ABSTRACT

Title of thesis: ECTOCELLULAR ENZYME ACTIVITIES IN THE MIXOTROPHIC DINOFLAGELLATE *PROROCENTRUM MINIMUM* (DINOPHYCEAE)

Monica Salerno, Master of Science, 2006

Thesis directed by: Professor Diane K. Stoecker
Horn Point Laboratory, UMCES

The activities of the enzymes α and β-glucosidase, leucine aminopeptidase and alkaline phosphatase were measured in cultures of the dinoflagellate *Prorocentrum minimum* (Pav.) J. Schiller, and in field samples collected during dinoflagellate blooms in tributaries of the Chesapeake Bay. Activities were measured using fluorogenic artificial substrates and partitioned among different size fractions. Little to no glucosidase activity was found associated with the dinoflagellate size fraction in either cultures or in field samples. A large fraction of the total aminopeptidase (LAP) activity in cultures was found in the dinoflagellate size fraction. LAP activity was positively correlated with dinoflagellate abundance and chl *a*, and negatively correlated with ammonium

concentration. Alkaline phosphatase exhibited a strong response to P-deficiency, increasing up to 330 times in P-depleted cells compared to P-replete conditions, and decreasing after addition of phosphate. *P. minimum* can make a substantial contribution to ectocellular hydrolytic activity in the water column during blooms.

ECTOCELLULAR ENZYME ACTIVITIES IN THE MIXOTROPHIC DINOFLAGELLATE *PROROCENTRUM MINIMUM* (DINOPHYCEAE)

by

Monica Salerno

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Advisory committee:

Dr. Diane K. Stoecker, Chair

Dr. D. Wayne Coats

Dr. Margaret R. Mulholland

Dr. Judy O'Neil

To Gio` and my parents

for their support, encouragement and love.

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CHAPTER 1

Introduction

The goal of my thesis was to quantify the activity of ectocellular enzymes in the bloom forming dinoflagellate *Prorocentrum minimum* (Pav.) J. Schiller. My research consisted of two parts: one conducted in the laboratory on cultures of *P. minimum*, and the other one on field assemblages. In the first part of my research I investigated the effect of *P. minimum* growth and various environmental parameters on enzyme activities. In the second part I used samples collected during dinoflagellate blooms in tributaries of Chesapeake Bay to determine if bloom forming dinoflagellates can contribute significantly to *in situ* ectoenzyme activities and to determine under what conditions dinoflagellate contribution could be important to total activities.

Importance of studying mixotrophic dinoflagellates, particularly *Prorocentrum minimum*

Mixotrophy is the combination of autotrophy with heterotrophy (Sanders 1991; Raven 1997; Stoecker 1998, 1999). Heterotrophy includes phagotrophy (Sanders 1991; Raven 1997; Stoecker 1998), and osmotrophy (Flynn and Butler 1986; Carlsson and Granéli 1998; Antia et al. 1991). Osmotrophic mixotrophy, also called photoheterotrophy that is the uptake of organic compounds, has been recognized in a number of algae (Droop 1974, Lewitus and Kana 1994, Doblin et al. 1999, Stolte et al. 2002).

Mixotrophic dinoflagellates are an important component of plankton assemblages in Chesapeake Bay and its tributaries (Bockstahler and Coats 1993a, b, Li et al. 1996, Smalley et al. 1999, Stoecker et al. 1997). They can form blooms and some are potentially toxic. Increases in the occurrence and persistence of harmful algal blooms, particularly dinoflagellate blooms, have been attributed to dissolved organic

nitrogen enrichment of estuarine waters in the mid-Atlantic region of United States (Glibert et al. 2001).

Among harmful algal bloom species, *P. minimum* has been largely studied because it has a pan-global distribution and its blooms appear to be undergoing a geographical expansion over the past several decades (reviewed in Heil et al. 2005). *P. minimum* is able to persist at high densities over changing environmental conditions and to survive and grow under low light and/or nutrient stress (Stoecker et al. 1997). In Chesapeake Bay, blooms of *P. minimum* usually occur with varying intensity in mid-salinity waters during spring resulting in areas of mahogany tides (reviewed in Tango et al. 2005). The high biomass of *Prorocentrum* in blooms may severely reduce the amount of oxygen resulting in local fish kills and loss of habitat (Heil et al. 2005, Tango et al. 2005). All these effects may produce local changes in the dynamics of the food web.

