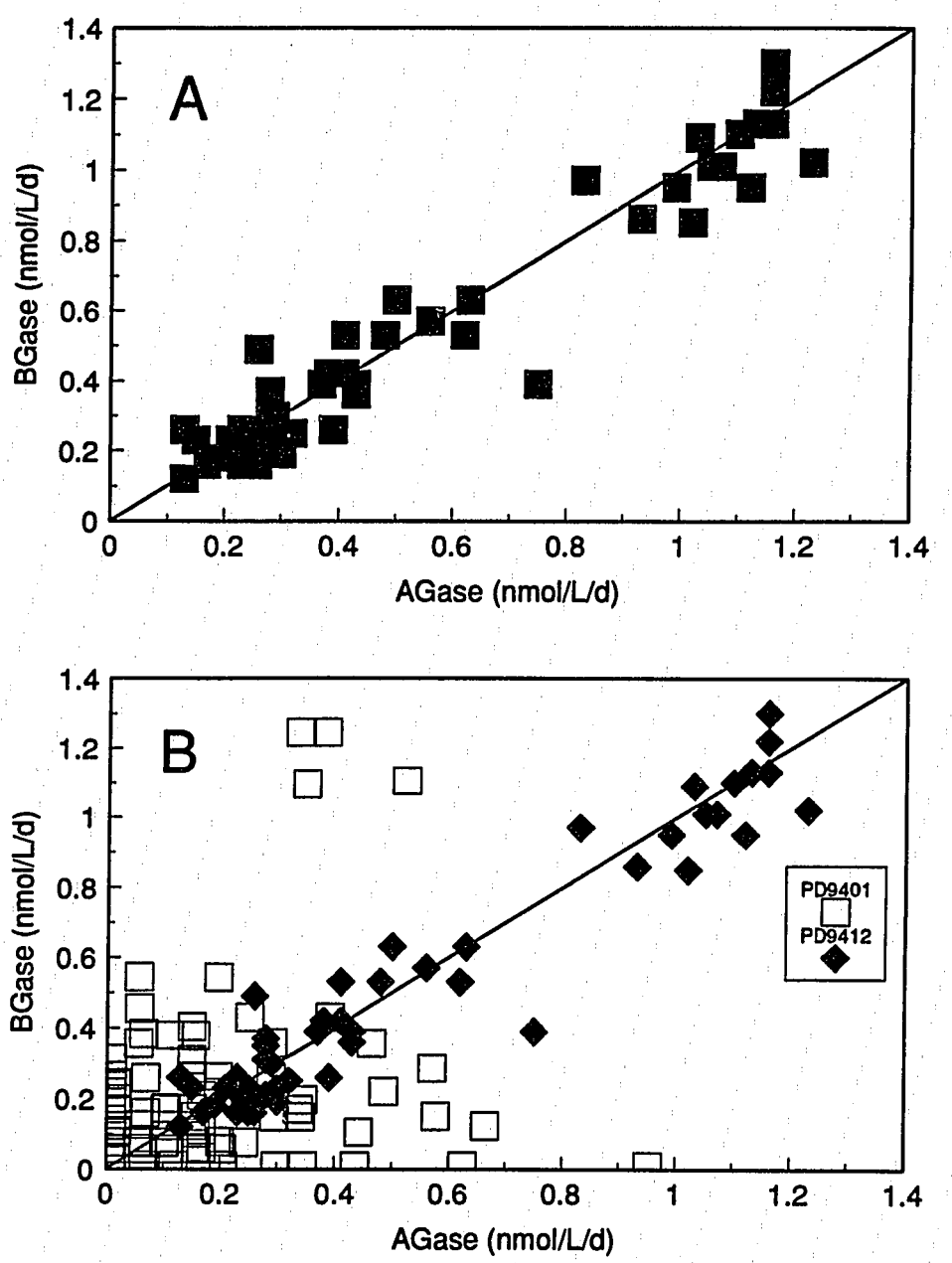


Figure 3.14. (a) AGase vs. BGase (nmol $l^{-1}$ $d^{-1}$) for water samples collected in December 1994. Line shown is 1:1 line. Regression equation (Model II, geometric mean method) is BGase = -0.0067 + 0.984(AGase) ($r^2$=0.922, P<0.001, n=49). (b) same samples shown relative to those collected in January-February 1994, again with 1:1 line.

Table 3.4. Ratios of BGase to AGase derived from several regression methods. GM = Model II regression (geometric mean method); ZI = zero-intercept regression; RM = ratio of mean values. "All samples" includes only natural water samples; "bottle bloom" experiment is illustrated in Figure 3.15; ice samples included brown ice collected from a zodiac on the ice surface as well as algal mat samples collected from underneath the ice by SCUBA divers. All ice samples are from Crystal Sound; samples in "Crystal Sound" row include only water column samples. GM slope is significantly different from 1 only for ice samples.

| Sample source | Slope (GM) | Slope (ZI) | n | r | RM |
|---|---|---|---|---|---|
| All samples | 0.98 | 0.96 | 49 | 0.960 | 0.97 |
| Paradise Harbor | 0.98 | 0.96 | 33 | 0.951 | 0.97 |
| Crystal Sound | 1.23 | 0.95 | 6 | 0.871 | 0.94 |
| Bottle Bloom | 1.07 | 1.09 | 9 | 0.984 | 1.10 |
| Ice | 1.11 | 1.10 | 11 | 0.998 | 1.08 |

intercept is not significantly different from 0 (based on 95% confidence limits for regression statistics). The ratio of AGase to BGase associated with the ice microbial community was also near unity in contrast to the previous year (Table 3.4). Hydrolysis of MUAG and MUBG was inhibited to a similar extent by cellobiose in both ice and water (data not shown), in contrast to results at Station ALOHA (Chapter 2) and in Antarctica in autumn 1993. It is hypothesized that a single enzyme with broad specificity for α- or β-glucose moieties was widespread during the 1994-95 austral summer, but was not present in previous years.

In mid-December, nine 12 l polycarbonate bottles were filled with surface seawater collected near Victor Hugo Island. These were then incubated on the deck of the ship at sea surface temperature and light intensity, and after 4 days one was emptied each day for chemical and biological analysis. Over nine days, both AGase and BGase activities increased by an order of magnitude, but the two activities varied in concert (Figure 3.15). The two activities were strongly positively correlated ($r^2$=0.967, P<0.001), and the ratio remained near unity (Table 3.4).

*3.3.2.8 LAPase activity relative to leucine incorporation*

LAPase activity was significantly correlated (P<0.001) with bacterial uptake of radiolabelled leucine on cruises in January-February 1994 and January-February 1995 (Figure 3.16). The correlation was stronger in 1994 ($r^2$=0.729) than in 1995 ($r^2$=0.362). The slopes of the regression equations (Model II, Geometric Mean method) indicate a ratio of 600-750 moles of potential hydrolytic activity for each mole of leucine assimilated into bacterial protein. Hydrolysis of dissolved combined amino acids (DCAA) can therefore account for all of the supply of amino acids to the

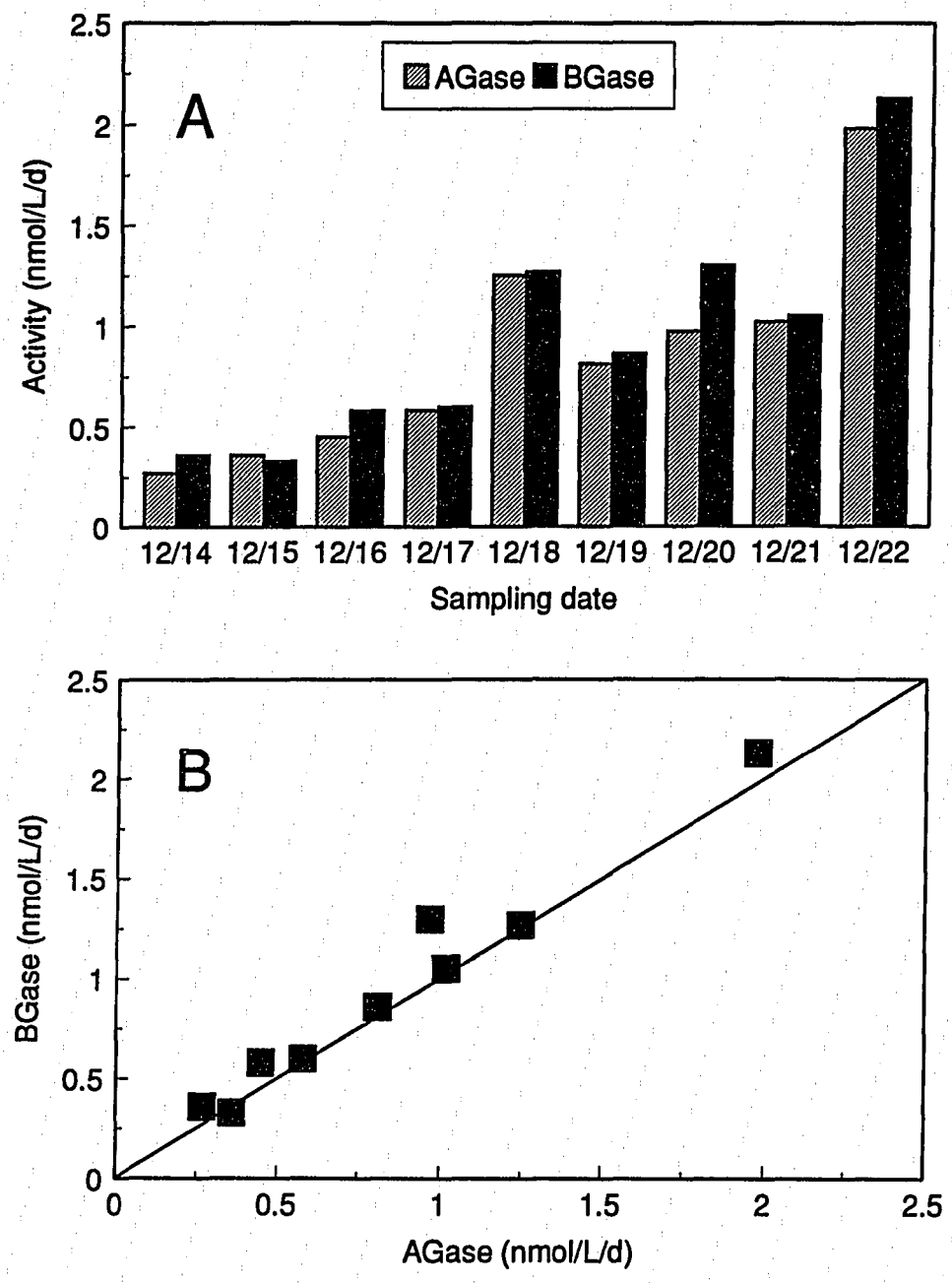


Figure 3.15. (a) AGase and BGase (nmol $l^{-1}$ $d^{-1}$) over 9 days in seawater incubated at sea surface light and temperature in 12 l containers (sampled each day at noon) (b) AGase vs. BGase for all samples from the experiment shown in (a).

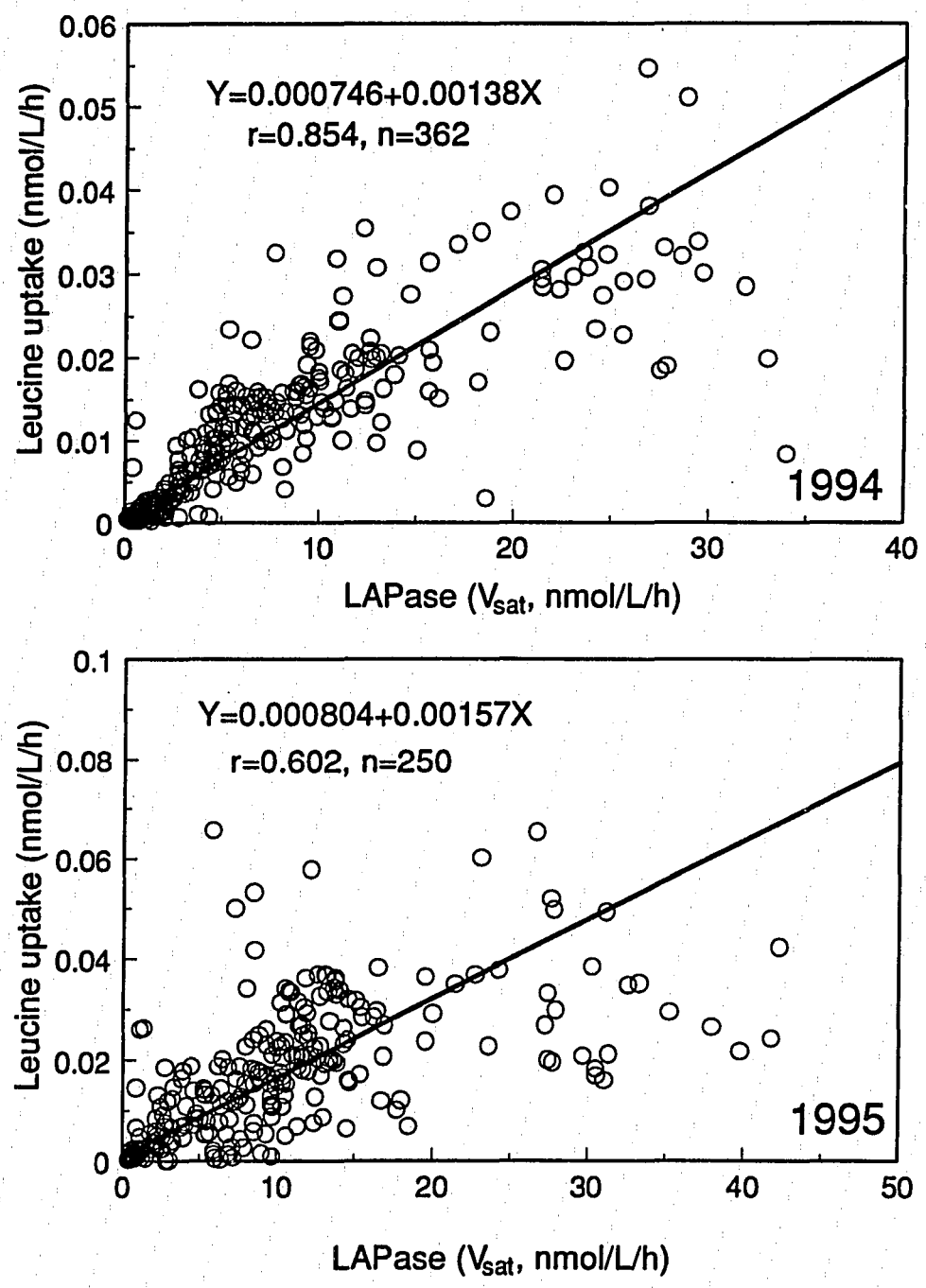


Figure 3.16. LAPase activity relative to assimilation of $^{3}$H-leucine, with Model II regression lines (geometric mean method). Data from Antarctica in January-February 1994 (top) and January-February 1995 (bottom).

bacteria at substrate concentrations of less than 0.5 μM (using $K_m$ =100 μM). However, this calculation does not account for the entire range of membrane transport systems. If these are specific for particular amino acids (Kirchman and Hodson, 1984) the actual rate of uptake may be significantly greater than the observed rate of assimilation of leucine. When standard conversion factors are applied to convert leucine incorporation to bacterial carbon production, LAPase accounts for less than 10% of bacterial carbon demand at these low substrate concentrations (see Chapter 6).

## 3.4 DISCUSSION

The latitudinal pattern of activity ratios described here bears an interesting resemblance to patterns observed by Kriss et al. (1963), who isolated more than 4,000 bacterial strains on extensive cruises in all of the major ocean basins (Figure 3.17). Qualitatively, these results show a similar trend with latitude, with glucosidase activity predominating in the equatorial zone and proteases increasing in importance with increasing latitude. However, the data of Kriss et al. (1963) depict a more or less continuous latitudinal gradient in activity ratios, whereas these results show a relatively small difference in the LAP/BG ratio between subtropical and polar study waters, with an altogether unique situation in the equatorial zone.

The oligotrophic subtropical gyres may represent something approaching global minima in both activities. Moving away from this minimum it appears generally true that LAPase increases towards the poles and BGase increases towards the equator. However, this conceptual model must be qualified by emphasizing that these data come only from the extremes of latitude, and these trends may not hold in the mid-latitudes. In mid- and high-latitude environments, moreover, these trends are complicated by the

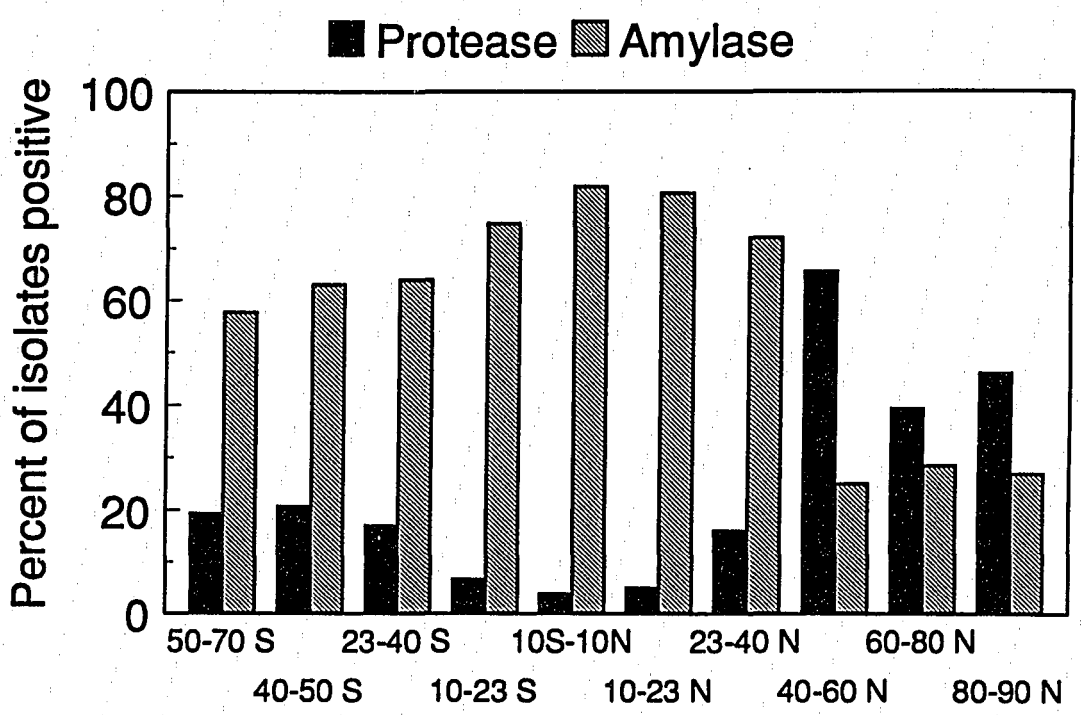


Figure 3.17. Percent of bacterial strains isolated from seawater hydrolyzing starch (amylase) and gelatin (protease) (from Kriss et al., 1963).

effects of seasonality. If these data have elucidated the true global minima for the activities of these enzymes in seawater, they are to be found in the Antarctic winter rather than in the subtropical gyre (Table 3.1). Both BGase and LAPase in Antarctica in summer can be as great as or greater than at Station ALOHA; however, the ratio of LAPase to BGase is generally greater in Antarctica.

Although there are few data from oceanic waters, LAPase activities in the mid-latitudes of the Northern hemisphere appear to be comparable to those in Antarctica (Hoppe, 1991). This may imply a dominant role for peptides and amino acids in the nutrition of bacterioplankton in temperate to polar waters. However, LAPase has a much higher $K_m$ (see Chapter 2) suggesting that LAPase activity *in situ* is significantly less than $V_{sat}$, while BGase may be active at or near $V_{sat}$ much of the time.

Recent data suggest that dissolved polysaccharide concentrations in the ocean are much greater than previously believed (Benner et al., 1992; Pakulski and Benner, 1994) . However, neutral sugars may constitute only a small fraction of this pool and the enzymes hydrolyzing 4MUF-glucosides do not necessarily hydrolyze the bonds present in naturally occurring polysaccharides, especially those involving acidic and amino sugars (see Chapter 2). Whatever the abundance of glucose or other neutral sugars in the dissolved polysaccharide pool, a low level of constitutive BGase activity may be sufficient to supply the bacteria with all of the glucose that they can utilize given the mixture of other substrates available and the biosynthetic pathways that are active. For example, while bacteria may be genetically capable of synthesizing amino acids from glucose and ammonium, this pathway is not necessarily active, and in practice most cell protein may be synthesized from amino acid precursors. Respiration of significant amounts of amino acid carbon by Antarctic bacteria (Tupas et al., 1994) suggests that this is the case.

Wheeler and Kirchman (1986) found that bacterial growth in Gulf Stream water was stimulated by glucose and ammonium to a greater extent than by stoichiometrically equivalent (with respect to C and N) amounts of an algal amino acid mixture. Kirchman (1990) found the opposite to be true in the subarctic Pacific, where glucose and ammonium stimulated growth but to a lesser extent than amino acids. These results are consistent with the hypothesis of a latitudinal trend in bacterial utilization of sugars and polysaccharides, relative to amino acids and proteins. The subarctic Pacific shares certain oceanographic characteristics with waters of the southern ocean (e.g., low temperatures, salinity stratification, permanently high inorganic nutrient concentrations). Gulf Stream water is largely derived from the Sargasso Sea and other tropical and subtropical seas (Gulf of Mexico, Caribbean Sea), with some indirect input from the South Equatorial Current (Froehlich et al., 1978; Pickard and Emery, 1982).

While the studies quoted above offer some evidence in support of the biogeographic patterns hypothesized in this paper, the actual patterns are likely to be complex, and much remains to be learned about the rates of acquisition of various substrates by marine bacteria and their fates once assimilated. Bacterial uptake of $^{3}H$- or $^{14}C$-labeled glucose is easily demonstrated, but quantitative information on partitioning between respiration and synthesis of carbohydrate and noncarbohydrate cell constituents is scarce for oceanic systems. Similarly, amino acids may be respired or used in protein synthesis, and the percent respired varies significantly among the few amino acids that have been studied (Tupas et al., 1994). For most amino acids, particularly those not abundant in seawater, the partitioning between assimilation and respiration, and between *de novo* synthesis and synthesis from intermediate precursors available in seawater is largely unknown for native aquatic bacteria. It is probably reasonable to assume that many, if not most, natural bacterioplankton are auxotrophic

for at least some substrates, as are many bacteria in culture (Guirard and Snell, 1962; see Chapter 4).

The different temperature responses at each study site indicate that distinct bacterioplankton populations with different enzymes or isozymes have evolved in each region. Antarctic populations do not display a temperature optimum for $k_2$ near *in situ* temperatures, and weak temperature-dependence at low substrate concentration suggests that a $k_2$ optimized at low temperatures offers little selective advantage. However, enzymes from Antarctic microenvironments where substrate concentrations may be much higher (sediments, sea ice, decaying macrophytes) also do not display temperature optima approaching *in situ* temperature (Reichardt and Dieckmann, 1985; Helmke and Weyland, 1991) so this may also reflect fundamental physicochemical constraints that natural selection can not overcome. The slope of the temperature response can also be interpreted as an adaptation to low temperature; i.e., a lowered activation energy barrier, which is generally observed in cold-adapted enzymes (Low et al., 1973; Somero, 1978).

Activities from the different regions can be normalized to a constant temperature, but because the slopes of the temperature-activity relationships are different for the three environments, the activity ratio depends on the temperature chosen. Normalized activities can not, therefore, be interpreted in terms of relative $E_0$. However, it should be noted that, although the surface water temperature in the Equatorial Pacific during March-April 1992 was high, the BGase activities observed would still be much greater than those at Station ALOHA if normalized to a temperature nearer the climatological mean.

Very little is known about the evolution and taxonomy of free-living oceanic bacterioplankton. The bacterial community in the equatorial Pacific appears to be

physiologically quite distinct. Whether they are taxonomically distinct is unknown, and it is possible that these physiological adaptations occur within the taxonomic groups delineated by molecular methods such as ribosomal RNA analysis (Ward et al., 1992) and are not presently detectable using such methods. It is also possible that the extremely temperature-sensitive isozymes expressed by equatorial bacterioplankton during March-April 1992, within the El Niño warm water pool, are not present at other times. Although it appears that these enzymes are ideally adapted to rapid "shift up" in a region of variable temperature, it is also possible that these bacteria are the culmination of a successional process following a change in water temperature and are not representative of the community in this region at other times. BGase activities in August 1992, when the study site was no longer within the warm water pool (Murray et al., 1994), remained orders of magnitude above those at the other two study sites, but temperature experiments were not conducted.

The extremely high levels of BGase activity near the equator have important implications for the role of DOM in the global carbon cycle. Initial reports of a low C:N for persulfate-resistant DOM appear to be incorrect (Hedges and Lee, 1993), and oceanic DOM contains a large amount of polysaccharide (Benner et al., 1992; Pakulski and Benner, 1994). If, for example, 10 μM dissolved organic carbon is hydrolyzable by the enzymes that hydrolyze MUBG, this fraction will have a turnover time of 1-2 d at the equator and ~6 years in the subtropical North Pacific. Little is known about the composition of oceanic DOM and the biological turnover rates of the various fractions. If the turnover rates of significant fractions vary by several orders of magnitude, simulations of the global carbon cycle incorporating DOM (e.g., Najjar et al., 1992) will be made much more realistic if the rates of these processes are better constrained.

Weak correlations between BGase and LAPase suggest that these enzyme activities are not a simple function of bacterial biomass. LAPase activity has been shown to be correlated with bacterial biomass and is believed to be constitutively expressed by most marine bacteria (Billen, 1991), implying that BGase is not. Billen and coworkers concluded that BGase is not in general constitutively expressed (G. Billen, pers. comm.). It was shown in Section 3.3.1 that over the entire multiyear data set there is a significant positive correlation between the activities of these two enzymes. However, this data set spans a large activity range from winter (oligotrophic) to summer (eutrophic) conditions, and the results from the fast grids show that the degree of covariance within a given area and season can be very weak, and can change very quickly. The repeat profiles at Station A show that this is a highly dynamic system, and the decomposer community is far from uniform in time and space.

LAPase activity in the shelf and oceanic waters of the ACC are surprisingly high and extend to significant depths. LAPase activity tends to decline sharply around the pycnocline. In Gerlache Strait during austral summer 1991-92, LAPase activities declined sharply at depths of 10 to 40 m (Figure 3.9). On the seaward edge of Anvers Island in Autumn 1993, activities comparable to those at the surface extended to depths as great as 100 m. Although summer mixed layer activities in coastal waters can be greater than those in the ACC (Table 3.1), the depth-integrated activity may be greater in outer shelf and oceanic waters where wind mixing extends to greater depths.

The absence of a strong onshore-offshore gradient has significant implications for the remineralization of organic matter in the southern ocean. If turnover rates of polymeric DOM (PDOM) are high, there will be little horizontal advection of phytoplankton-derived detritus. If turnover rates are low, production and consumption of dissolved organic matter may be uncoupled in time and space, and attempts to "close

the loop" and balance photosynthesis and respiration must integrate over fairly large space and time scales. The absence of an onshore-offshore gradient of LAPase activity implies that PDOM produced in eutrophic inshore waters may be respired in more oligotrophic waters.

Eutrophic coastal waters show consistent patterns of activity. In December 1994 an intense phytoplankton bloom (15-20 µg chlorophyll *a* $l^{-1}$) was encountered in Paradise Harbor. The activities and activity ratios were quite similar to those on RACER 3 (December 1991), which was the last time such a bloom was encountered (Table 3.1). These activities were among the highest observed in five cruises, but the degree of enrichment during blooms was much less than for phytoplankton, as is the case for other indicators of microheterotrophic activity (Karl et al., 1991; 1995). This implies spatial and temporal uncoupling of production and consumption of organic matter, but whether this "excess" organic matter helps to sustain the populations in the open shelf waters is uncertain. Surface currents in the Gerlache and Bransfield Straits flow northeast (Niiler et al., 1990), so it is unlikely that production in the inshore waters sampled in RACER supply organic matter to the offshore waters of the Palmer LTER grid. There may be a flux from coastal waters further south, but there has been no documentation of intense phytoplankton blooms of the type observed in RACER in that region. Moreover, climatic conditions (i.e., persistence of ice well into summer) make such blooms unlikely, although ice-edge blooms do occur (Karl et al., 1992). These blooms appeared to give rise to very active decomposer communities (Dore et al., 1992) and ectoenzyme activities associated with such communities were much greater than any others observed in Antarctica.

The relative activities of AGase, BGase, and LAPase suggest that the bacterial community goes through several as yet poorly defined stages of succession as the

annual pack ice recedes. The water is initially seeded with bacteria, as well as receiving substantial inputs of organic matter that may be quite different in its chemical composition than the allochthonous organic matter in the water column. Phytoplankton blooms at the receding ice edge provide additional sources of organic matter, through excretion, lysis, and grazing. These processes are likely to play an important role in the adaptation of marine bacteria to this seasonally variable environment. The coincidence of AG/BG ratios in ice and seawater suggests that a substantial fraction of the summer bacterial community originates from the ice. On PD94-12 the covariance of AGase and BGase was geographically widespread, occurring in both ice and water in Crystal Sound and in (ice-free) Paradise Harbor, approximately 400 km away.

The existence of a glucosidase with little or no specificity for α- or β-anomers is unexpected and intriguing. Although these field results do not conclusively demonstrate that a single enzyme is involved, the strength of the correlation ($r^2$=0.922 for natural water samples) and the similar degree of inhibition by cellobiose suggest that this is the case. Microbial β-glucosidases tend to have strong specificity for β anomers even when they have broad specificity among β-hexoses (Plant et al., 1988; Ware et al., 1990; Nucci et al., 1993; Paavilainen et al., 1993; Copa-Patiño and Broda, 1994). However, AGase at Station ALOHA was more strongly inhibited by cellobiose than β-xylosidase or β-glucuronidase (see Chapter 2). Other enzymes such as glucose-6-phosphate dehydrogenase and phosphoglucoisomerase generally show specificity, or at least selectivity, for α or β anomers (Malaisse et al., 1985; Kayser et al., 1993).

The contrasting patterns in AGase and BGase activities observed in two successive years is also intriguing. The coincidence of these contrasting patterns in ice and water may indicate that the ice has a significant influence on water column communities in summer. If so, the presence of the nonspecific α/β-glucosidase in

Paradise Harbor after ice breakup and during an intense phytoplankton bloom suggests that this effect is long lived.

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# CHAPTER 4

# REGULATION OF ECTOENZYME ACTIVITY

## 4.1 INTRODUCTION

Expression of aminopeptidases and glucosidases by marine heterotrophic bacteria must in some sense be regulated by substrate availability. Activities of these two groups of enzymes will reflect the carbon and nitrogen requirements of the bacteria, or more precisely, the energy demand given the availability of nitrogen and phosphorus for biosynthesis. An important control on the availability of N and P is the activity of microzooplankton grazers; seawater microbial communities are tightly coupled systems in which the isolation of a particular trophic group is likely to alter the nutrients available to it. Several experiments were carried out in which seawater was passed through a 1.0 μm membrane filter to remove grazers and incubated to determine the effects of grazing on ectoenzyme expression. In other experiments various organic compounds were added in order to elucidate the nature of substrate effects on ectoenzyme expression and identify those compounds that play a significant role in regulation.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Study areas

Experiments were conducted at Station ALOHA and in Antarctica. Cruise dates and designations are described in Chapter 3.

### 4.2.2 Ectoenzymes

Methods for ectoenzyme analysis are described in Chapters 2 and 3. Saturating substrate concentrations were used for leucine aminopeptidase (LAPase), α-glucosidase (AGase), and β-glucosidase (BGase) (1 mM, 1.6 μM, and 1.6 μM respectively).

### 4.2.3 Enumeration of bacteria

Bacteria were enumerated by epifluorescence microscopy using 4'6-diamidino-2-phenylindole (DAPI, Porter and Feig, 1980). A Zeiss Model 16 standard microscope equipped with a IV FI epifluorescence condenser and 100 W mercury vapor lamp was used. Filters were a standard Zeiss UV filter set (#487702; 365 nm broad band excitation, 420 long pass). Ocular and objective lenses were 16X and 100X respectively. Bacteria were stained with 1.0 μg $ml^{-1}$ DAPI for a minimum of 1 h. Filters with 0.2 μm pore size (25 mm) were used, either commercially prestained (Poretics Corp.) or stained with Irgalan Black (2 g $l^{-1}$ in 2% acetic acid).

### 4.2.4 Sample preparation for seawater cultures

To incubate bacterioplankton in the absence of grazers, seawater was filtered through 1.0 μm polycarbonate membrane filters using an enclosed polycarbonate collection vessel (Nalgene), except on HOT 53 when a glass collection vessel and frit

were used. These were washed thoroughly with 10% HCl and distilled water, then rinsed 2-3 times with sample water.

The effect of solar ultraviolet radiation at Station ALOHA on the bioavailability of dissolved organic matter (DOM) to bacteria was investigated by incubating filtered seawater in quartz or borosilicate glass (BSG) tubes on board ship (i.e., at sea surface light intensity). This was then inoculated with a small amount of freshly collected seawater (~1:4) and incubated for 10-40 h in the dark before determining ectoenzyme activities. In an initial experiment (HOT 45, February 1993), quartz and BSG tubes were exposed for one day (dawn to dusk). In a subsequent experiment (HOT 54, June 1994) no BSG controls were used, and the quartz tubes were exposed for 0, 4, 6, 8, and 12 h in full sunlight beginning at dawn (i.e., the samples received 0%, 25%, 50%, 75%, and 100% of the daily integrated UV irradiance, assuming sinusoidal variation over 12 h). On HOT 45 both the filtered seawater and the live inoculum were collected from a depth of 25 m; the filter used was a Whatman GF/F. On HOT 54 seawater was collected from a depth of 25 m, filtered, exposed to sunlight and frozen; the filter used was a 0.2 μm polycarbonate membrane. These samples were stored (-20 °C) for ~1 month, thawed simultaneously and inoculated with freshly collected seawater (30 m) on HOT 55 (July 1994).

## 4.3 RESULTS

### 4.3.1 Effect of grazing

Recycling of nitrogen by micrograzers appears to play an important role in regulating bacterial ectoenzyme activities. In a seawater sample from Station ALOHA,

the relative activities of these enzymes changed significantly when grazers were excluded by passing the water through a 1.0 µm filter. BGase activity stayed roughly constant while LAPase activity and the BG/LAP ratio decreased by an order of magnitude (Figure 4.1). In the unfiltered treatment, the BG/LAP ratio remained unchanged over 72 h, although both activities increased by a factor of more than two. Changes in AGase were similar to those in BGase. Bacterial numbers remained approximately constant in both filtered and unfiltered treatments. In a similar experiment in Antarctica in January 1994 (PD94-01), bacterial numbers increased threefold while LAPase activity increased tenfold (Figure 4.2). This suggests grazer control of bacterial biomass. The increased LAPase activity per cell may imply nitrogen limitation, but may also result from an increase in mean cell size in the absence of grazers with no change in LAPase activity per unit biomass. In Antarctica in December 1994 (PD94-12), trends in LAPase, AGase and BGase activities following filtration were more or less consistent with those observed at Station ALOHA.

### 4.3.2 Nitrogen limitation

In Antarctica in austral spring (November) 1992 (PD92-09), LAPase activity was determined in 1.0 µm filtered seawater incubated 50 h with added ammonium chloride (200 µM) and with glycine (100 µM) plus proline (100 µM). In both cases LAPase activity increased markedly following filtration, as in Figure 4.2a. This increase was observed even in the presence of high concentrations of supplemental nitrogen as $NH_4^+$ or amino acids (Figure 4.3). Therefore nitrogen availability alone is a poor predictor of LAPase expression.

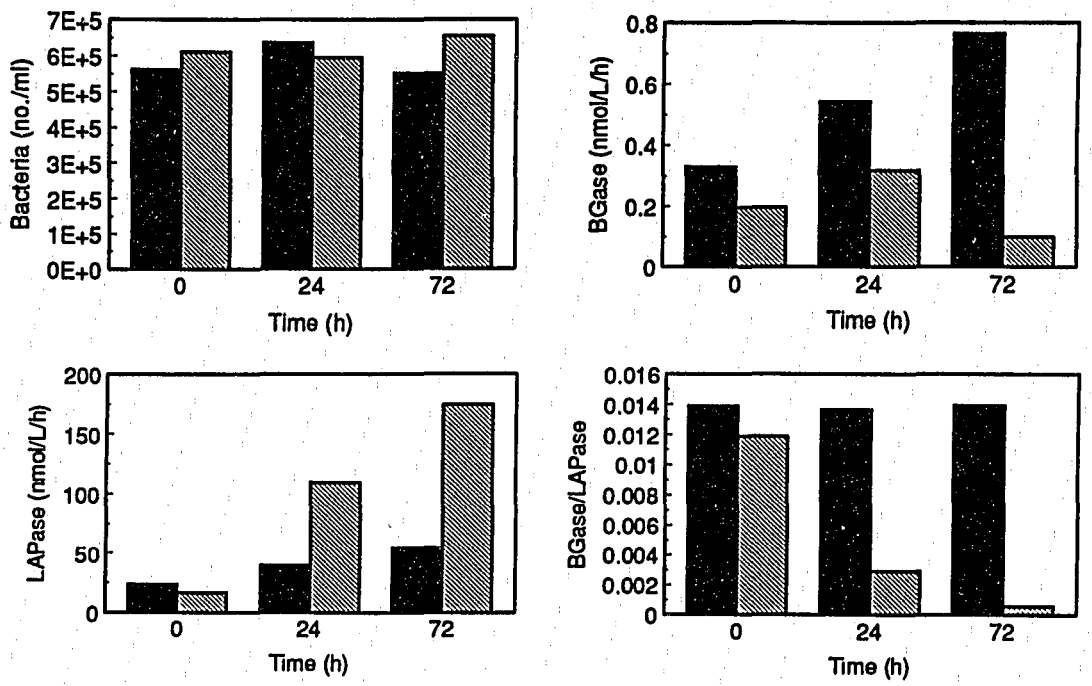


Figure 4.1. Changes in bacterial abundance (cells $ml^{-1}$), BGase (nmol $l^{-1}$ $h^{-1}$), LAPase (nmol $l^{-1}$ $h^{-1}$), and BGase/LAPase over 72 h in 1.0 µm filtered (light bars) and unfiltered (dark bars) surface seawater collected at Station ALOHA in March 1994 (HOT 53).

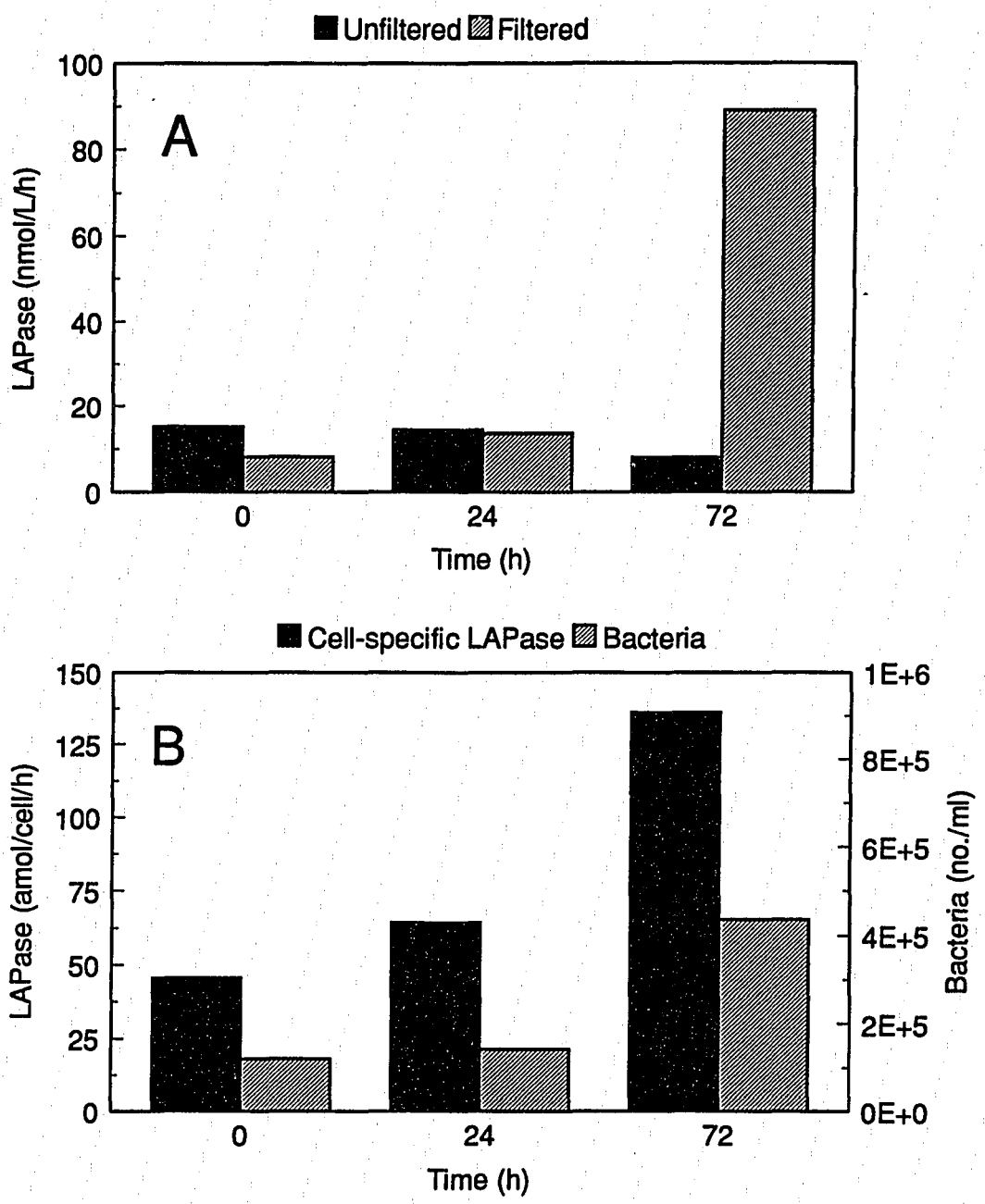


Figure 4.2. (a) Evolution of LAPase activity (nmol $l^{-1}$ $h^{-1}$) in 1.0 µm filtered and unfiltered surface seawater; (b) evolution of cell-specific LAPase activity (amol $cell^{-1}$ $h^{-1}$) and bacterial abundance (cells $ml^{-1}$) in 1.0 µm filtered surface seawater; both over 72 h. The experiment was conducted in Antarctica in January 1994 (PD94-01).

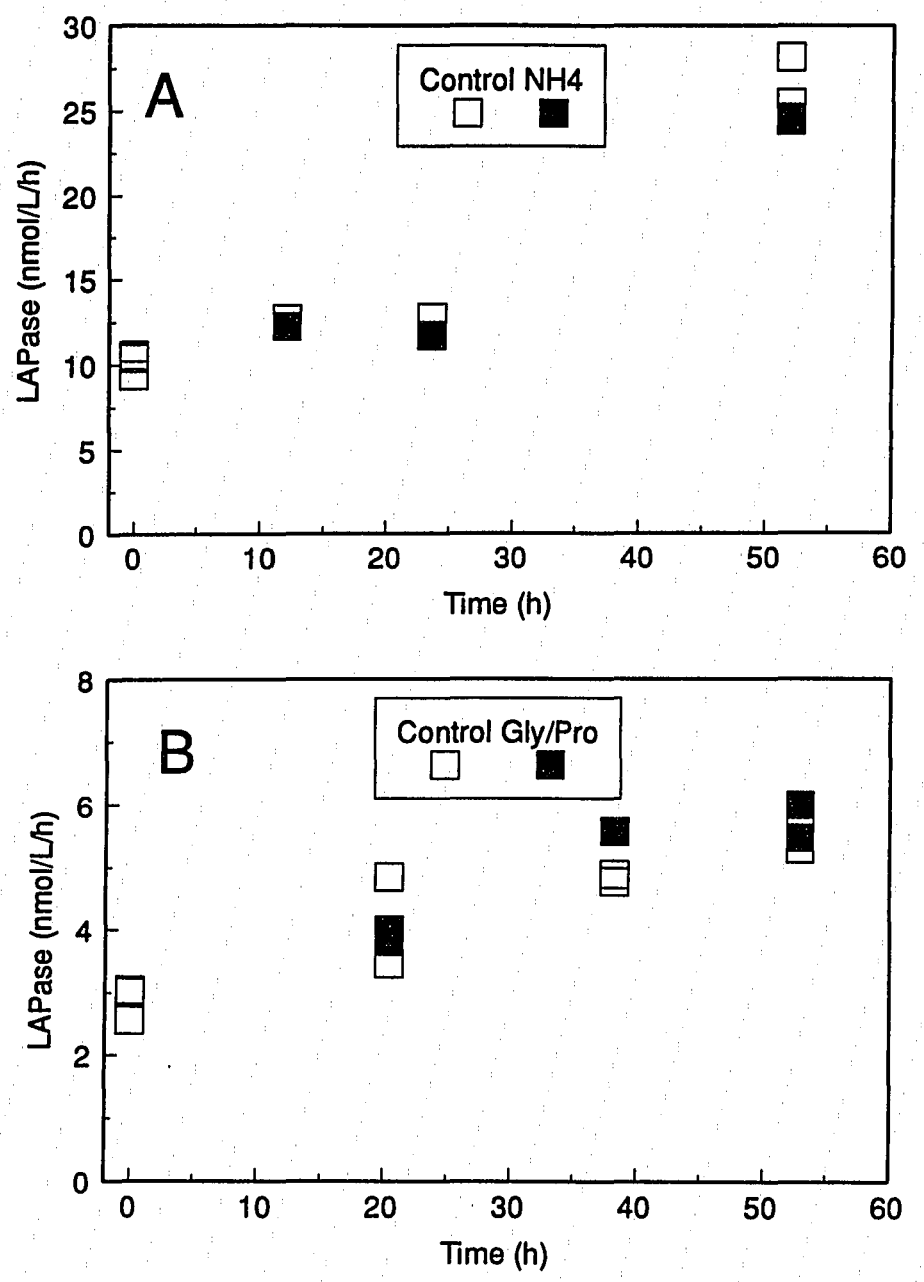


Figure 4.3. Time course of LAPase activity (nmol $l^{-1}$ $h^{-1}$) in 1.0 µm filtered surface seawater with and without added (a) $NH_4Cl$ (200 µM) and (b) glycine + proline (each 100 µM). All replicate subsamples shown. The experiment was conducted in Antarctica in November 1992 (PD92-09).