P. minimum has a range of physiological adaptations that make it responsive to eutrophication, including rapid growth, ability to use inorganic and organic nutrients, tolerance to a large range in irradiance, salinity, and temperatures (reviewed in Heil et al. 2005). Although *P. minimum* is known to be capable of using both inorganic and organic nutrient sources (reviewed in Heil et al. 2005), little information is available on its mixotrophic nutrition, including the range of dissolved organic compounds that it is able to consume, the regulation of osmotrophy and mixotrophy by environmental factors, and the role that nutritional mode plays in bloom dynamics (Heil et al. 2005).

Importance of enzymes

Enzymes are important in degrading dissolved organic matter (DOM), making low molecular weight compounds available for cells. The dissolved organic matter in aquatic environments is a complex mixture of compounds not totally characterized (Hansell and Carlson 2002). While inorganic nutrients, when present, are immediately available for phytoplankton, only a very small part of DOM is directly available to organisms (Chróst et al. 1989). The major part of DOM is composed of polymeric high-molecular-weight (HMW) compounds (Wheeler 1976). Although HMW DOM is not readily available for microorganisms because it can not be transported across the cytoplasm membrane (Nikaido and Vaara 1985, Antia et al. 1991), it is thought to be the more bioreactive fraction of the bulk DOM (Benner 2002). In order for HMW DOM to be biologically available, taken up and used by cells, it has to undergo preliminary transformation involving enzymatic depolymerization and hydrolysis (Chróst 1991). Since DOM often represents a significant portion of total dissolved nutrient (nitrogen and phosphorus) pool (Antia et al. 1991, Karl and Björkman 2002), its remineralization is a potentially important source of bioavailable nutrients for the entire microbial community. Because of the heterogeneity of DOM, a number of substrate-specific enzymes are necessary to remineralize DOM (Hoppe 1991).

Enzymes are classified based on their location (Fig.1.1). Endoenzymes are present inside the cells, exoenzymes are released by cells and occur free in the water or adsorbed to surfaces other than those of their producers. Ectoenzymes are bound to the cell surface or are present in the periplasmic space (Chróst 1991). Both exo- and ecto-enzymes are important in degrading dissolved organic matter in natural waters (Azam et al. 1983, Hollibaugh and Azam 1983, Hoppe 1983, Billen 1991, Chróst 1991, Boetius and Lochte 1994, Karner and Rassoulzadegan 1995, Martinez et al. 1996). Both types

of enzymes are able to catalyze the cleavage of covalent bonds. They can act at one end of polymers releasing monomeric products, or they can split bonds located in the interior of polymers releasing oligomers. Due to the location of ectoenzymes, monomers and oligomers released are concentrated near the cell surface and thus they may be preferentially taken up by cells. Since ectoenzymes can transform DOM producing more available compounds, they are important in influencing not only the growth of microorganisms (Azam et al. 1995), but also the chemical processing and biogeochemical cycling in the ocean (Azam et al. 1983, Chróst 1991, Martinez et al. 1996).

Enzyme activities in seawater are usually ascribed to bacterial activity, but recently the contribution of phytoplankton has been recognized (Sankievicz and Colepicolo 1999, Sala et al. 2001, Mulholland et al. 2002, Stoecker and Gustafson 2003, Dyhrman 2005). The utilization of organic compounds seems to be particularly important in ecology of mixotrophic bloom-forming species, including dinoflagellates. Organisms able to use DOM particularly as a source of nitrogen or phosphorous, may have a competitive advantage in organically enriched environments where dissolved inorganic nitrogen (DIN) or phosphorous are in short supply. Therefore, the ability of expressing enzyme activity could be an important factor controlling phytoplankton abundance and species composition. Ectoenzyme activity associated with phytoplankton may facilitate degradation of DOM and nutrient recycling during blooms. In fact, it is generally suspected that phytoplankton may be responsible for an important fraction of ectocellular enzymes particularly during blooms (Uchida 1992, Mulholland et al. 2002, Stoecker and Gustafson 2003).