A further experiment in austral autumn 1993 (NBP93-02) shows the effect of nitrogen (as $NH_4^+$) and substrate (cellobiose) availability on BGase activity. Addition of cellobiose (100 nM) repressed BGase activity, while $NH_4^+$ (6 µM) did not stimulate it to any significant extent (Figure 4.4). Utilization of carbohydrates by the bacteria does not appear to be limited by nitrogen availability, and the demand for glucose appears relatively fixed. Thus the addition of supplemental cellobiose represses BGase activity.

### 4.3.3 Effects of organic N sources on LAPase

In January-February 1994 (PD94-01) and December 1994 (PD94-12), a wider variety of nitrogen compounds were assayed for effects on ectoenzyme activities. Histidine, and to a lesser extent phenylalanine, significantly repressed LAPase expression. Other amino acids such as glycine and tyrosine had little or no effect even at concentrations that should provide more than adequate nutrients to the bacteria (Figure 4.5). This response to histidine was highly consistent and reproducible. It was observed in unfiltered seawater (i.e., in the presence of grazing microzooplankton, Figure 4.6), but the percent difference, relative to the controls, was less than in 1.0 µm filtered seawater.

Histidine has a unique and complex biosynthetic pathway, incorporating an imidazole ring, and in some ways is more similar to purine biosynthesis than to that of other amino acids (Umbarger, 1978; Dawes and Large, 1982). For this reason, several purine and pyrimidine compounds (adenine, uracil, and imidazole) were also assayed for repression of LAPase activity. The results were negative (Figure 4.7), although

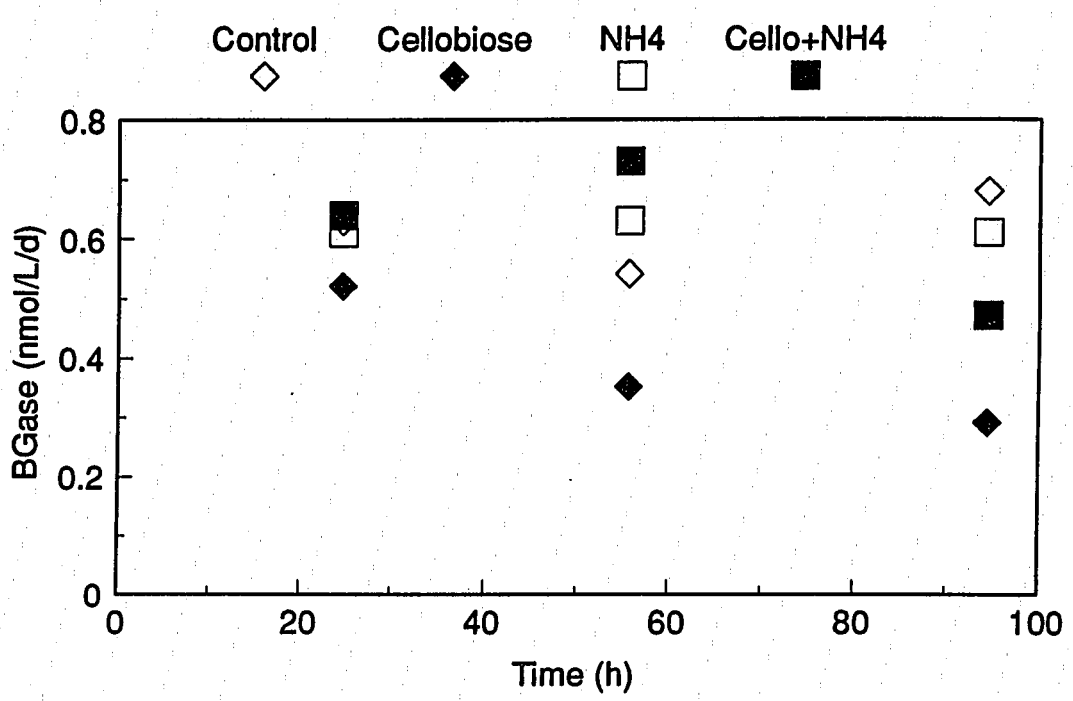


Figure 4.4. Time course of BGase activity (nmol $l^{-1}$ $d^{-1}$) in 1.0 µm filtered surface seawater with added $NH_4Cl$ (6 µM) and/or cellobiose (100 nM). The experiment was conducted in Antarctica in May 1993 (NBP93-02). Each point is the mean of two subsamples.

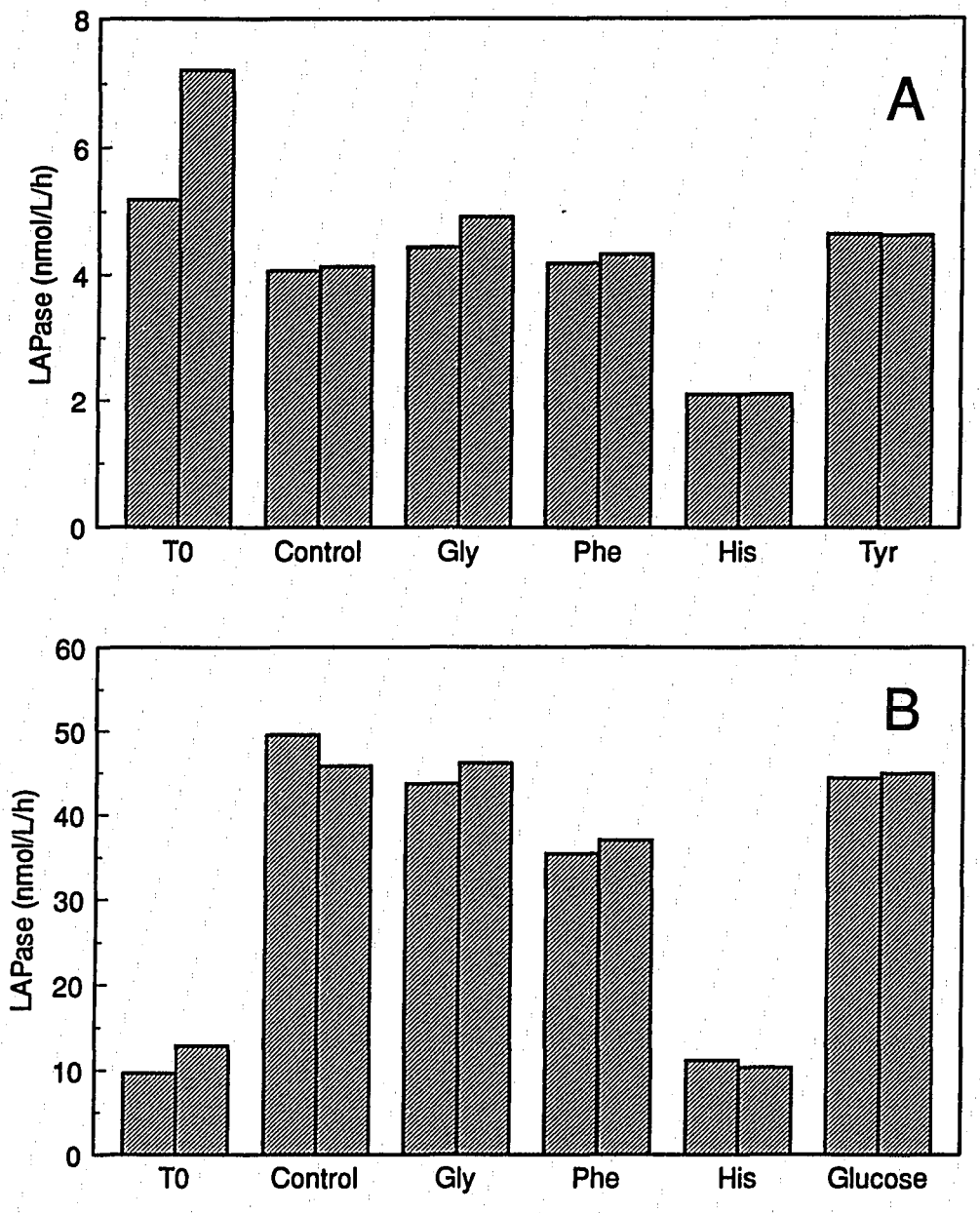


Figure 4.5. LAPase activity in 1.0 µm filtered surface seawater after 48 h incubation with added organics. (a) All supplemental compounds added at 10 µM. (b) All supplemental compounds added at C and N molar equivalent of 10 µM histidine, with additional nitrogen added as $NH_4Cl$. The experiment was conducted in Antarctica in December 1994 (PD94-12). Adjacent bars are duplicate subsamples.

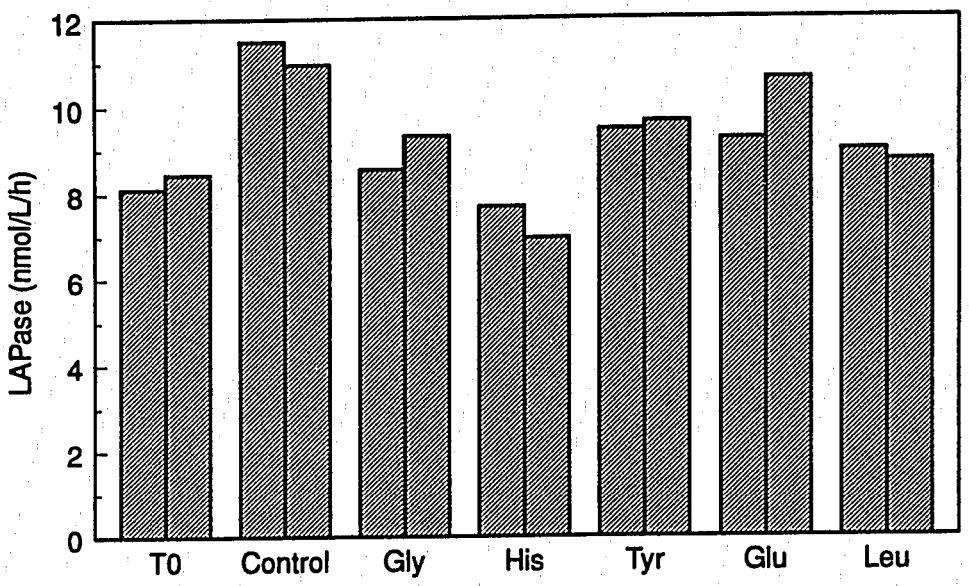


Figure 4.6. LAPase activity in unfiltered surface seawater after 48 h incubation with added organics. All supplemental compounds added at 10 µM. The experiment was conducted in Antarctica in December 1994 (PD94-12). Adjacent bars are duplicate subsamples.

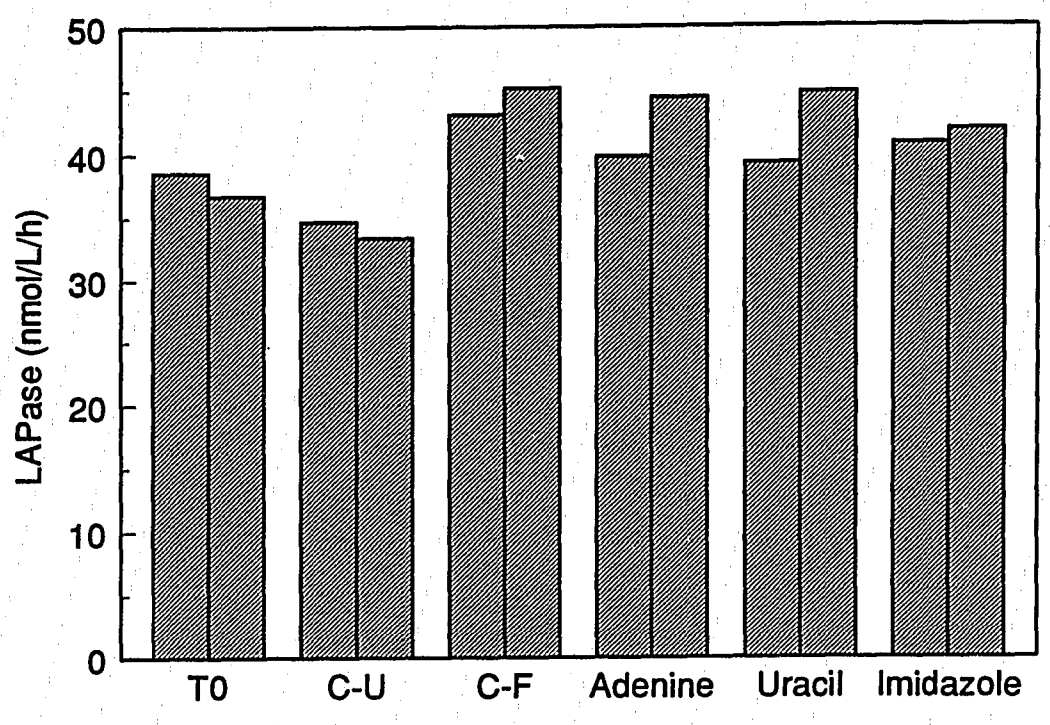


Figure 4.7. LAPase activity in 1.0 µm filtered surface seawater after 48 h incubation with nucleotide-related compounds (2 µM). C-U = unfiltered control; C-F = filtered control (all other treatments filtered). The experiment was conducted in Antarctica in December 1994 (PD94-12). Adjacent bars are duplicate subsamples.

there may have been a slight effect with imidazole. However, the concentration of the nucleotide-related compounds (2 µM) was less than for the amino acids.

### 4.3.4 Effects of organic N sources on AGase and BGase

The effects of organic N compounds on AGase and BGase were more difficult to interpret, because the activities of these enzymes in seawater, at Station ALOHA and in Antarctica, were much lower than that of LAPase. The method was often near the limit of its sensitivity, and variance among replicate subsamples was frequently high. Effects of additional organic nitrogen compounds were generally small. In oligotrophic waters near Victor Hugo Island, Antarctica, addition of amino acids resulted in BGase activities in 1.0 µm-filtered seawater that were greater than those in the controls (Figure 4.8a). This effect was most pronounced for phenylalanine and tyrosine. In unfiltered seawater, by contrast, BGase activity decreased in the presence of amino acids (Figure 4.8b). These results are consistent with the hypotheses (1) that bacterial utilization of glucose depends on grazer recycling of nitrogen, (2) that Antarctic heterotrophic bacterioplankton utilize amino acids as a carbon source for biosynthesis and respiration, and (3) that glucose utilization depends on the availability of these alternative substrates. However, these results should be interpreted cautiously, because of the high variance among duplicate subsamples, and because the primary incubation containers were not replicated.

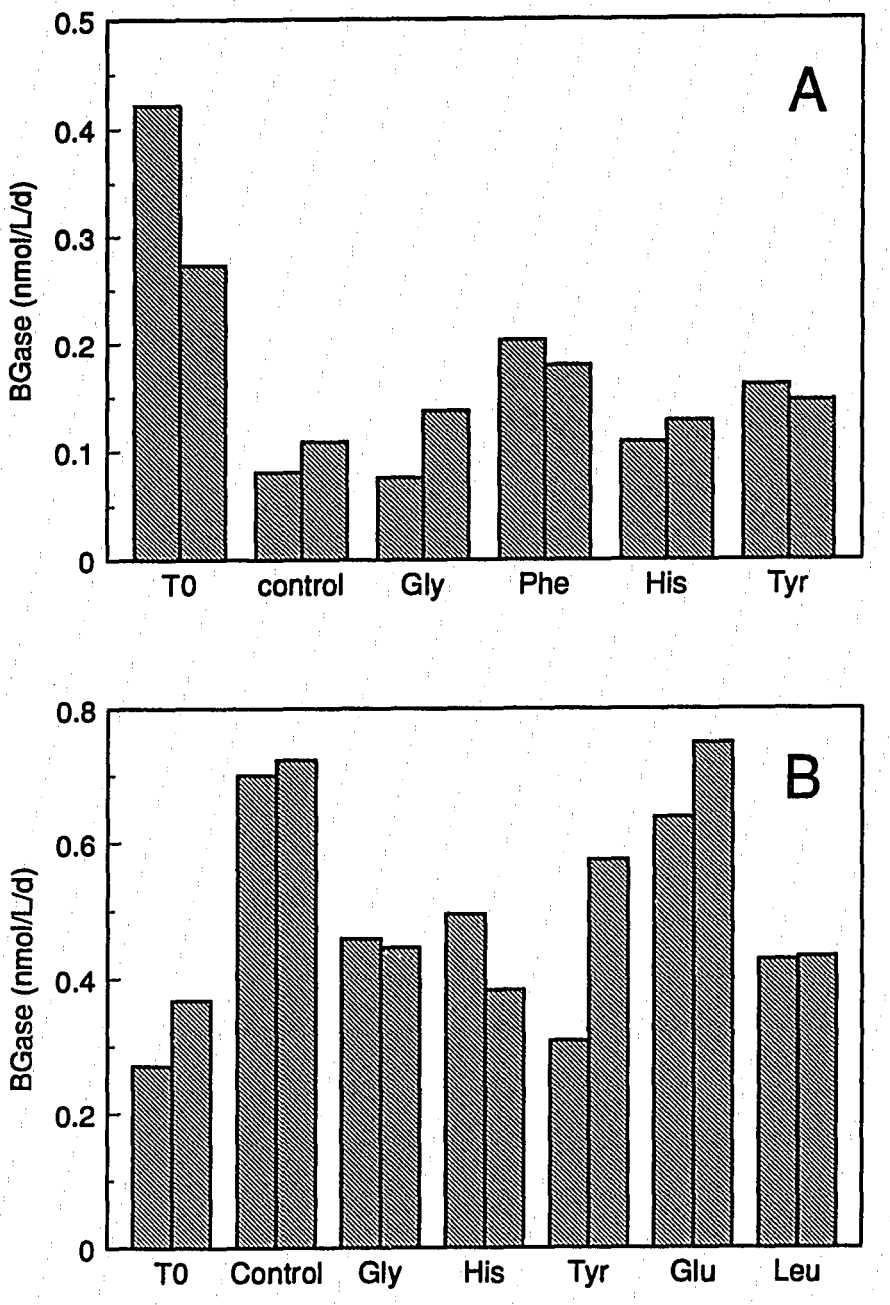


Figure 4.8. BGase activity in (a) 1.0 μm filtered and (b) unfiltered surface seawater after 48 h incubation with added organic compounds. The experiment was conducted in Antarctica in December 1994 (PD94-12). Adjacent bars are duplicate subsamples.

### 4.3.5 Effect of solar ultraviolet radiation

LAPase activity in filtered seawater incubated in full sunlight for 1 day in quartz containers at Station ALOHA and inoculated with freshly collected seawater showed an approximately twofold increase relative to borosilicate glass controls (Figure 4.9a). BGase was similar in both treatments (data not shown). When samples were incubated for periods corresponding to 4 approximately equal fractions of the daily integrated UV irradiance, the 12 h incubation showed a large increase in LAPase, as in the previous experiment. Shorter incubations caused activity to decrease with increasing exposure time, with a reversal of the overall effect occurring somewhere between 8 and 12 h of exposure (Figure 4.9b).

### 4.3.6 Effect of organic matter from sea-ice algae

Sea ice rich in organic matter ("brown ice") was melted and heat-sterilized, and the effects of the organic matter present on water column bacteria was determined. Effects were inconsistent and generally small. In January 1994 (PD94-01) there was a slight repression of LAPase activity; a much greater decrease occurred when histidine (80 μM) was added as well (Figure 4.10). In December 1994 (PD94-12) no effect on LAPase was observed. BGase activity was slightly stimulated in January 1994, whereas in December 1994 it was slightly repressed (data not shown).

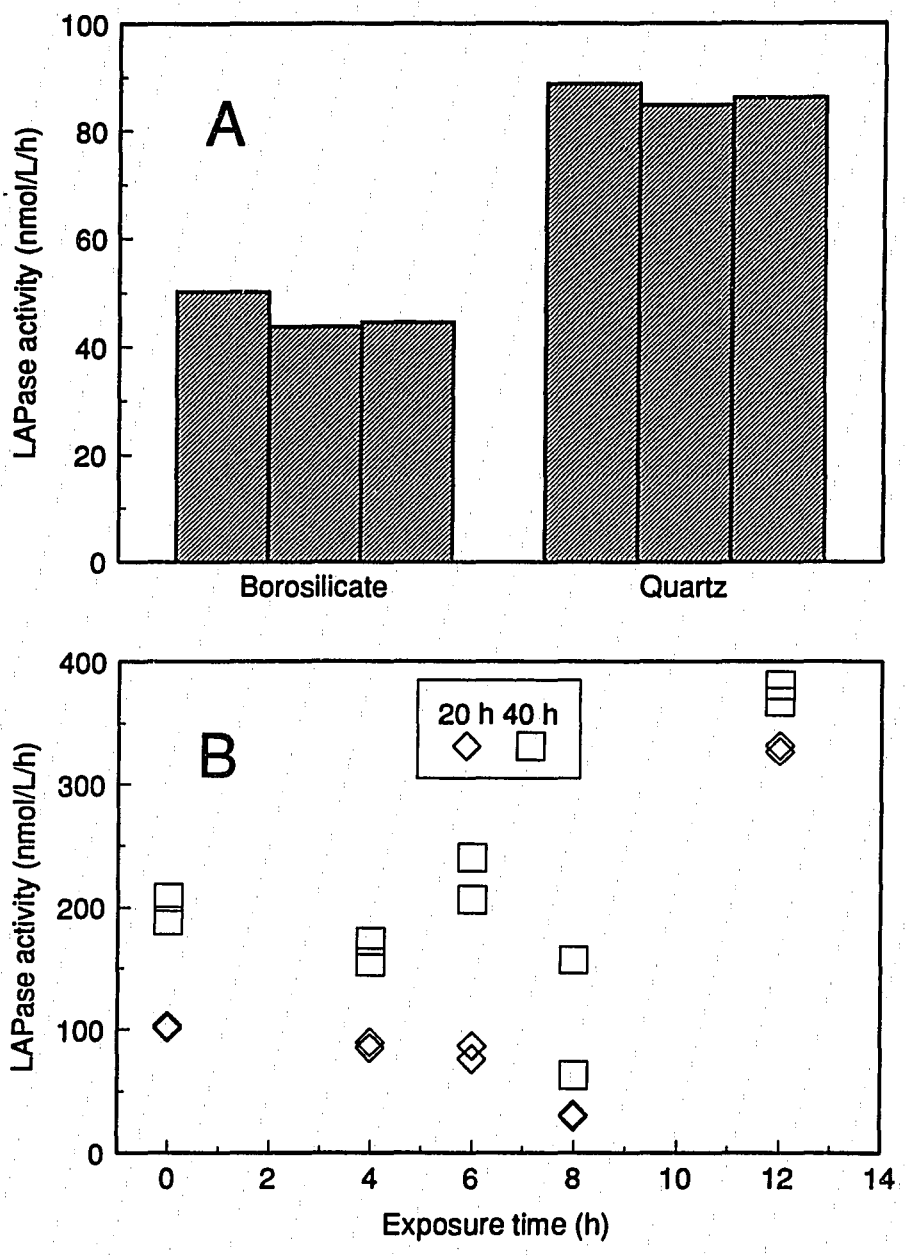


Figure 4.9. LAPase activity in filtered Station ALOHA surface seawater, exposed to sunlight, and inoculated (1:4) with fresh surface seawater. (a) Filtered seawater exposed to sunlight in quartz and borosilicate containers for one full day (HOT 45, February 1993), 14 h incubation in the dark following inoculation. (b) Filtered seawater exposed to sunlight in quartz containers for varying periods (HOT 54, June 1994), 20 or 40 h incubation in the dark following inoculation.

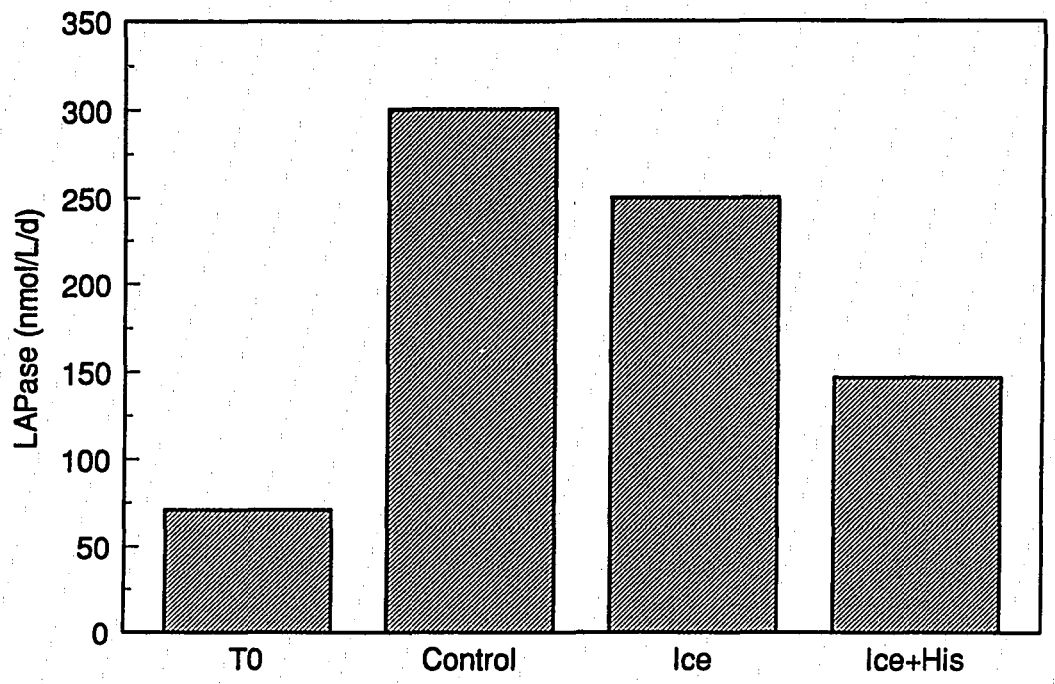


Figure 4.10. LAPase activity in 1.0 µm filtered surface seawater after 48 h incubation with heat-sterilized sea ice algae with and without added histidine (80 µM). The experiment was conducted in Antarctica in January 1994 (PD94-01). Each bar is the mean of two subsamples.

## 4.4 DISCUSSION

Seasonality is a significant consideration for interpreting the substrate addition experiments described above in terms of nitrogen limitation (Figures 4.3 and 4.4). In the Antarctic spring, prior to the annual phytoplankton bloom, $NH_4^+$ did not significantly repress LAPase activity. Since bioavailable dissolved organic nitrogen is expected to be low at this time of year, this shows that nitrogen limitation alone does not regulate expression of this enzyme. In the fall, when the BG/LAP ratio *in situ* is relatively high (Christian and Karl, 1995; see Chapter 3), $NH_4^+$ did not stimulate BGase activity. This also implies that the bacteria are not nitrogen limited.

The consistent repression of LAPase by histidine suggests that a large fraction of the bacterial community is auxotrophic for this amino acid. If histidine requirements cause this enzyme to be expressed at higher levels than are required to satisfy basic carbon and nitrogen requirements, the flux of amino acids to the cell should exceed that required for biosynthesis, so that a substantial fraction is respired (Tupas et al., 1994). This may help to explain why the ratio of aminopeptidase to glucosidases is high in Antarctica. Rivkin et al. (1991) hypothesized that Antarctic bacterioplankton are "substrate sufficient" and that growth is limited by some other factor, such as temperature. However, they assayed only substrates shown to have little or no effect on LAPase activity (glucose, glutamic acid, glycine). Further experimentation with a broader range of compounds (e.g., histidine and phenylalanine) is necessary to determine whether bacteria in this environment are substrate sufficient. Histidine is among the rarest amino acids both in seawater (Hubberten et al., 1995) and in phytoplankton (Müller et al., 1986). Müller et al. (1986) found that histidine mole fractions in netplankton (diatoms) were significantly less in Antarctic than in

subantarctic waters. Why this should be so is not clear. Histidine plays an important role in adaptation of enzymes to varying temperature (Somero, 1978), and seasonal temperature changes in Antarctic waters are small. The relatively constant temperature may also decrease selective pressure for histidine biosynthesis among heterotrophic bacteria.

The Western Antarctic Peninsula (WAP) region is an upwelling zone, and recently upwelled deep water is unlikely to be rich in biochemically labile organic matter. Concentrations of dissolved organic nitrogen in the WAP region are among the lowest ever observed in surface seawater (<4 μM; Karl et al., 1995), and a large fraction of "dissolved" amino acids are associated with humic substances (Hubberten et al., 1995). This may help to explain the low biomass ratio of bacteria to phytoplankton (Karl et al., 1991). Bacterial numbers observed in the Benguela upwelling zone by Linley et al. (1983) also fall consistently below those predicted by the regression equations of Bird and Kalff (1984) and Cole et al. (1988).

Auxotrophy for amino acids is widespread among bacteria (Guirard and Snell, 1962). Of 15 bacterial strains isolated from seawater by Ostroff and Henry (1939), only 5 could grow with ammonium as the sole nitrogen source. Two hyperthermophilic heterotrophic Archaea, isolated from deep-sea hydrothermal sulfide deposits, were auxotrophic for 11 different amino acids (Hoaki et al., 1993). These two strains are quite distantly related (one being from each of the major kingdoms within the Archaea), and their auxotrophic requirements are very similiar to those of animals. So it is possible that auxotrophy is an ancestral condition and widespread among the Bacteria as well, although it may be among the Bacteria that evolution of a more diverse set of biosynthetic pathways (as in e.g., *Escherichia coli*) is most widespread. Most of the energy cost of protein synthesis is in the polymerization (Stouthamer, 1973), so it is

somewhat surprising that more species have not evolved to overcome auxotrophic amino acid requirements.

The biosynthetic pathways for histidine and the aromatic amino acids are complex and quite different from those of the major amino acid families. The imidazole group in histidine is normally derived from ATP (Umbarger, 1978), which is ubiquitous in the marine environment and rapidly turned over in Antarctic seawater (Nawrocki and Karl, 1989). Perhaps salvage pathways for histidine biosynthesis are active in Antarctic bacterioplankton, but adenine, uracil, and imidazole do not appear to be among the precursors. Other possible precursors are purine catabolism products such as hypoxanthine, xanthine, uric acid, and allantoin (Paul 1983). Hypoxanthine is excreted by ciliates (Antia et al., 1980); uric acid is excreted by many organisms, including the pygoscelid penguins that are common in our Antarctic study area (Staley and Herwig, 1993). Uric acid is metabolized by a variety of bacteria and cyanobacteria and is probably the most biochemically labile of these purine-related compounds (Paul 1983; Staley and Herwig, 1993).

Selection for auxotrophy may be result from the presence of compounds that mimic intermediates in the pathways of amino acid biosynthesis. Partial catabolism of aromatic amino acids, leaving low molecular weight (LMW) aromatic compounds likely to be refractory to further decomposition, is known among both bacteria (Pometto and Crawford, 1985) and eukaryotic algae (Landymore et al., 1978). Excretion of such partial catabolism products would help to explain the apparent paradox of the LMW fraction of DOM being apparently more biologically refractory than the high molecular weight fraction (Amon and Benner, 1994). In an upwelling zone with a short production season, the ratio of such long-lived substances to the more labile products of contemporaneous phytoplankton production would frequently

be high. The products of purine catabolism discussed above may play a similar role for the histidine biosynthetic pathway. Another possibility is imidazole compounds produced for protection against oxygen radicals (Dahl et al., 1988; Hartman et al., 1990). During blooms, dissolved oxygen in the WAP reaches concentrations that are among the highest ever observed in the ocean (Karl and Hebel, 1990; Karl et al., 1995), and even under non-bloom conditions dissolved oxygen concentrations are relatively high because of low temperatures and strong winds. If a large fraction of bacterioplankton are in fact histidine or phenylalanine auxotrophs, such inhibition of biosynthetic pathways may help to explain why they evolved in this way.

The observed effects of ultraviolet light are somewhat paradoxical and point to a complex phenomenon that these limited experiments barely begin to elucidate. Ultraviolet light inhibits bacterial growth (Sieracki and Sieburth, 1986; Herndl et al. 1993) but also increases the biological lability of DOM (Kieber et al., 1989; Lindell et al., 1995). The short (<12 h) incubations resulted in a decrease of LAPase activity, which may indicate monomer production that reduces the need for ectoenzymes. Full-day incubations, however, stimulated LAPase activity. This may indicate that prolonged exposure to sunlight destroys amino acids, decreasing substrate supply to periplasmic LAPase and therefore stimulating enzyme production. The results of the organic-addition experiments suggest that photodegradation of particular compounds, such as histidine, could strongly affect ectoenzyme activities even if these are a small fraction of the DOM pool. However, 12 hours of exposure to full surface sunlight is a large dose of solar radiation. In the ocean this would be moderated by mixing, so the shorter incubations may more accurately simulate processes occurring in nature.

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# CHAPTER 5
# DECOMPOSITION OF PARTICLES

## 5.1 INTRODUCTION

Azam and Cho (1987) introduced the idea that much DOM originates from bacterial solubilization of particulate organic matter (POM). This DOM, being more labile than bulk DOM, is utilized more rapidly than the ambient DOM pool. Supporting evidence was presented by Smith et al. (1992) who demonstrated that marine snow aggregates were solubilized by hydrolytic enzymes at rates much greater than the DOM produced could be assimilated by the particle-associated bacteria. Living organisms contain many substances more labile biochemically than most DOM in the ocean. They also have components, particularily cell walls and exopolysaccharides (glycocalyx), that may be refractory (Cowen and Bruland, 1985; Lindberg, 1990) and therefore contribute to the long-lived DOM pool.

A variety of types of nonliving particles are found in the ocean: fecal pellets of zooplankton, particles formed from dissolved and colloidal organic matter by aggregation and surface forces (e.g., on bubbles), abandoned feeding structures such as larvacean houses, cell walls of phytoplankton lysed by viruses or bacteria, bacterial and algal exopolysaccharides (EPS) released or dislodged from the cells, and amorphous aggregates of all of these. In the last case the particles are not strictly nonliving, as living organisms are an integral component of aggregates. All types of particles, in fact, contain microbial communities. Particles are referred to as nonliving only to distinguish them from the component of POM that is single, intact organisms, and are more accurately described as nonreproducing. Particles in the ocean are diverse, and much

remains unknown about either the origin or fate of even the best-studied classes. Particles collected by sediment traps may include all of the different types discussed above. It is also important to note that the definition of "particulate" is operational (generally defined by a filter type), and organic matter in seawater forms a continuum from truly dissolved in the physical sense to particulate, with a substantial fraction falling in between (colloids) (Sharp, 1973).

Hydrolytic enzymes were thought to play an important role in bacterial utilization of POM long before there were any direct measurements of these activities (Joint and Morris, 1982). In recent years several papers have appeared confirming that high activities of hydrolytic enzymes are associated with particles (Karner and Herndl, 1992; Smith et al., 1992). However, these investigators dealt only with particles collected by SCUBA divers. This places two fundamental contraints on the kinds of particles that may be included: they must be large enough to be visible to divers, and they must be found within the depth range accessible to divers (generally <20 m; but see Orzech, 1984; Orzech and Nealson, 1984). Amy et al. (1987) collected marine snow samples from a small manned submersible in Howe Sound, British Columbia and determined activities of several enzymes. The maximum depth in their study area was 325 m, but the depth at which the samples were collected was not specified.

Relative rates of bacterial utilization of organic compounds in particles are crucial to the notion of a "biological pump" transporting carbon from the surface to the deep ocean via sedimenting organic particles (Volk and Hoffert, 1985). Net downward fluxes of carbon occur in certain parts of the ocean, balancing upward fluxes in other areas (upwelling zones). A net flux of carbon across the thermocline via sinking particles requires uncoupling of the C, N and P cycles: if the upward flux of nutrients is in Redfield ratio (Eppley and Peterson, 1979), the downward flux must be non-Redfield

for a net downward carbon flux to exist. This does not apply, however, if there is significant flux of N or P from the atmosphere (Karl et al., 1992; 1995a).

Bacterial decomposition of DOM and POM is to a large extent a non-Redfield process. Bacterial biomass itself contains the major bioelements in non-Redfield ratio (Nagata, 1986). Ectoenzymatic hydrolysis of DOM and POM is clearly a non-Redfield process, and the relative rates of hydrolysis of nitrogen-rich (protein) and nitrogen-poor (polysaccharide) fractions vary considerably (Chapter 3). EPS is nitrogen-poor and probably biochemically refractory. Bacterial interactions with POM through these cell surface structures are potentially important mechanisms for decoupling of elemental cycles in the decomposition process. These processes therefore have significant effects on biogeochemical cycles in the ocean.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Sample collection

#### *5.2.1.1 Particle interceptor traps*

Particle interceptor traps (PITs) were of the MULTITRAP design (Knauer et al., 1979). These are polycarbonate cylinders approximately 70 cm high and 7 cm in diameter (collection cross-section 0.0039 $m^2$). The traps are attached to frames suspended by a polypropylene line from a set of surface floats. Deployment depths were 15-30 m below the standard HOT depths of 150, 300 and 500 m. HOT sediment trap protocols are described at length by Karl et al. (1995b). Trap solutions were made up in surface seawater from Station ALOHA, amended with 50 g NaCl $l^{-1}$ and passed

through a 0.5 µm cartridge filter. Samples for bacterial enumeration were taken from traps poisoned with 1% formalin; samples for ectoenzyme analysis were taken from unpreserved traps. Upon recovery, the overlying seawater was siphoned off to 5 cm above the interface, and the solution was poured through 202 µm Nitex® mesh to remove zooplankton.

The traps were mixed to randomize particles, and subsamples were collected for ectoenzyme analysis and bacterial enumeration. For ectoenzyme analysis, 2 ml subsamples were diluted to 6 ml with a solution of KCl (11 mM), $MgCl_2$ (53 mM), and $CaCl_2$ (10 mM) to dilute $Na^+$ and create a solution with concentrations of these four cations at approximately seawater concentration. Samples for enumeration of bacteria (10 ml) were placed in 15 ml polypropylene centrifuge tubes and stirred vigorously on a vortex mixer for 15-30 s to remove bacteria from particles. A 1 ml subsample was then placed in a cryovial and frozen under liquid $N_2$ for flow cytometric analysis. The remainder was stained with 4'6-diamidino-2-phenylindole (DAPI), and filtered and mounted for epifluorescence microscopy (see Section 5.2.3).

*5.2.1.2 Fecal pellets*

Fecal pellets were collected, in most cases, by placing zooplankton collected in net tows in a "fecotron," i.e., a container equipped with a screen through which fecal pellets can sink but animals can not swim. All tows were done at night. In the equatorial Pacific, pellets were provided by Dr. Hans Dam (University of Conneticut). These were collected from the fecotron and concentrated on a 30 µm screen. Both pellets trapped on the screen and the water from which they were collected were assayed for ectoenzymatic activity. At Station ALOHA, mixed zooplankton were

collected with a 202 µm mesh net, and the zooplankton placed in a container with similar mesh. Off the windward coast of Oahu, a 505 µm mesh net was used; the mesh in the fecotron was also 505 µm. In this instance, however, a large number of animals were observed to have passed through the mesh, and following pellet collection the water was rescreened through 335 µm mesh. After screening, the pH of the water was determined to ensure that respiration of the animals did not alter the pH sufficiently to significantly affect 4MUF fluorescence yield (Chróst and Krambeck, 1986). In Antarctica, pellets were collected from monospecific collections of the Antarctic krill, *Euphausia superba*, collected by Drs. Langdon Quetin and Robin Ross (University of California, Santa Barbara). Krill are large (3-8 cm), as are their pellets, so in this case no screens were used. Pellets were collected visually by pipette from a large vessel containing the animals.

*5.2.1.3 Other particle types*

Samples of sea-ice rich in microbiota ("brown ice") were collected by hand from a Zodiac® placed on the ice by a ship's crane, in Crystal Sound and the Grandidier Channel (see Chapter 3). *Trichodesmium* colonies were collected at Station ALOHA with a 75 µm mesh net and separated from the remainder of the catch under a dissecting microscope. Marine snow aggregates were collected off the coast of Oahu near Kewalo Basin by SCUBA divers, using a 50 cm length of plastic tubing attached to a syringe.

### 5.2.2 Ectoenzymes

Methods for ectoenzyme analysis are described in Chapters 2 and 3. Saturating substrate concentrations were used for leucine aminopeptidase (LAPase), α-glucosidase (AGase), and β-glucosidase (BGase) (1 mM, 1.6 μM, and 1.6 μM respectively). Other 4MUF-glycosides were added at 1.6 μM. Hydrolytic enzymes in the interstitial spaces of particles are unlikely to be entirely cell-surface associated, so in this context the term "ectoenzyme" is not used. It is still used in reference to the general method. Samples containing particles were incubated 5-10 h to reduce variance from diffusion-limitation. When high fluorescence required dilution prior to fluorescence determination, dilution was with the same matrix as the original sample (seawater, with 4 mM $HgCl_2$ if used in the sample).

### 5.2.3 Enumeration of bacteria

Bacteria were enumerated by epifluorescence microscopy using DAPI (Porter and Feig, 1980) and by dual-laser flow cytometry using Hoechst 33342 (Monger and Landry, 1993). The flow cytometer was a Coulter EPICS 753 operated by Hector Nolla (Department of Oceanography, University of Hawaii). A dual-laser (UV/visible) flow cytometer permits separate enumeration of heterotrophic (unpigmented) bacteria and picophytoplankton (cyanobacteria) (Monger and Landry, 1993; Campbell et al., 1994). Cyanobacteria were not observed in trap samples, but this may be a result of pigment bleaching by formalin in the traps. Data analysis was carried out using CYTO-PC software (Vaulot, 1989). For epifluorescence microscopy, a Zeiss Model 16 standard microscope equipped with a IV FI epifluorescence condenser and 100 W

mercury vapor lamp was used. Filters were a standard Zeiss UV filter set (#487702; 365 nm broad band excitation, 420 long pass). Ocular and objective lenses were 16X and 100X respectively. Bacteria collected in PITs were stained with 0.5 µg $ml^{-1}$ DAPI for a minimum of 1 h; for free-living bacteria the concentration was 1.0 µg $ml^{-1}$. Filters with 0.2 µm pore size (25 mm) were used, either commercially prestained (Poretics Corp.) or stained with Irgalan Black (2 g $l^{-1}$ in 2% acetic acid).

### 5.2.4 Ruthenium Red staining

Ruthenium Red (Sigma) was dissolved in distilled water at a concentration of 5 mg $ml^{-1}$ (Strycek et al., 1992) and filtered (0.2 µm) before each use. Approximately 50 µl of the Ruthenium Red solution was added to 10 ml of trap material and filtered onto a Whatman GF/F filter. The filters were examined under a Zeiss standard compound microscope at 100X magnification and photographed using standard 35 mm 400-speed film.