It is also possible that extracellular hydrolysis has functions other than nutrition, such as defense against parasites, cell signaling, metabolic regulation, or toxin degradation and production in phytoplankton (Berges and Falkowski 1996, Wolfe 2000, Ryan et al. 2002). Ecto- and exo-enzymes and their products may also result in competition or mutualistic interactions between phytoplankton and bacteria.

Several types of ectocellular enzymes have been measured in seawater including α and β-glucosidase (Hoppe 1983, Vrba et al. 1993, Karner et al. 1994, Bochdansky et al. 1995), leucine aminopeptidase (Hoppe 1983, Hoppe et al. 1988, Karner and Rassoulzadegan 1995, Berges and Falkowski 1996, Lamy et al. 1999, Sala et al. 2001, Stoecker and Gustafson 2003), and alkaline phosphatase (Halemejko and Chróst 1984, Gage and Gorham 1985, Paasche and Erga 1988, Hernández et al. 1993, Nausch and Nausch 2000, Rengefors et al. 2001).

α and β-glucosidases

Carbohydrates constitute up to 35% of the dissolved organic carbon in most marine systems (Naush and Kerstan 2003). Polymeric carbohydrates can be degraded by α and β-glucosidases (Münster 1991, Vrba 1992, Hoppe et al. 1993). More than 95% of glucosidase activities have been found associated with the bacterial-size fraction both in seawater and freshwater (Hoppe 1983, Somville 1984, Chróst and Overbeck 1990). Polysaccharides represent the substrate of glucosidases (Fig. 1.2) that release monosaccharides. Glucosidases have been thought to be mainly bound to bacteria cell walls, however, dissolved enzymes can also play a significant role in hydrolysis of

dissolved organic carbon (DOC) (Azam and Smith 1991, Smith et al. 1992). No information on their production (or lack of) by phytoplankton species is available.

Leucine aminopeptidase

In many temperate and polar coastal water ecosystems, primary production by phytoplankton is limited by nitrogen (N). N is also the most important nutrient that limits primary production at the interface between marine and freshwater habitats (Anderson et al. 2002). Besides new inorganic nitrogen (i.e. NO_3), different forms of recycled N including ammonium, urea and amino acids, may be important sources of N for microorganisms. Bacteria are thought to be the major consumers of dissolved organic nitrogen (DON) since they typically have high uptake rates (Wheeler and Kirchman 1986). Several authors have studied the utilization of DON by bacteria particularly in coastal and estuarine ecosystems, and they estimate that DON accounts for about 50% of bacterial N demand (Keil and Kirchman 1991, 1993, Middelboe et al. 1995) and 25% of bacterial C demand (Middelboe et al. 1995). In addition to bacteria, a variety of phytoplankton species can use DON to supplement their nutrition (Paerl 1988, Berg et al. 1997, Lewitus et al. 1999, Glibert et al. 2001, Mulholland et al. 2003). In particular, some phytoplankton species are known to use urea (Glibert et al. 2001) or dissolved free amino acids (DFAA) (Wheeler et al. 1974, John and Flynn 1999) as N sources. However concentrations of DFAA are fairly low, even if their turnover can be high (Keil and Kirchman 1991, Middelboe et al. 1995).

Dissolved combined amino acids (DCAA) are present in relatively high concentrations, and in estuarine systems they may account for up to 13-20% of total

DON (Keil and Kirchman 1991, Mulholland et al. 2003). DCAA include peptides and proteins that are too large for direct uptake by phytoplankton cells, therefore they require enzymatic cleavage before they can be assimilated. One process involved in degradation of large amino acid chains, peptide hydrolysis, has been reported for both oceanic (Hollibaugh and Azam 1983, Keil and Kirchman 1992, Taylor 1995) and coastal systems (Hoppe 1983, 1991, Pantoja and Lee 1999). This reaction can produce free amino acids and smaller peptides. Extracellular amino acid oxidation can further degrade amino acids and can liberate ammonium (Palenik and Morel 1990a, 1990b, Pantoja and Lee 1994, Mulholland et al. 1998, 2002).