### 5.2.5 Sample preparation for seawater cultures

To incubate seawater bacteria in the absence of grazers, seawater was filtered through 1.0 µm polycarbonate membrane filters. The collection vessel and frit were washed thoroughly with 10% HCl and distilled water, then rinsed twice with sample water. Particulate matter (PITs collections, phytoplankton, zooplankton) was frozen and thawed, then autoclaved at 120°C for 60 min. Zooplankton (mixed collection from coastal Oahu using a 505 µm net) were pulverized with a mortar and pestel and filtered through a 202 µm screen prior to autoclaving.

## 5.3 RESULTS

### 5.3.1 Decomposition of particles

#### *5.3.1.1 Hydrolytic enzyme activities associated with marine particles*

Activities of hydrolytic enzymes were determined on several categories of particles, including fecal pellets, particles collected in sediment traps, particles hand-collected by SCUBA divers, and decomposing colonies (trichomes) of *Trichodesmium* spp. Activities were also determined in ice-algal communities ("brown ice"). While not strictly "particles," this microenvironment is greatly enriched in organic matter and bacterial activity relative to ambient seawater, as are the four types of particles mentioned.

Ratios of LAPase to BGase in particles collected in sediment traps at Station ALOHA (135-515 m) are consistently less than those in fecal pellets (Figure 5.1 and Table 5.1). They are also much less than the ratio in seawater samples at ALOHA (Christian and Karl, 1995a; see chapter 3). The difference in the LAP/BG ratio between fecal pellets and particles collected in PITS is statistically significant ($P<0.005$ by Wilcoxon test, and 95% confidence limits for model II regression slopes do not overlap). Activities associated with fecal pellets were determined in a number of different locations: at Station ALOHA, near the windward coast of Oahu, in the equatorial Pacific and in Antarctica. The ratios on fecal pellets from these four locations show remarkable consistency, except in the equatorial Pacific where the ratio

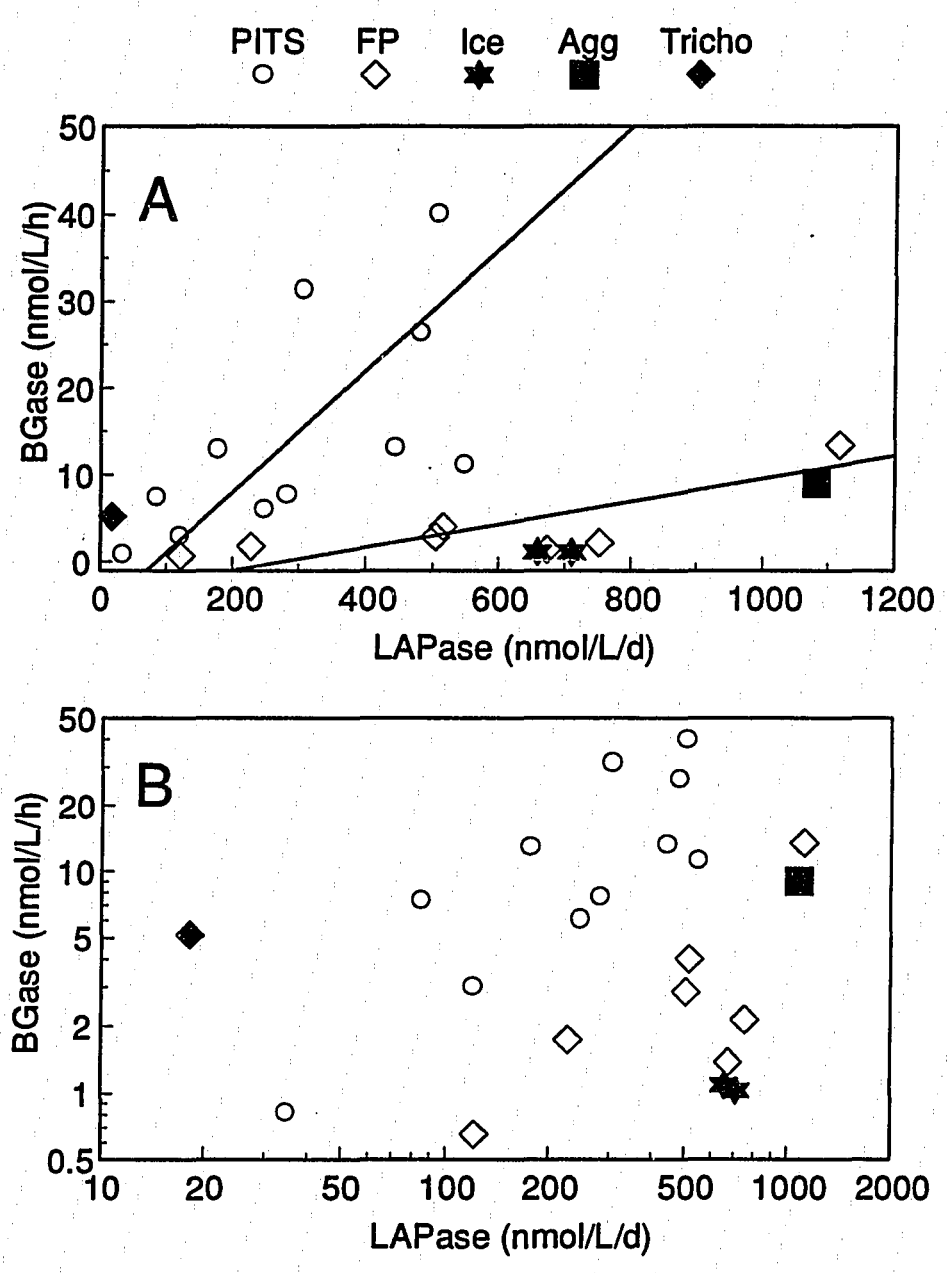


Figure 5.1. BGase vs. LAPase on several types of marine particles. PITS = particle interceptor traps, deployed at 135-515 m at Station ALOHA; FP = fecal pellets from crustacean zooplankton; Ice = sea ice microbial community; Agg = aggregates hand-collected by SCUBA divers at Kewalo Basin, Oahu; Tricho = colonies of *Trichodesmium* spp. (a) linear plot with Model II regression lines for PITS (n=11) and FP (n=7) (b) log-log plot.

Table 5.1. LAPase/BGase and AGase/BGase ratios on several types of marine particles. PITS = particle interceptor traps, deployed at 135-515 m at Station ALOHA. On HOT 50 one of 3 traps at 165 m (called b) had much higher activities of all enzymes than the others; these are presented separately for comparison. Aggregates were hand-collected by SCUBA divers at Kewalo Basin, Oahu.

| Sample Source | | LAP/BG | AG/BG |
|---|---|---|---|
| PITS | Depth (m) | | |
| HOT 41 | 135 | 18.2 | |
| HOT 45 | 165 | 12.6 | |
| HOT 45 | 330 | 9.76 | |
| HOT 44 | 165 | 33.7 | |
| HOT 44 | 330 | 11.5 | |
| HOT 50 | 165 (mean) | 4.48 | 0.299 |
| HOT 50 | 165 (b) | 3.54 | 0.300 |
| HOT 50 | 165 (a+c) | 13.7 | 0.286 |
| HOT 54 | 165 | | 0.583 |
| HOT 54 | 315 | | 0.446 |
| HOT 55 | 165 | | 0.675 |
| HOT 55 | 315 | | 0.747 |
| HOT 55 | 515 | | 0.223 |
| Fecal pellets | | | |
| Equator >30 µm | | 83.2 | |
| Equator < 30 µm | | 129 | |
| *E. superba* | | 489 | |
| *E. superba* | | 187 | |
| Windward Oahu | | 131 | |
| *E. superba* | | 177 | 2.26 |
| Station ALOHA | | 353 | 1.31 |
| Brown Ice | | | |
| PD9401 | | 606 | |
| PD9401 | | 691 | |
| PD9412 | | | 0.992 |
| PD9412 | | 1418 | 0.885 |
| PD9412 | | 1058 | 0.913 |
| Aggregates | | 120 | |
| Trichomes | | 3.51 | |

is less than in the other three sites. Overall the highest ratios of LAPase to BGase were observed in brown ice, and the lowest in decomposing trichomes.

On HOT 50 (Oct.-Nov. 1993) and HOT 55 (June 1994), a wider variety of hexosidases were assayed on particles collected in PITs. BGase was the predominant enzyme in all cases, followed approximately by chitobiase, AGase, β-xylosidase, β-galactosidase and β-glucuronidase (Table 5.2). β-glucuronidase activity was extremely low and frequently not detectable. On HOT 50, one of 3 traps deployed at 165 m had much greater activities than the other 2, but the ratios among the different enzymes were fairly consistent.

*5.3.1.2 Temperature responses*

Temperature responses of particle-associated enzymes were determined in only a few instances. On fecal pellets collected from mixed zooplankton off windward Oahu, LAPase and BGase had weak temperature responses in comparison to water column bacteria at Station ALOHA (Table 5.3; see Chapter 3). AGase in this microenvironment showed an apparent temperature optimum at <25°C, which made calculating a meaningful semilog regression equation for the 3 temperatures assayed impossible. BGase on particles collected in PITs at Station ALOHA showed a stronger temperature response than that of water column bacteria (Table 5.3, see Chapter 3).

*5.3.1.3 Exopolysaccharides associated with marine particles*

Bacterial and algal EPS are important components of marine POM (Biddanda, 1985; Kirchman, 1992) and potentially very significant biogeochemically because they

Table 5.2. Activities of various hexosidases relative to BGase on PITs-collected particles and in one water column sample. Abbreviations are as follows: MUAGlu = 4MUF-α-glucoside, MUBGlu = 4MUF-β-glucoside, MUBGal = 4MUF-β-galactoside, MUBGluA = 4MUF-β-glucuronic acid, MUBXyl = 4MUF-β-xyloside, MUNAGA = 4MUF-N-acetyl-β-glucosamine. All substrate analogues added at 1.6 μM. nd = not detected. See Table 5.1 for explanation of codes for 165 m traps on HOT 50.

| Substrate Analogue | HOT 50 | | | HOT 55 | | | HOT 50 |
|---|---|---|---|---|---|---|---|
| | 165b | 165a,c | 330 m | 165 m | 315 m | 515 m | wc 25 m |
| MUAGlu | 0.38 | 0.36 | 0.33 | 0.67 | 0.75 | 0.22 | 0.07 |
| MUBGlu | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| MUBGal | 0.04 | 0.12 | 0.04 | 0.32 | 0.41 | 0.01 | 0.19 |
| MUBGluA | 0.001 | nd | 0.001 | 0.001 | nd | 0.007 | 0.001 |
| MUBXyl | 0.08 | 0.18 | 0.02 | 0.19 | 0.22 | nd | 0.01 |
| MUNAGA | 0.48 | 1.07 | 0.31 | 0.62 | 3.47 | 1.15 | 0.04 |

Table 5.3. Regression statistics for temperature responses of particle-associated bacterial communities. Model I regression of natural logarithm of activity in nmol $l^{-1}$ $h^{-1}$ on incubation temperature in degrees celsius. PITS were deployed at Station ALOHA; fecal pellets were collected from mixed zooplankton off windward Oahu. nd = not determined.

| | LAPase | | | BGase | | |
|---|---|---|---|---|---|---|
| Sample type | Intercept | Slope | $r^2$ | Intercept | Slope | $r^2$ |
| PITS | nd | nd | nd | -2.26 | 0.260 | 0.95 |
| Pellets | 8.70 | 0.0267 | 0.95 | 3.03 | 0.0541 | 0.99 |

have high C/N ratio and are not easily degraded. Microscopic examination of particles collected in PITs at Station ALOHA and stained with Ruthenium Red showed abundant EPS of both algal and bacterial origin. Although particles may have become compacted during screening and subsampling, few resembled aggregates in which the primary matrix was EPS. The most common types of Ruthenium Red positive particles were outer mucoid layers of diatoms (Figure 5.2a) and epicolonies of bacteria attached to larger particles (Figure 5.2b).

It was also observed that a small sample of trap material added to surface seawater rapidly scavenged a large percentage of the bacteria from suspension (Figure 5.3). Multiple field counts could not account for the number of cells present initially (not counting those present in the trap material). A large fraction of the cells were in aggregations that were extremely patchy in their distribution and too dense to count accurately. The variance among field counts was much greater than in the unamended seawater (coefficient of variation of 71.5% vs. 8.8%).

*5.3.1.4 Response of free-living bacteria to particulates*

Water-column bacterial communities inoculated with autoclaved particulate matter showed varying responses. The 330 m trap material did not stimulate ectoenzymatic activity to any significant extent, and activity ratios resembled the unfiltered control (Table 5.4). The 165 m trap material and the *Pavlova lutheri* culture resulted in LAP/BG ratios similar to the unfiltered control and therefore alleviated the nitrogen limitation that caused large increases in the LAP/BG ratio in the filtered control (see Chapter 4). As expected, zooplankton tissue resulted in a high LAP/BG ratio and also stimulated chitobiase activity (data not shown). Trap material stimulated

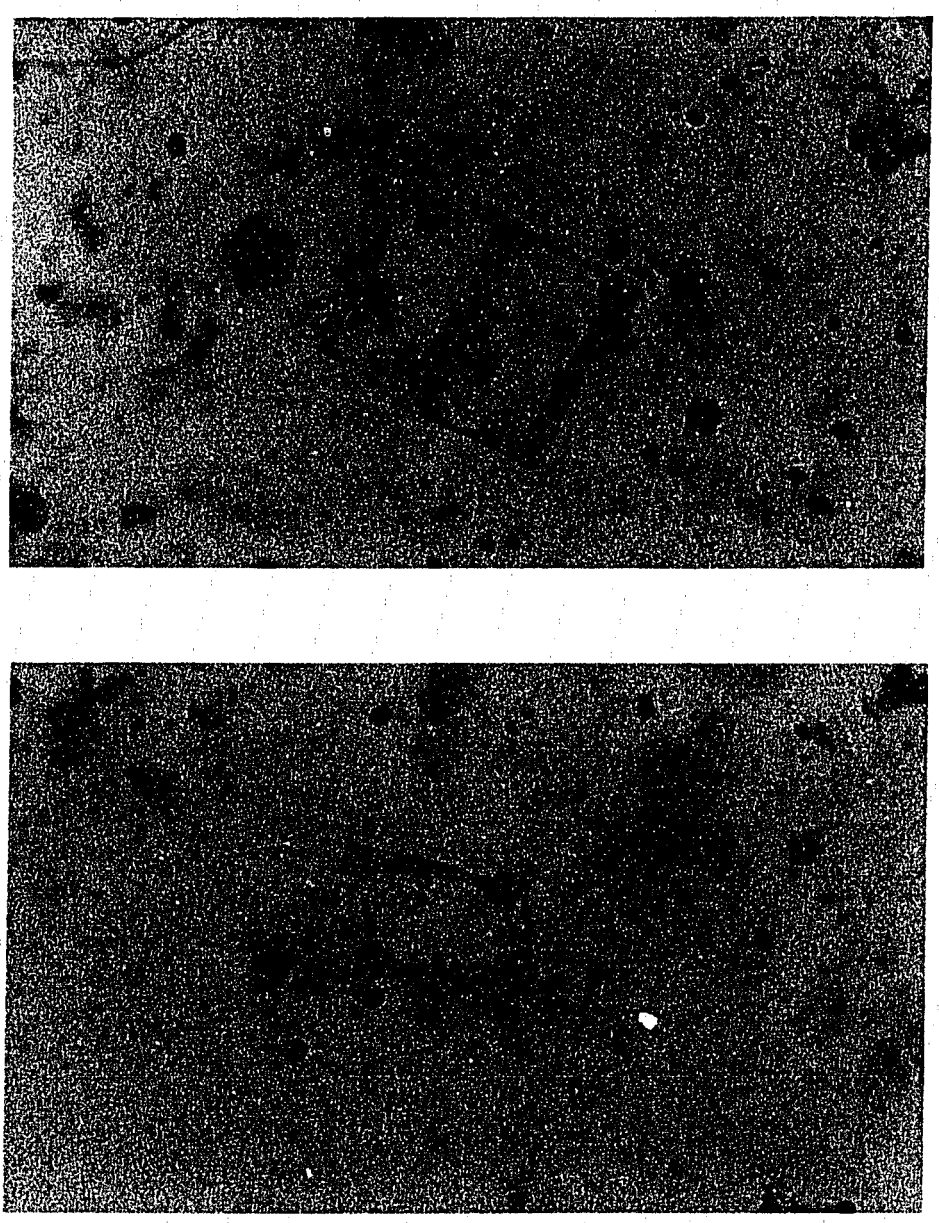

Figure 5.2. Photomicrographs of particles collected in particle interceptor traps at Station ALOHA, stained with Ruthenium Red (100X magnification). (top) mucopolysaccharide matrix from diatoms (bottom) epibacterial colonies on diatom mucopolysaccharide matrix.

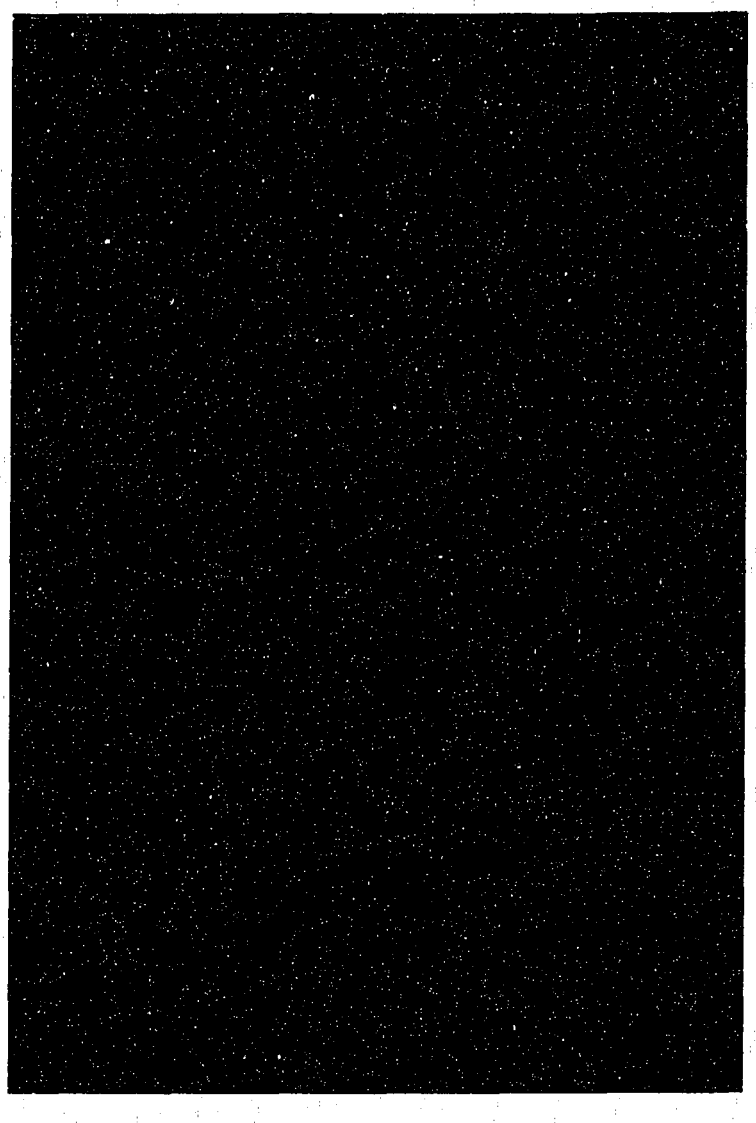

Figure 5.3. Epifluorescence photomicrograph of bacterial cells stained with DAPI adhering to material collected in particle interceptor traps at Station ALOHA (1600X magnification).

Table 5.4. LAPase/BGase ratios in Station ALOHA surface seawater inoculated with dissolved and particulate organic matter from various marine sources. All treatments except the unfiltered control were filtered through a 1.0 µm membrane filter. All added organics autoclaved 1 h at 120 °C.

| | Time (h) | | |
|---|---|---|---|
| Treatment | 0 | 24 | 72 |
| Unfiltered Control | 72 | 73 | 72 |
| Filtered Control | 84 | 345 | 1765 |
| PITS - 165 m | | 84 | 93 |
| PITS - 330 m | | 306 | 1282 |
| *Pavlova lutheri* | | 145 | 92 |
| Zooplankton | | 4542 | 385 |

increases in β-xylosidase and β-glucuronidase relative to the filtered control, but activities of these enzymes were much less than those of AGase and BGase. AG/BG ratios generally increased over the 72 h incubation but remained less than 1 in all treatments. Small (1-5 μm) Ruthenium Red positive colonies of epibacteria were observed on the *P. lutheri* fragments, but there were no aggregates composed primarily of Ruthenium Red stainable polysaccharides. Epifluorescence microscopy showed that most bacteria in the *P. lutheri* treatment occurred in such epibacterial colonies on cell fragments rather than in aggregates.

*5.3.1.5 Formation of proteolytic microzones*

Hydrolytic enzyme activities in the interior of aggregates are not completely inhibited by mercuric chloride because of diffusion limitation and scavenging of $Hg^{2+}$ ions by reactive groups in the floc (Christian and Karl, 1995b). In the autoclaved particle experiment described above, high fluorescence was observed in $Hg^{2+}$-killed controls for the LAPase assay, but not in AGase or BGase assays (Figure 5.4). It appears, therefore, that the hydrolytic enzymes associated with the interior spaces of newly-formed aggregates are largely proteases, while the glucosidases are associated with free-living bacteria or the less well protected outer layers of the floc. In several experiments conducted in Antarctica in December 1994, a similar result was obtained with unamended 1.0 μm filtered seawater, suggesting that bacteria can spontaneously form such proteolytic microaggregates. This was observed in the midst of a dense phytoplankton bloom (15-20 mg chlorophyll *a* $m^{-3}$), so colloidal organic matter may have been abundant. In the equatorial Pacific in 1992, several water samples appeared

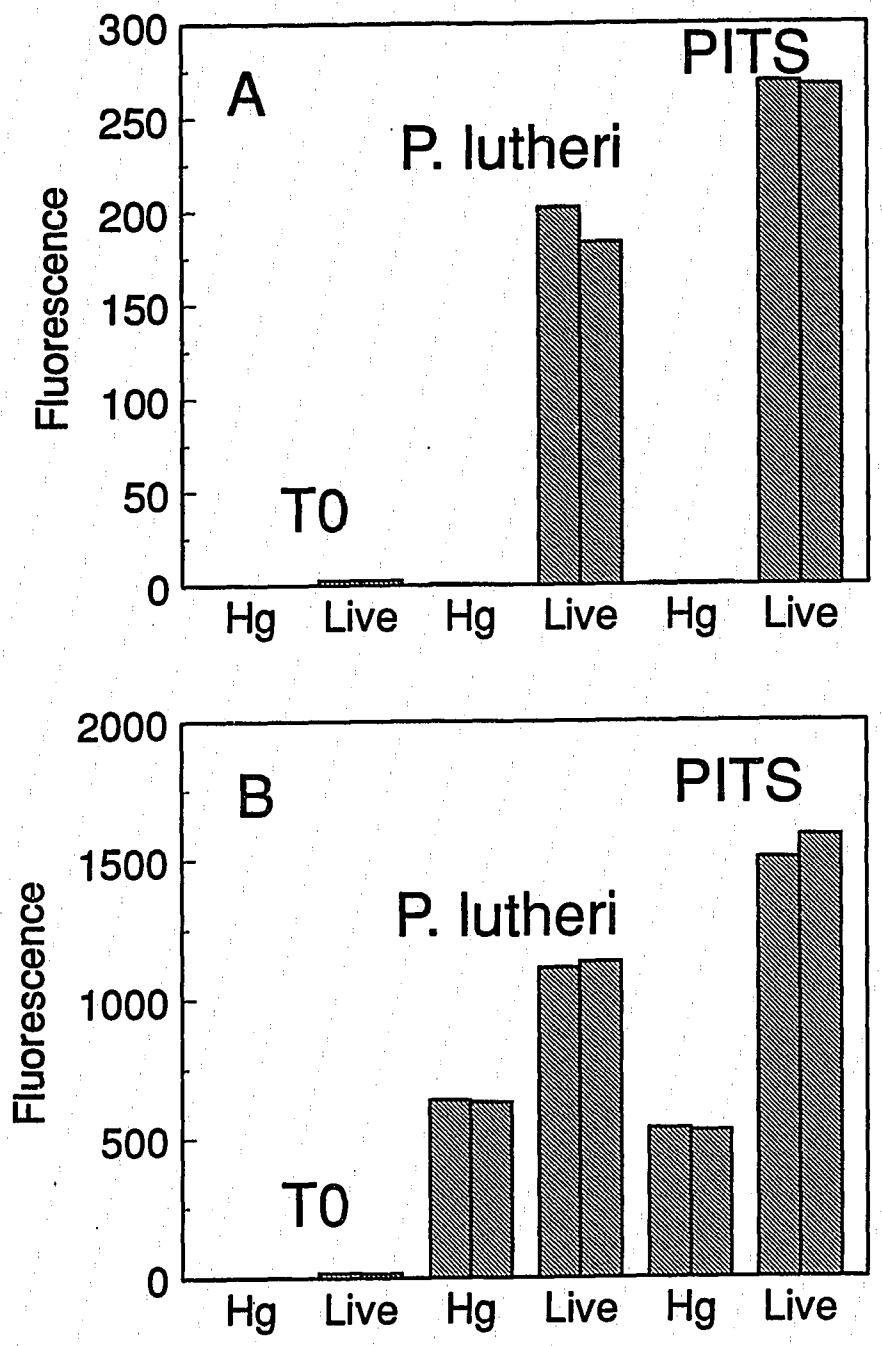


Figure 5.4. Fluorescence (arbitrary units) liberated from (a) MUBG and (b) LLBN in 1.0 µm filtered seawater incubated 72 h with freeze-thawed and autoclaved *Pavlova lutheri* culture and PITs-collected (165 m, on HOT 50) material, with and without $HgCl_2$ (4 mM). T0 = 1.0 µm filtered seawater control at time zero.

to contain such protease-rich aggregates, as they had LAPase activities much greater than the cruise mean (see Chapters 2 and 3).

### 5.3.2 Bacteria and biogeochemical fluxes at Station ALOHA

To put some of the observations described above into oceanographic context, I will compare results from three HOT cruises during which different hydrographic and biogeochemical conditions prevailed. HOT 44 (January 1993) occurred during calm conditions but in the wake of a mixing event that created a very deep wind-mixed layer (90 m, based on a maximum density gradient of $< 5$ g $m^{-4}$) which, however, contained little nitrate (Figure 5.5a). On HOT 45 (February 1993) the mixed layer was only ~40 m but the nitrate isopleths were translated upward by ~30 m, relative to the mean condition (Figure 5.5b), resulting in a large input of nitrate to the upper 100 m. HOT 50 (October-November 1993) occurred during calm conditions in the wake of the annual minimum in the trade winds (Wyrtki and Meyers, 1976; see also Karl et al., 1992; 1995a). Density, nitrate, and chlorophyll profiles on this cruise (Figure 5.5c) were nearer to "typical" conditions at Station ALOHA than on HOT 44 or 45. On all three of these cruises carbon and nitrogen fluxes at 150-500 m were high relative to the mean condition. Examination of the microbiology of the particles and anomalies in the water column biology may help to explain the mechanisms controlling the flux in each case.

It appears that HOT 44 occurred in the wake of a mixing event of the classical Ekman-pumping variety (Eppley and Renger, 1988). At Station ALOHA this type of mixing event results in little input of nitrate to the mixed layer because of the great depth of the nitracline (Dore, 1995). The nonuniform chlorophyll profile within the

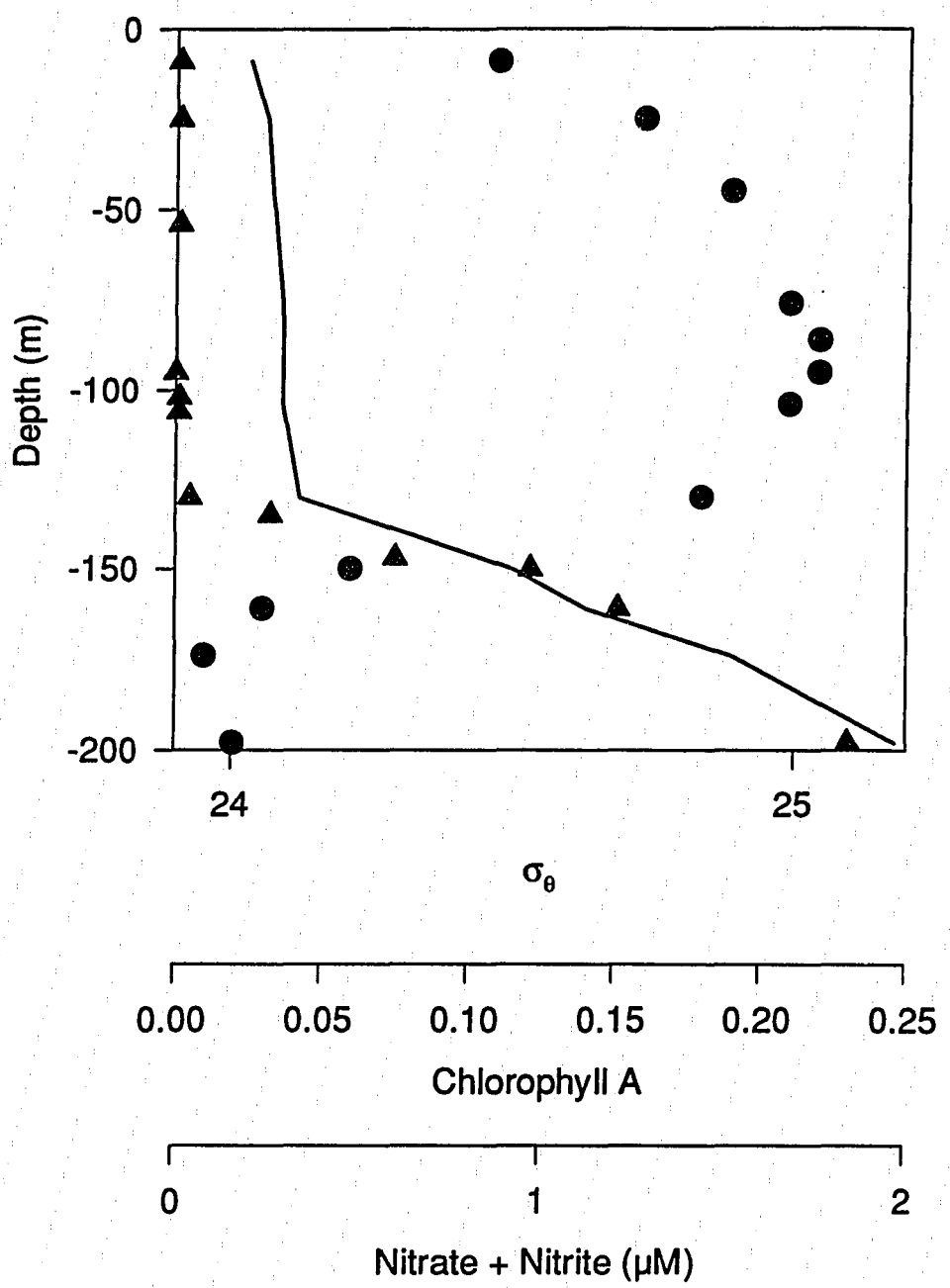


Figure 5.5a. Depth profiles of density ($\sigma_\theta$, solid line), $NO_3^-$+$NO_2^-$ (μM, triangles), and chlorophyll *a* (μg $l^{-1}$, circles) at Station ALOHA on HOT 44 (January 1993).

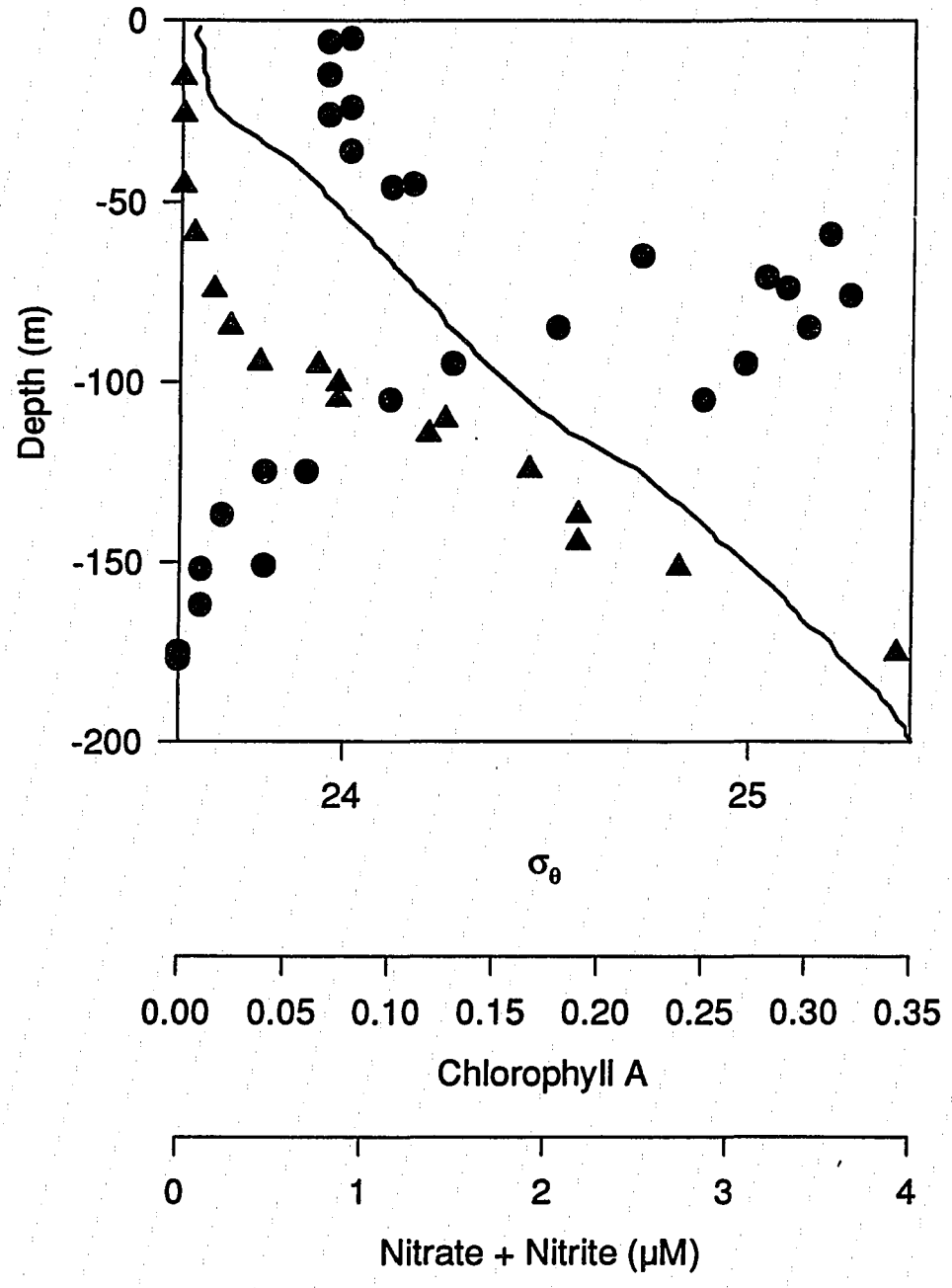


Figure 5.5b. Depth profiles of density ($\sigma_\theta$, solid line), $NO_3^-$+$NO_2^-$ (μM, triangles), and chlorophyll *a* (μg $l^{-1}$, circles) at Station ALOHA on HOT 45 (February 1993). Two chlorophyll profiles (different hydrocasts) are shown.

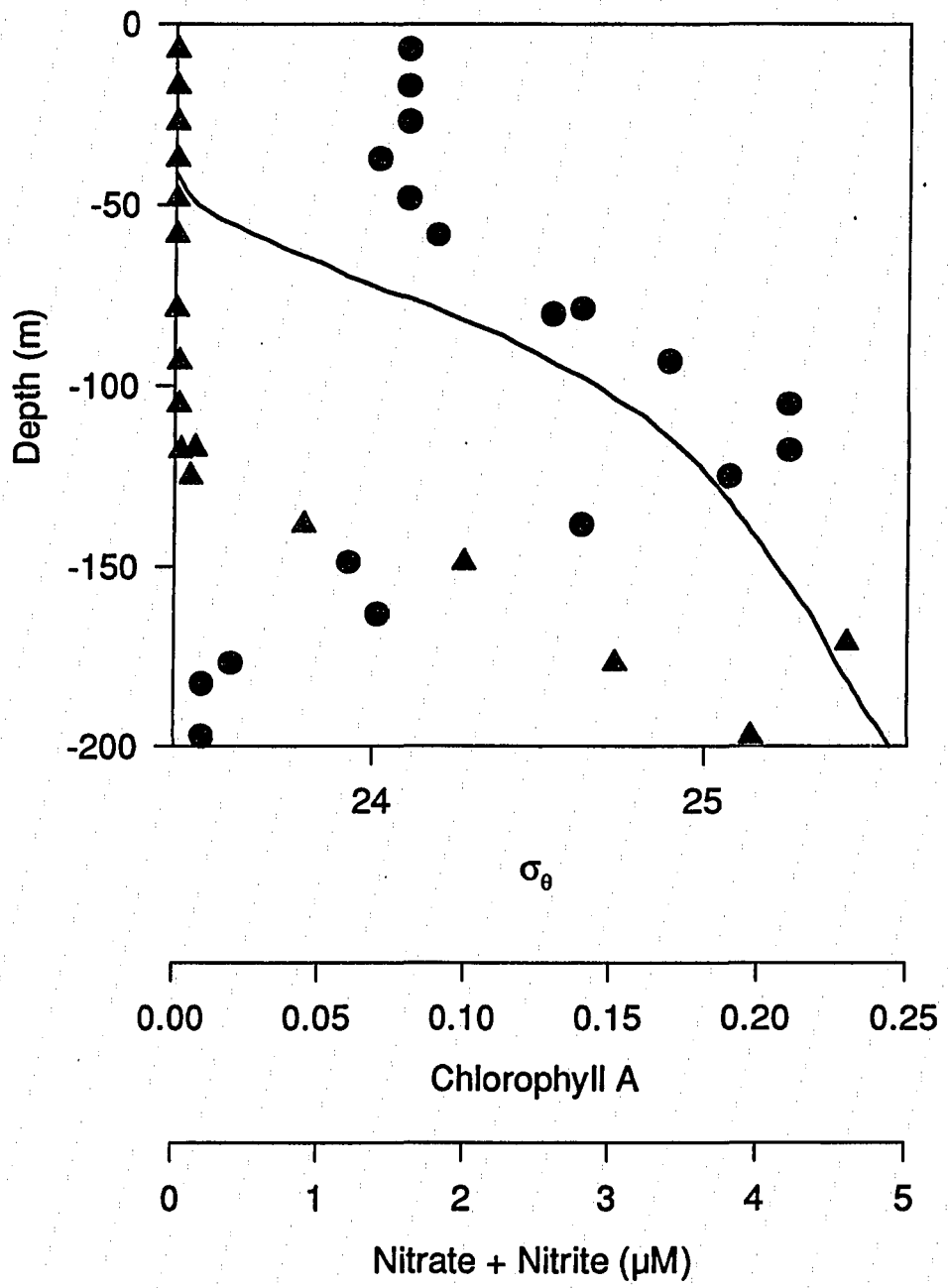


Figure 5.5c. Depth profiles of density ($\sigma_\theta$, solid line), $NO_3^-$+$NO_2^-$ (μM, triangles), and chlorophyll *a* (μg $l^{-1}$, circles) at Station ALOHA on HOT 50 (Ocober-November 1993).

isopycnal layer shows that mixing was weak at the time of the cruise (Figure 5.5a). HOT 45, in contrast, appears to have encountered a mixing event forced from below, either by breaking of internal waves (McGowan and Hayward, 1978) or by the passage of a mesoscale eddy (Falkowski et al., 1991). Chlorophyll concentration in the mixed layer on HOT 45 (Figure 5.5b) was low for this time of year, showing no evidence of photoadaptation as would be expected had the wind-mixed layer recently been deep enough to have caused this large input of nitrate (Winn et al., 1995).

On HOT 44 and 45 there was substantial enzymatic activity associated with sinking particles at 165 and 330 m, but few bacteria and high variance of bacterial counts among replicate 1 ml subsamples (Table 5.5). On HOT 50 the number of bacteria was much greater than on HOT 44 or 45 but the enzyme activities were similar or less. At 330 m on HOT 50, there was no measurable LAPase associated with the sinking particles. In addition, the particles used in the autoclaved-particle experiment were collected on HOT 50, suggesting that there was little biologically labile organic matter present in the 330 m collections on this cruise, although the bulk carbon and nitrogen fluxes at 300 m were high. It is hypothesized that these particles were in a relatively advanced state of degradation or microbial succession, characterized by low ratios of LAPase to BGase and AGase and low cell-specific activities. In contrast, those observed on HOT 44 and 45 were "fresh," reflecting recent inputs of nitrogen to the surface layer. Absolute activities were greatest on HOT 45 (see Discussion).

Several anomalous results were observed in the water column ectoenzyme activities on these cruises. On HOT 44, LAPase activity was approximately constant to a depth of greater than 100 m. On all other cruises, it decreased from the surface downwards, sometimes showing a subsurface maximum (Figure 5.6). This may indicate a role for ultraviolet photolysis of dissolved protein in regulating LAPase activity (see

Table 5.5. Enzyme activities and bacterial numbers in PITs deployed at Station ALOHA. Total activities determined in live traps at end of 3-day deployment. nd = not detected. Coefficient of variation (CV) for bacteria is aggregate of within-trap and between-trap variability.

| Cruise | Depth (m) | Total activity (nmol/l/h) | | Cell-specific (amol/cell/h) | | Bacteria flux (cells $m^{-2}$ $d^{-1}$) | CV (%) |
|---|---|---|---|---|---|---|---|
| | | LAP | BG | LAP | BG | | |
| HOT 44 | 165 | 444 | 13.2 | 82154 | 2435 | 6.50E+08 | 181 |
| HOT 44 | 330 | 84.9 | 7.38 | 3669 | 319 | 2.55E+09 | 94.9 |
| HOT 45 | 165 | 507 | 40.2 | 18135 | 1436 | 3.24E+09 | 175 |
| HOT 45 | 330 | 306 | 31.4 | 16809 | 1723 | 2.14E+09 | 246 |
| HOT 50 | 165 | 413 | 92.1 | 2260 | 504 | 2.00E+10 | 31.8 |
| HOT 50 | 330 | nd | 10.1 | nd | 61 | 1.85E+10 | 31.4 |
| Mean | 165 | 455 | 48.5 | 34183 | 1458 | 7.95E+09 | |
| Mean | 330 | 195 | 16.3 | 10239 | 701 | 7.73E+09 | |

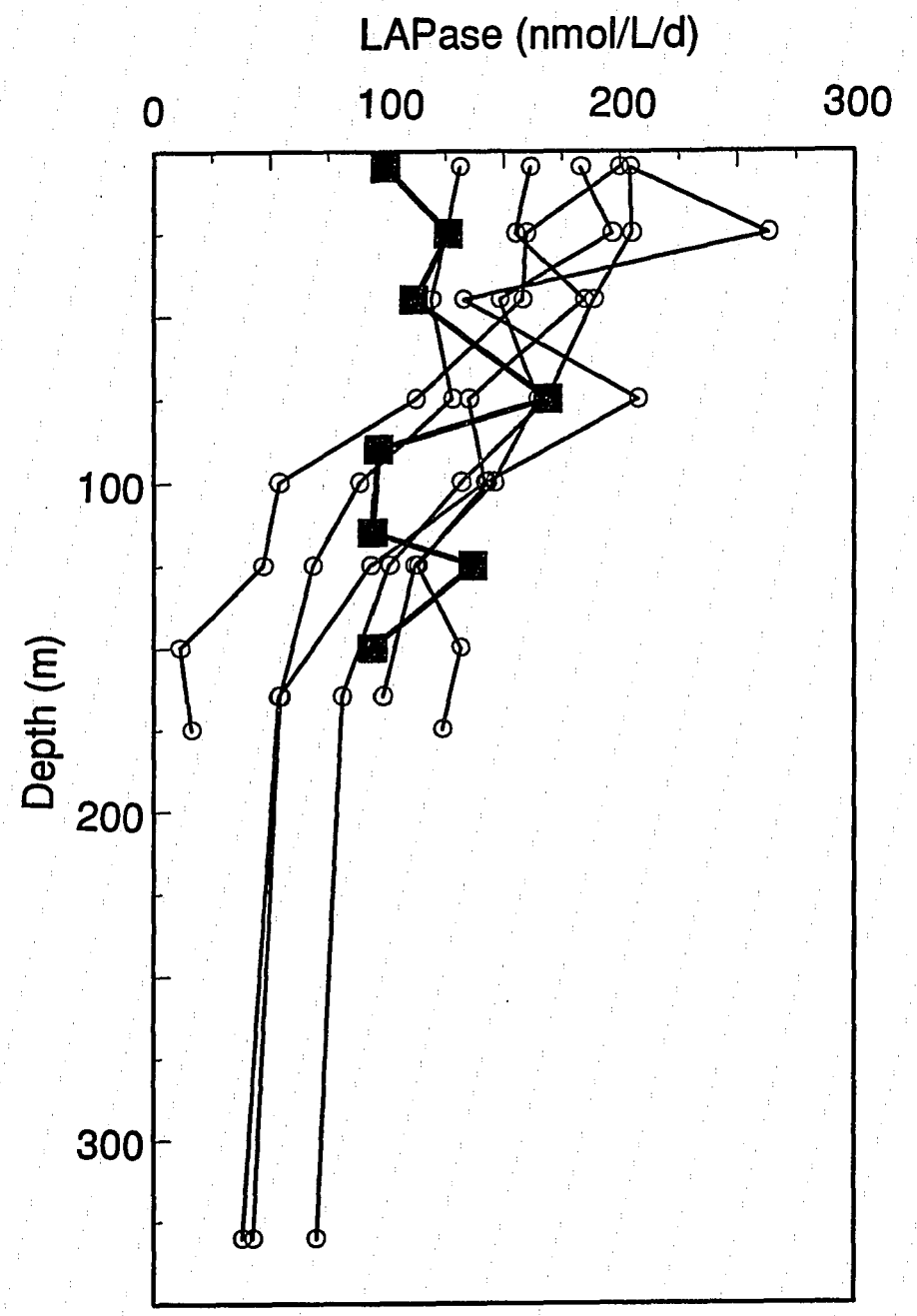


Figure 5.6. Depth profiles of LAPase at Station ALOHA, from Oct. 1992 through June 1994. Solid squares: HOT 44 (January 1993); circles: all other cruises.

Chapter 4). On HOT 45 the variance of triplicate 6 ml subsamples incubated for BGase activity was unusually low. Of 30 such sets of triplicates from depths less than 100 m (HOT 43 through HOT 54), 8 had coefficients of variation of less than 20%. Of these, 5 were from HOT 45 (all of the samples from this cruise). It is hypothesized that allochthonous input of nitrogen stimulates BGase activity of free-living bacterioplankton, whereas under N-depleted conditions BGase activity is primarily associated with microzones with high rates of N regeneration (Goldman, 1984).

## 5.4 DISCUSSION

The relative activities of the hydrolytic enzymes studied, and the temperature responses of the enzymes, are quite different on PITs-collected particles and fecal pellets. The difference in LAP/BG ratios on PITs-collected particles from Station ALOHA over 4 cruises and on zooplankton fecal pellets from a variety of habitats is statistically significant even though there are few data in each group. Fecal pellets consistently have higher AG/BG ratios than other types of particles. Fecal pellets may be a significant component of sinking particles in the oligotrophic ocean, but their native bacterial communities are a small fraction of the total community when they are incorporated into aggregates.

LAP/BG ratios on autoclaved PITs particles incubated with water column bacteria are also greater than those observed on the freshly collected particles (Tables 5.1 and 5.4), suggesting that the activity ratios *in situ* are the product of a succession of bacterial species or phenotypes. However, the higher LAP/BG ratios in the autoclaved-particle experiment may also result from the absence of grazers in the incubation containers, which could inhibit this community succession. The generally

low LAP/BG ratios on sinking particles suggest that micrograzers are an important component of the microbial community in such particles (Taylor et al., 1986; Biddanda and Pomeroy, 1988) and that the physiology of particle-associated bacteria is regulated by feedback from the rest of the microbial community (see Chapter 4).

Particle-associated bacterial communities show enzyme activity ratios and temperature responses that distinguish them from free-living bacterioplankton. Fecal pellet associated enzymes show a reduced temperature response relative to water-column communities at Station ALOHA, suggesting adaptation to low temperatures. The reduced temperature response of LAPase and BGase is consistent with the adaptation to low tempertature observed in Antarctic bacterioplankton communities (Christian and Karl, 1995a; see Chapter 3). It could also be an adaptation to varying temperature associated with diel vertical migration of zooplankton in a region of strong temperature stratification. AGase, however, appears adapted to low rather than variable temperatures. It may be that the gut bacteria are optimally adapted to the daytime (subthermocline) habitat of the animals, i.e., when the gut is full following nightime feeding in the euphotic zone.

Bacteria on sinking particles below 100 m at Station ALOHA inhabit an environment where temperature is constantly changing. Because the temperature response on these particles was determined only for BGase and the slope of the regression differed from that for the water column community, no attempt was made to normalize activities to a constant temperature. It is important to note that the incubation temperature on HOT 45 (20°C) was less than on HOT 44 or 50 (25°C). Relative activities on HOT 45 are therefore greater than the values in Table 5.5 indicate, and the measured activities reflect those *in situ* more accurately than on the other cruises.

Rates of hydrolysis *in situ* are difficult to estimate because of uncertainties about enzyme specificity and substrate concentration. However, high LAPase activities observed on PITs-collected particles suggest that this enzyme is a very significant factor in the increase of C/N with depth, as consistently observed in PITs (Martin et al., 1987; Karl et al., 1995b). Solubilization of proteins by bacterial aminopeptidases can potentially account for all of the observed decrease in N flux; this implies a loss to the suspended fraction of material with a high C/N (Karl et al., 1988; Smith et al., 1992) if substrate concentrations approach saturation. If the effective substrate concentration is substantially less than saturating while those experienced by AGase and BGase are saturating or nearly so (see Chapters 2 and 3), material solubilized by these enzymes may have C/N ratios near Redfield. Potential daily rates of hydrolysis from LAPase alone exceed 100% of the C and N at the shallower depths (165-330 m), so the half-lives of labile components of particles are likely to be short.

BGase was consistently the predominant hexosidase on particles, except on fecal pellets where AGase was frequently as great or greater. As this study was conducted in areas with little or no input of vascular plant material, there is likely to be a marine source of β-glucose polymers distinct from cellulose. Chitobiase showed relatively high activity in sediment traps, which may imply a zooplankton-derived component of the sinking flux, However, other organisms such as fungi also contain chitin, and bacterial cell wall peptidoglycans contain N-acetylglucosamine (Reynolds, 1982). Chitobiase, AGase, and β-xylosidase were higher, relative to BGase, in sinking than in suspended particles. These results are consistent with those obtained by other investigators on different types of particles. Smith et al. (1992) found LAP/BG ratios of 25-50 on larvacean houses and diatom floc and >500 on fecal pellets, in the Southern California Bight. They also found that chitobiase was similar to BGase, and that

AG/BG was <1 on particles other than fecal pellets, but their AG/BG ratios on fecal pellets were much greater than mine. Karner and Herndl (1992) observed AG/BG ratios of 0.14-0.54 and LAP/BG ratios of 94-547 on marine snow aggregates. The results of Smith et al. (1992) suggest that the microbial communities occurring on their (suspended) aggregates are quite distinct from fecal pellets, and more similar to particles collected in PITs at Station ALOHA. The results of Karner and Herndl (1992) are somewhat difficult to interpret, as they used substrate concentrations (2.5 μM) that may be saturating for AGase and BGase, but not for LAPase. Their study site (northern Adriatic Sea) also has substantial terrestrial and anthropogenic influences. The results of Amy et al. (1987) are also somewhat difficult to relate to mine. They determined protease at only 2 stations, at both of which BGase was undetectable. This makes calculating a protease/glucosidase ratio impossible, although it is certainly high (>100). However, their protease assay (casein hydrolysis) is not analogous to that used here, and their BGase numbers may be unreliable. Cellulose acetate (Millipore) filters were used to concentrate samples, and these filters may inhibit hydrolysis of BGase model substrates (Christian, unpublished data). In addition, *p*-nitrophenyl-β-glucoside was used as a model substrate for BGase, and *p*-nitrophenyl is subject to enzymatic decomposition under low oxygen conditions (Chadwick et al., 1995).

β-glucuronidase activity was consistently low, so acidic sugars are likely to play a small role in the nutrition of bacteria in open ocean environments, even in aggregates. Ruthenium Red staining shows the presence of acidic mucopolysaccharides (AMPS) associated with sinking particles. Such polysaccharides are thought to be biologically refractory in part because of the great diversity of components and linkages (Lindberg, 1990). However, β-glucuronidase is a reasonable choice for a diagnostic enzyme, and its consistently low activity confirms that this fraction of the sinking flux is refractory.

Low activities of familiar enzymes such as β-glucuronidase and β-xylosidase suggest that turnover times are very long for the more "exotic" sugars in bacterial EPS (Lindberg, 1990).

Particle-associated bacteria are likely to include specialists in degradation of AMPS. DeLong et al. (1993) found a high proportion of *Cytophaga* spp. among bacteria associated with marine snow aggregates. *Cytophaga* are gliding bacteria. Since their peculiar form of motility makes them unable to move in a liquid medium (Reichenbach and Dworkin, 1981), they are unlikely to be found as free-living cells in the ocean. Several *Cytophaga* species were among the agar-liquifying bacteria isolated from seawater by Stanier (1941), so they are potential consumers of AMPS. However, *Cytophaga* have been observed to lyse or kill a variety of phytoplankton (Stewart and Brown, 1969; Imai et al., 1993), and it is not clear whether they possess enzymes for hydrolysis of AMPS in order to utilize these as a carbon source, or to attack algae. The particles analyzed by DeLong et al. (1993) were phytoplankton aggregates consisting primarily of *Rhizosolenia* sp. and *Phaeocystis* sp., both of which produce abundant AMPS. Other marine bacteria observed to digest agar and/or alginic acid, or from which agarases or alginases have been isolated, include species of *Bacillus*, *Pseudomonas*, and *Vibrio* (Stanier, 1941; Mody and Chauhan, 1993; Sugano et al., 1993), the latter two being gram-negative genera common in seawater.

The source and nature of the EPS component of sinking particles has important implications for the biogeochemistry of the upper ocean. EPS of apparent bacterial origin did not form the primary matrix of most aggregates observed (cf. Biddanda, 1985). Much of the EPS appeared to be of diatom origin, consisting of intact cells and frustules with surface slime layers or slime layers dislodged from cells. Diatom EPS may be much more labile to microbial decomposition than that of bacteria (Smith et al.,

1995; see Chapter 6). Given the substantial agitation experienced by samples during screening and subsampling it is difficult to speculate about the presence of large, low-density aggregates such as the "transparent exopolymer particles" of Alldredge et al. (1993). Much of the stained material was amorphous and impossible to identify.

The patterns of ectoenzyme activity and bacterial abundance on sedimenting particles during HOT 44, 45, and 50 have been interpreted in terms of an hypothesized succession of bacterial communities. According to this hypothesis, cell-specific hydrolytic enzyme activities decrease and glucosidases become more important, relative to proteases, as particles decompose and the more labile fractions are consumed. This hypothesis is speculative, but it gives some insight into the relationship of decomposer communities on particles to the hydrography, ecology and biogeochemistry of the subtropical oceanic environment. The data supporting this hypothesis can be summarized as follows. On HOT 50 (stable, stratified conditions; late successional stage of bacterial communities on particles), the particle flux at 330 m had abundant bacteria, AGase, and BGase, but little labile organic material and no measurable LAPase activity. Two of three traps at 165 m also had little LAPase activity. On HOT 44 and 45 (during or following mixing events that brought new N into the euphotic zone; early successional stage of bacterial communities on particles), there were few bacteria, high cell-specific activities, and higher LAP/BG ratios than on HOT 50.

On HOT 50, integrated (0-200 m) primary production was the lowest in 6 years of observations at Station ALOHA (219 mgC $m^{-2}$ $d^{-1}$). Carbon flux, however, was relatively high. Carbon flux increased, relative to the mean, with increasing depth: 112% of the 1991-93 mean at 150 m, 121% at 300 m, and 157% at 500 m. On HOT 45, the carbon flux at 150 m was high (161% of the 1991-93 mean), but the flux declined more rapidly with depth than on HOT 44 or 50, or in the multiyear mean

profile. These observations are interpreted as indicating that the particles collected at 150-350 m on HOT 45 were "fresh," with active decomposer communities (Karl et al., 1988) while those at similar depths on HOT 50 were in a relatively advanced state of decomposition. Low primary production and the depth profile of carbon flux on HOT 50 suggest that the sinking particles did not originate from contemporaneous primary production, while the nitrate and density profiles on HOT 45 suggest a recent pulse of phytoplankton production and particle flux. It may be that all of these three cruises are "anomalies" (cf. Weatherhead, 1986), though for different reasons. The relationship between upper water column hydrography, primary production and particle flux at Station ALOHA is complex and still poorly understood (Karl et al., 1995a,b). The observations discussed here show distinctive patterns with respect to the decomposer communities on particles that are not apparent from the bulk C and N fluxes. However, a more definitive interpretation is not possible with such a small sample size.

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# CHAPTER 6

# CONCLUSION AND PROSPECTUS

## 6.1 SUMMARY OF CONCLUSIONS

There are several conclusions that can be drawn from this work, both about the enzymes and their roles in the biogeochemistry of the upper ocean. With regard to the enzymes, leucine aminopeptidase (LAPase) and β-glucosidase (BGase) are ubiquitous and their half-saturation coefficients are similar in widely separated habitats. Temperature responses are adapted to *in situ* temperature in each environment, in terms of a lowered activation energy barrier, but the ectoenzymes of Antarctic bacterioplankton do not display temperature optima less than 20°C. "LAPase" in seawater is a fairly nonspecific aminopeptidase but may be more similar to glutamate aminopeptidases. Specificities of α-glucosidase (AGase) and BGase for α or β anomers are variable; enzymes hydrolyzing neutral sugars appear specific for neutral sugars.

With regard to the ecology and biogeochemistry of the upper ocean, it is clear that rates of hydrolysis of the high C/N and low C/N fractions of polymeric dissolved organic matter (DOM) are substantially uncoupled in space and time. Relative activities of aminopeptidases and glucosidases vary significantly and *systematically* among regions and among microenvironments. Particle-associated bacterial communities have different assemblages of enzymes than free-living bacteria, and the enzymes have different temperature responses. Assemblages on fecal pellets appear distinct from those of bulk particulate matter collected by drifting sediment traps. Turnover times for dissolved polysaccharides vary widely, with acidic polysaccharides turned over slowly, and significant variation among neutral polysaccharides. These long turnover times are

consistent with the vertical and geographical distribution of dissolved polysaccharide (Pakulski and Benner, 1994) and imply a significant role for acidic polysaccharides in ocean biogeochemistry.

Histidine plays a significant role in regulation of LAPase expression in Antarctic bacterioplankton, and many bacterioplankton in Antarctica may be histidine auxotrophs. High LAP/BG ratios in Antarctica may be a result of this auxotrophy rather than nitrogen limitation. Recycling of nitrogen by micrograzers appears crucial to bacterial utilization of dissolved polysaccharides. Microbial communities are tightly coupled systems, in which bacterial physiology is regulated by the activities of other organisms. Isolation of the bacterial community by filtration causes rapid divergence from natural conditions.

The characteristics of, and spatial and temporal variations in, the activities of the ectoenzymes raise several further questions. Why do activities vary so much among different environments, and in particular, why is BGase activity so great near the equator? The ubiquity of these enzymes indicates that the flux of carbon and nitrogen from dissolved combined amino acids (DCAA) and dissolved polysaccharides (DPCHO) to heterotrophic bacteria via ectohydrolases is an important fraction of substrate supply. However, the quantitative importance of this fraction remains unknown, and depends on the effective substrate concentration, which is difficult to quantify. What is the effective substrate concentration and the rate of hydrolysis *in situ*? Why is the saturating substrate concentration for LAPase so much greater than substrate concentrations likely to be found in the ocean? What is the relationship of the flux of carbon and nitrogen to the cells via hydrolysis by these enzymes to bacterial growth requirements and to other sources of substrate? All of these questions are

interrelated and tied to the broader question of how expression of these enzymes is regulated by the composition of the medium.

## 6.2 WHY DOES LEUCINE AMINOPEPTIDASE HAVE SUCH A HIGH $K_m$, AND WHAT IS THE EFFECTIVE SUBSTRATE CONCENTRATION IN THE OCEAN?

The high $K_m$ for LAPase in planktonic bacteria is intriguing given the low concentrations of dissolved peptides and proteins in seawater (< 1 μM, e.g., Druffel et al. 1992). Maintaining high constitutive levels of an enzyme that consistently operates at only a small fraction of its capacity does not appear energetically optimal. The structure of the enzyme may prevent isozymes with a lower $K_m$ from evolving, or for reasons unrelated to energetics there may little selective advantage to such an adaptation. Also, a high $k_{cat}$ may permit high rates of hydrolysis at substrate concentrations much less than saturation. In experiments with natural water samples it is not possible to determine $k_{cat}$ and $E_o$ independently (only the product of the two, $V_{sat}$, is determined), so this possibility can not be excluded. The apparent half-saturation constants for solubilization of dissolved proteins are much lower than $K_m$ for LAPase (Hollibaugh and Azam 1983), suggesting that liberation of individual amino acid residues by terminal aminopeptidases (exohydrolases) may be the rate-limiting step in bacterial utilization of concentrated patches of proteinaceous matter (see also Kirchman and Hodson, 1984; Hoppe et al., 1988). This implies a flux of peptides of intermediate size to the surrounding medium (Azam and Cho 1987).

It is interesting that many free-living marine bacteria appear to have evolved along an opposite course with respect to the glucosidases: low $K_m$ and synthesis or

induction only in response to certain environmental conditions. BGases with much higher $K_m$ have been reported for eutrophic lakes (Chróst 1989), but $K_m$ values similar to those observed in oligotrophic oceanic waters (Chapter 2) have also been reported from eutrophic environments (Somville 1984). AGase and BGase may also operate at subsaturating substrate concentrations at most times. DPCHO concentrations are large enough to provide saturating substrate concentrations for these enzymes (Pakulski and Benner, 1994). However, little is known about the composition of DPCHO and much of it may not be hydrolyzable by these enzymes.

Many intracellular enzymes operate at subsaturating concentrations to facilitate feedback regulation, the rule of thumb being that $K_m \sim S$ (Somero, 1978). The environment of ectoenzymes and their apparent conservatism (i.e., the near constancy of $K_m$ over a wide range of environments) suggests very different selective pressures than intracellular enzymes adapted to a relatively constant substrate concentration. However, the periplasm of gram-negative bacteria is a poorly understood microenvironment, especially for natural populations. It is not clear how much more variable and less well regulated this microenvironment is than the cytoplasm itself. It is reasonable to postulate a regulatory role for ecto-aminopeptidase. If a high $K_m$ causes the flux of hydrolysis products (free amino acids, FAA) to the plasma membrane to vary according to the substrate concentration in the periplasm, the cell will have a mechanism by which it can "sense" fluctuations in the flux of DCAA to the cell surface.

It is generally accepted that nonregulatory enzymes operate near $V_{max}$ while regulatory enzymes operate near $V_{max}/2$ (Somero, 1978). The ecology and biochemistry of LAPase and BGase is consistent, at least in Antarctica, with a regulatory function for LAPase but not for BGase. LAPase appears to be expressed at a near constant level relative to biomass (Billen, 1991). BGase, however, is only

weakly correlated with LAPase, and so is hypothesized to be an induced rather than a constitutive enzyme. The seasonal variation in LAP/BG ratios suggests that a decreased flux of FAA to the cell via hydrolysis by periplasmic LAPase plays a role in inducing BGase activity.

## 6.3 WHAT IS THE EFFECTIVE SUBSTRATE CONCENTRATION *IN SITU* AND THE RELATIONSHIP OF ECTOENZYMATIC HYDROLYSIS TO BACTERIAL SUBSTRATE REQUIREMENTS?

The effective substrate concentration for exohydrolases is, by definition, less than the molar concentration of dissolved amino acid or sugar residues present in polymeric form (DCAA or DPCHO). If the mean length of peptide chains is y and the total DCAA is x, the concentration of N-terminal residues is x/y. The total DCAA concentration in seawater is normally much less than $K_m$ for hydrolysis of LLBN, and the effective substrate concentration is lower still. However, y may be small as much DCAA occurs in small peptides (<1000 daltons) in at least some marine environments (Coffin, 1989). In addition, not all of the DCAA in seawater occurs in chemical forms available to bacteria (Keil and Kirchman, 1993; Hubberten et al., 1995; Tanoue et al., 1995).

Estimates of *in situ* activities at Station ALOHA (Table 6.1) suggest that the fraction of substrate supply to the heterotrophic bacteria that is mediated by BGase and LAPase is fairly large at Station ALOHA. However, the bacterial production estimates for Station ALOHA are based on a single profile, and they are near the low end of the expected range for this environment (Cho and Azam, 1988; Ducklow and Carlson, 1992). In the equatorial Pacific, LAPase plays a minor role in both C and N flux, but

Table 6.1. Ectoenzyme activities in relation to bacterial production (BPRO) and carbon and nitrogen budgets for the equatorial Pacific and Station ALOHA. Station ALOHA bacterial production values from Karl, unpublished; equatorial values from Ducklow et al., 1995. The first set of equatorial values are for the El Niño cruise in March-April 1992, the second for October 1992. Ectoenzyme activities for October are estimated from activities in March-April extrapolated to 25°C using the temperature-activity relationships in Chapter 3. C and N from LAPase assume a mean of 4.5 C and 1.3 N atoms per amino acid residue; bacterial N production assumes a C:N of 5. Percent C and N are the percent of C and N requirements for growth accounted for by each ectoenzyme.

| | Equator | | Equator (25°C) | | ALOHA | |
|---|---|---|---|---|---|---|
| | LAP | BG | LAP | BG | LAP | BG |
| $V_{sat}$ (nmol/l/d) | 400 | 1500 | 170 | 297 | 160 | 0.75 |
| Estimated S (µM) | 0.5 | 10 | 0.5 | 10 | 0.5 | 10 |
| $K_m$ (µM) | 100 | 0.1 | 100 | 0.1 | 100 | 0.1 |
| V (µmol/l/d) | 0.002 | 1.49 | 0.0008 | 0.29 | 0.0008 | 0.0007 |
| V (µg C/l/d) | 0.11 | 107 | 0.046 | 21 | 0.043 | 0.054 |
| V (µg N/l/d) | 0.031 | | 0.013 | | 0.012 | |
| BPRO (µg C/l/d) | 3 | | 1.2 | | 0.12 | |
| BPRO (µg N/l/d) | 0.6 | | 0.24 | | 0.024 | |
| Percent C | 3.7 | 3566 | 3.8 | 1750 | 35.8 | 45.0 |
| Percent N | 5.2 | | 5.4 | | 50.0 | |

carbon flux via BGase greatly exceeds bacterial carbon requirements. This implies that growth efficiency is low for growth on carbohydrates. Bacterial carbon production was estimated using conversion factors of 1.15 cells (mol leucine)$^{-1}$ and 20 fg C cell$^{-1}$ (Ducklow et al., 1995). Carbon and nitrogen fluxes from LAPase was estimated from $V_{sat}$ assuming S = 0.5 µM, $K_m$ = 100 µM, and 4.5 C and 1.3 N atoms per amino acid residue. Carbon flux from BGase assumes that S = 10 µM and $K_m$ = 0.1 µM. These estimates assume effective substrate concentrations for LAPase and BGase similar to concentrations of DCAA and DPCHO in surface seawater (Druffel et al., 1992; Pakulski and Benner, 1994), but these may be greater than the concentrations of substrates hydrolyzable by these enzymes.

In Antarctica, the rates of hydrolysis of DCAA and DPCHO at substrate concentrations similar to ambient concentrations account for only a small fraction of bacterial carbon demand derived from $^3$H-leucine uptake. The slopes of the regression equations for estimated carbon flux to the cells via LAPase relative to total bacterial carbon production (Figure 6.1) provide an estimate of the fraction of carbon demand accounted for by this enzyme. LAPase accounts for approximately 8.5% and 7.5% for 1994 and 1995, respectively. BGase accounts for about half as much carbon as LAPase based on activities averaged over several cruises (see Chapter 3).

If it is assumed that the substrate concentration for hydrolysis of peptides by LAPase is approximately equal to the ambient DCAA concentration, the turnover time of the DCAA pool should be long (>1 year at Station ALOHA). This result is contradicted by several reports in the literature of turnover times for DCAA on the order of hours to weeks (Hollibaugh and Azam, 1983; Hoppe et al., 1988; Coffin, 1989; Keil and Kirchman, 1993). Results from a simple model using observed $V_{sat}$ and $K_m$ for LAPase in Antarctica predict little or no attenuation of DCAA concentration in

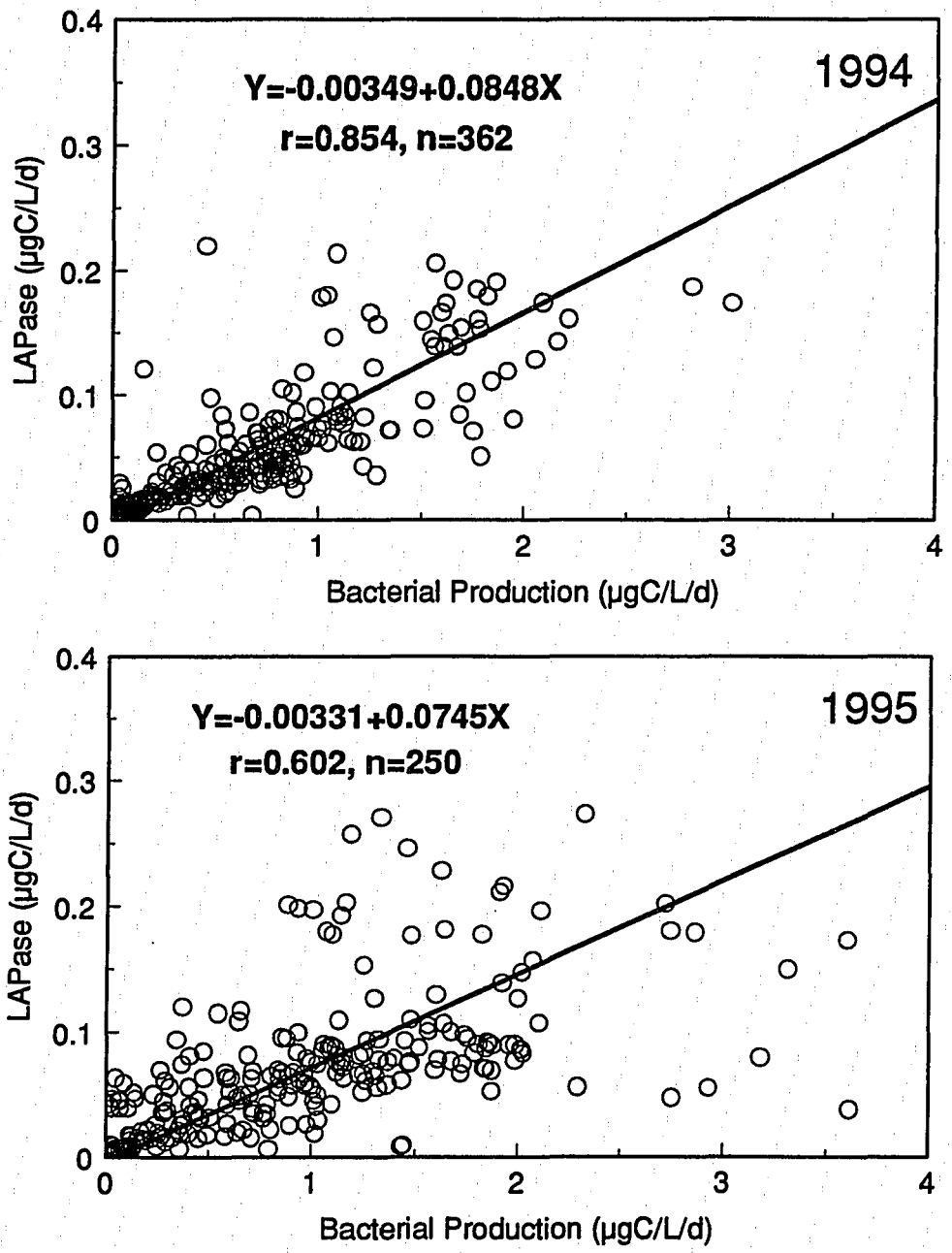


Figure 6.1. LAPase activity relative to bacterial production determined from assimilation of $^{3}$H-leucine, with Model II regression lines (geometric mean method). Data from Antarctica in January-February 1994 (top) and January-February 1995 (bottom); see text for conversion factors.

the post-bloom period, even in the absence of any further production, unless the effective substrate concentration is several times the ambient concentration (Figure 6.2 and Table 6.2). This result is independent of the initial concentration. The concentration factor (substrate concentration / ambient DCAA) required to produce a seasonal cycle in the model is similar to that required to account for bacterial carbon demand from LAPase (~10). DCAA can therefore account for <10% or as much as 80-90% of Antarctic bacterial carbon demand, but a reasonable case can be made for the latter.

The importance of ectoenzymes for bacterial nutrition is regionally variable. At Station ALOHA and at the equator, these enzymes can potentially provide a significant fraction of bacterial C requirements at ambient substrate concentration. However, estimates of ambient substrate concentration may be too high, and turnover times for DCAA are too long. In all of the environments sampled the effective substrate concentration for LAPase may be significantly greater than ambient DCAA. More thorough quantification of all of the carbon fluxes within the microbial food web, and especially rates of bacterial respiration (Jahnke and Craven, 1995), is required to resolve this question.

## 6.4 WHAT ARE THE REASONS FOR THE OBSERVED GEOGRAPHIC DISTRIBUTION OF ECTOENZYME ACTIVITIES?

A possible explanation for the extraordinarily high activity of BGase at the equator relates to the availability of the trace element activators of LAPase. Among the three metals expected to play this role in the ocean (Chapter 2), Co and Mn have surface-enriched profiles characteristic of a primarily aeolian source, whereas Zn has a

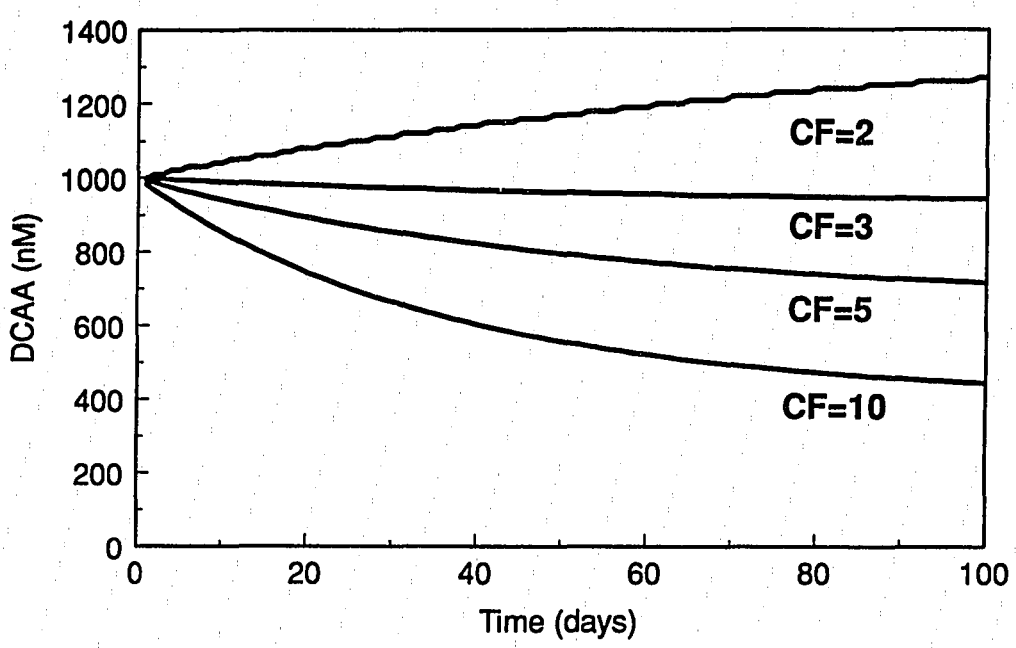


Figure 6.2. Model calculation of depletion of DCAA over 100 days with effective substrate concentration for LAPase equal to ambient DCAA multiplied by a "concentration factor" (CF). See Table 6.2 for model parameters.

Table 6.2. Coefficients used in model calculation of seasonal change in dissolved combined amino acids (DCAA). The concentration factor is the ratio of the effective substrate concentration for LAPase to the ambient DCAA concentration (non-dimensional). The last two (rates) decline linearly from initial ($T_o$) value to 50% of initial value over 100 days.

| | |
|---|---|
| Initial DCAA | 1 µM |
| Half-saturation coefficient | 100 µM |
| LAPase activity at saturation ($T_o$) | 230 nmol $l^{-1}$ $d^{-1}$ |
| Rate of production of DCAA ($T_o$) | 5 nmol $l^{-1}$ $d^{-1}$ |

nutrient-type profile suggesting that most input to surface waters comes from the pycnocline (Bruland, 1980; Landing and Bruland, 1980; Knauer et al., 1982). The flux of Zn to the mixed layer is therefore expected to be greater at the equator than at Station ALOHA, whereas the flux of Co and Mn would be significantly less (Duce and Tindale, 1991; Duce et al., 1991). Neritic Antarctic waters contain high concentrations of all three metals (Martin et al., 1990), while oceanic Antarctic waters are severely depleted in aeolian-source elements but should be fairly rich in zinc (Bruland 1980; Martin et al., 1990; Duce et al., 1991).

LAPase at Station ALOHA may be activated primarily by Mn. Although it is the weakest activator of the three, its concentration in the stratified waters of the north Pacific is much greater than those of Co and Zn. A hypothesis to explain the high BGase activities at the equator is as follows. The low aeolian flux of Co and Mn limits LAPase activity, and bacteria must therefore rely on carbohydrate as a carbon source to a greater extent. Because growth efficiency is low for growth on carbohydrate, rates of hydrolysis of dissolved polysaccharide must be much greater than the carbon requirement for bacterial growth (Table 6.1). The weakness in this hypothesis is that LAPase activity at the equator is not much lower than at Station ALOHA. However, it is significantly lower in relation to bacterial production (Table 6.1).

If trace element availability is a significant determinant of bacterial substrate selection, it is not necessarily the ectohydrolases themselves that are activated by the ions in question. Enzymes involved in biosynthesis may also be limited by the availability of necessary cofactors. However, given the distribution in the oceans of the ions activating LAPase, a plausible case can be made for trace element limitation of the activity of this enzyme in equatorial waters. It is difficult to imagine an element or elements activating the enzymes of amino acid biosynthesis whose distribution would

predict a greater tendency towards *de novo* synthesis in equatorial waters. Because our Antarctic study area includes neritic as well as oceanic waters, elements with both nutrient type (e.g., Ni, Zn) and aeolian-source type (e.g., Co, Fe, Mn) profiles are relatively abundant (Martin et al., 1990). If a trace element activator of amino acid biosynthesis had a primarily aeolian source, LAP/BG ratios would be expected to be less at Station ALOHA than at the equator, and least in neritic Antarctic waters. If it were a nutrient-type element, this ratio should be least in Antarctic waters. The geographic pattern of LAP/BG ratios is not consistent with the distribution of any conceivable activator of such pathways, but it is consistent with the known activators of LAPase. The coherence of this pattern with the distribution of Co, Mn and Zn fluxes is quite remarkable given that there are three elements involved, with quite different sources and distributions. Thus it is hypothesized that bacterial protein utilization in the ocean is limited by availability of the trace element activators of LAPase.

Another possible explanation for the equatorial BGase anomaly is that high rates of microzooplankton grazing on phytoplankton near the equator produce a flux of ammonium and other LMW nitrogen compounds to the bacteria that permits expression of pathways for *de novo* synthesis of amino acids that are not normally expressed by free-living bacterioplankton. Grazing rates are very high near the equator (Landry et al., 1995), but are also expected to be high at Station ALOHA, where daily primary production greatly exceeds the supply of "new" nitrogen (Dore, 1995; Karl et al., 1995). The role of compounds other than ammonium is largely unknown, but is potentially important if auxotrophy for particular amino acids or their biosynthetic precursors is widespread. The hypothesis that bacteria at the equator have evolved a more complete set of biosynthetic pathways than is usual among free-living

bacterioplankton is made more tenuous by the apparently low growth efficiencies estimated for growth on carbohydrate (Table 6.1).

The role of organic compounds excreted by microzooplankton in bacterial nutrition is a major unknown in aquatic microbial ecology, but the importance of this nutrient source appears to be great in oligotrophic waters (Hagström et al., 1988). It is important in this context to note that growth of microzooplankton should in general be limited by energy rather than nutrients. Microzooplankton excrete large amounts of nitrogen (Goldman and Caron, 1985); their high specific respiration rates and the low C:N ratio of their prey imply a continuous flux of N from the cells (Caron et al., 1990; Landry, 1993). It is unlikely that complete catabolism of amino acids or other LMW organic N compounds would occur in their feeding vacuoles given the low N demand. Energy yield per unit "investment" in catabolic enzymes should be greatest for polymers with repeating structures, such as peptidoglycan (González et al., 1993; Vrba et al., 1993). While microzooplankton probably make use of (cyano)bacterial protein as well, complete catabolism of amino acids is most likely for the more common amino acids such as glycine and glutamic acid. Maintaining enzymes for complete catabolism of the rarer amino acids does not appear energetically viable, but nonspecific enzymes may break down the basic amino acid skeleton, resulting in excretion of partial catabolism products.

Release of LMW organic N compounds by grazing microzooplankton is well documented (Antia et al., 1981; Andersson et al, 1985) but rates and chemical composition are poorly understood. A continuous flux of LMW organic N compounds to the heterotrophic bacteria in high turnover, rapid recycling communities implies a high probability of selection for salvage pathways utilizing these compounds, and little selective pressure for *de novo* synthesis where alternative precursors are available.

What this means for utilization of dissolved carbohydrate and for the biogeographic patterns described in Chapter 3 is less clear. Grazing could potentially relieve selective pressure for synthesis of protein from non-protein precursors, or alternatively make it possible for bacteria that would otherwise have auxotrophic amino acid requirements. In either case it probably can not explain the difference in BGase activity between the equatorial Pacific and the subtropical gyre, because the community at Station ALOHA is also a high turnover one.

## 6.5 WHAT DETERMINES THE COMPOSITION OF DISSOLVED ORGANIC MATTER IN THE OCEAN?

The composition of the DOM pool in the ocean is determined by the balance between production and decomposition of its various components. The high proportion of carbohydrates in this pool (Benner et al., 1992; Pakulski and Benner, 1994) is consistent with the low rate of turnover of this fraction estimated from hexosidase activities (Chapter 2). Within this pool the neutral sugars are likely to have shorter residence times than the acidic and amino sugars. It has long been known that the pool of organic substances that consists of LMW, readily assimilated substrates is small and rapidly cycled relative to the ambient DOM pool. It may be that there is also a component of the high molecular weight (HMW) pool that is rapidly turned over, while there are pools of both HMW and LMW compounds that are turned over slowly (Amon and Benner, 1994).

One hypothesis to explain the slow turnover of DPCHO is that many polysaccharides have immunological functions which preclude their utilization as nutrients by bacteria even after the death of the source organism (Rudbach and Baker,

1979; Garner et al., 1990). Lindberg (1990) hypothesized that the great diversity of sugars found in bacterial exopolysaccharides evolved as a defense against phage infection. The general concept extends to other organisms and other kinds of polysaccharides as well. However, diatom exopolysaccharide, or a large fraction of it, is apparently readily hydrolyzed by bacterial enzymes (Middelboe et al., 1995; Smith et al., 1995). There are also biochemically refractory proteins dissolved in seawater. The most abundant of these is a porin from gram-negative bacteria (Tanoue et al., 1995). A possible explanation for the persistence of this protein in the dissolved phase is the necessity of protecting it from the cell's own hydrolytic enzymes, which occur in close proximity to the outer membrane. This has important implications for the specificity of ecto-endohydrolases.

The concentration of a compound in seawater is a function of rates of both production and removal. If the rate of hydrolysis of a polymer is low, its steady state concentration may be great even if the rate of production is low. It is possible that heterotrophic bacteria, the primary consumers of DOM, could paradoxically be the source of most of the DOM in seawater (cf. Brophy and Carlson, 1989), with a smaller, rapidly cycled fraction being of phytoplankton origin. This is especially likely in oligotrophic waters where heterotrophic bacterial production may be 20-50% of primary production. The source of this DOM might be membrane or periplasmic structures that must necessarily exist in close association with hydrolytic enzymes, as suggested above for the porin described by Tanoue et al. (1995). Another potentially significant source is the glycocalyx. For example, a large fraction of endotoxic lipopolysaccharide, an ubiquitous and immunologically active component of the glycocalyx of gram-negative bacteria, occurs in the dissolved phase (Karl et al., unpublished data).

The energy that organisms expend on defenses against predators, parasites and pathogens is a necessary cost of survival and reproduction. Metazoans often create defensive structures of physically strong but biochemically simple polymers like chitin and keratin. Because they are composed of repeating units, these polymers are relatively easily hydrolyzed by enzymes, or rather enzymes hydrolyzing them are profitable for consumer organisms to evolve and express. Among microbes, recognition, attack and defense occur on a molecular level, and complexity of surface polymers is an important defense mechanism. What happens to such polymers in the detrital DOM and particulate organic matter (POM) pools is not clear, but it seems unlikely that maintaining enzymes for hydrolyzing rare linkages would be selectively advantageous for microheterotrophs. This applies both to heterotrophic bacteria and to microzooplankton, i.e., in the absence of enzymes capable of hydrolyzing them, polymers will survive ingestion by phagotrophs and be egested.

## 6.6 PROSPECTS FOR FUTURE RESEARCH ON BACTERIA AND ORGANIC MATTER IN THE OCEAN

There is much that remains unknown about the interaction of bacteria with DOM and POM. However, several general conclusions can be drawn from this work. The first is that a stoichiometric view of bacterioplankton physiology is inadequate. The fluxes of C and N to bacteria in different regions of the ocean may be similar, but the compounds comprising that flux are not (Chapter 3). Different compounds containing similar amounts of C and N affect bacterial expression of ectoenzymes very differently (Chapter 4). Enzymes hydrolyzing carbohydrates can be highly specific (Chapter 2) and the rates of hydrolysis of different polysaccharides vary significantly (Chapter 5).

Another important conclusion is that bacteria are part of a tightly coupled system and their physiology changes rapidly when they are isolated from it (Chapter 4). This has important implications for research in aquatic microbial ecology. Several groups of investigators have estimated rates of bacterial substrate utilization by measuring the change in dissolved substrate concentration in samples of seawater filtered through a 0.8 µM or 1.0 µM membrane filter to remove grazers (Kirchman et al., 1991; Rosenstock and Simon, 1993; Jørgensen et al., 1993). These results should be applied to natural mixed assemblages of microorganisms with great caution.

If feedback from the entire microbial community is an important determinant of bacterial species composition and physiology in the ocean, it is possible that many of the bacterial species that have not been cultured *can not* be grown in pure culture. The study of isolates has declined in importance in marine microbiology as it has become apparent that culturable strains represent only a small fraction of the bacteria present in seawater. However, the results of this research indicate that studies of pure cultures remain relevant in contemporary marine microbiology. The results of Kriss et al. (1963) on latitudinal trends in hydrolytic enzyme activities and of Ostroff and Henry (1939) on organic nitrogen requirements find strong parallels in these studies of mixed assemblages. In addition, the apparent evolutionary conservatism of ectoenzymes suggests that studies of isolates can provide useful information about the organisms and their enzymes. However, applying this information to mixed assemblages of microorganisms must still be done cautiously.

Marine microbial ecology remains a methods-limited science in many respects. There has been a tendency to focus on biomass and biomass production; there is much less information on the kinds of substrates that sustain natural populations in the ocean. Our inability to characterize much of the DOM in the ocean has encouraged a

"stoichiometric" as opposed to a "biochemical" approach. Uncertainty about specificity and about the suitability of natural DOM as substrate makes ectoenzyme activities uncertain predictors of C and N fluxes. However, they can provide an important complement to other relevant measurements, and provide information that is not provided by other commonly used methods. There are many fluxes within microbial food webs that are poorly constrained. Obtaining realistic estimates of rates requires both novel experimental approaches and statistical methods for analysis of complex systems where the precision and accuracy of different measurements is variable and often uncertain (Vézina and Platt, 1988; Karl, 1994).

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