Among proteases, leucine amino peptidase (LAP) is thought to produce small peptides and amino acids from proteins and polypeptides (Fig. 1.3) in the ocean and coastal systems (Chróst 1991), acting at the termini of a broad spectrum of substrates with a free amino group with preference for N-terminal leucine and related amino acids (Mahler and Cordes 1966). Both in fresh and seawater ectoproteolytic activity has been attributed primarily to heterotrophic bacteria (Rosso and Azam 1987, Sinsabaugh et al. 1997) but recently the contribution of phytoplankton to protease activity has been considered (Sala et al. 2001) and ectoproteolytic activity has been found in a number of dinoflagellate species including *P. minimum* (Sankievicz and Colepicolo 1999, Stoecker and Gustafson 2003).

Alkaline phosphatase

Phosphorus (P) is generally considered the most important nutrient that limits the growth of photosynthetic organisms in freshwaters (Schindler 1977). However,

recent studies demonstrated that phosphorous may be limiting not only in freshwater systems, but also estuarine and marine environments, particularly oligohaline regions of estuaries (Perry 1976, Smith 1984). In some areas, a seasonal phosphorous limitation of phytoplankton growth rate occurs as demonstrated for a number of estuarine and coastal systems including Chesapeake Bay (Fisher et al. 1992), Neuse River estuary (NC) (Rudek et al. 1991), and Chinese coastal waters (Harrison et al. 1990). Recently many authors have reported P limitation in oceanic regions including subtropical Pacific, Sargasso Sea, western tropical and subtropical Atlantic, northern and eastern Atlantic (Gruber and Sarmiento 1997, Guildford and Hecky 2000, Wu et al. 2000, Karl et al. 2001, Dyhrman et al. 2002, Ammerman et al. 2003, Sundareshwar et al. 2003), northeastern margin of the Gulf of Mexico (Smith 1984), Scandinavian fjords (Myklestad and Sakshaug 1983), and the Mediterranean Sea (Krom et al 1991, Thingstad et al. 1998, 2005, Zohary and Robarts 1998). Although dissolved inorganic phosphorus (DIP) (i.e. orthophosphate) is thought to be the most important source of P for phytoplankton, a number of phytoplanktonic species are known to be able to produce alkaline phosphatase in order to hydrolyze dissolved organic phosphorus (DOP) and thus use organic phosphorus compounds as P sources when DIN limited (Rivkin and Swift 1979, Cembella et al. 1984a, 1984b, Sakshaug et al. 1984, Boni et al. 1989, González-Gil et al. 1998, Yamaguchi et al. 2005). In surface coastal waters the concentration of dissolved organic phosphorus (DOP) is often higher than the concentration of dissolved inorganic phosphorus (DIP) (Kobori and Taga 1979, Hernández et al. 2000, Yamamoto et al. 2002, Yamaguchi et al. 2004a). Therefore, organic phosphorus may represent a significant portion of algal-available phosphorus and may be an important alternative source of phosphorus for phytoplankton (Malone et

al. 1996). Among DOP compounds, phosphomonoesters seem to be selectively remineralized (Clark et al. 1998), therefore phosphomoesterases can be important in DOP cycling (Karl and Björkman 2002).

Alkaline phosphatase (AP) is a cell-surface enzyme that hydrolyzes organic phosphorus compounds (Fig. 1.4) at alkaline pH, typically 7.2-9.8 (Kuenzler and Perras 1965). This enzyme acts on phosphate esters such as sugar phosphates, phospholipids, and other phosphomonoesters, releasing orthophosphate groups that may be taken up by cells and used as phosphorous supply. Most of AP activity in coastal waters is thought to be produced primarily by algae (Ammerman 1990, Yamaguchi et al. 2004b), and AP activity has been reported for a number of dinoflagellate species (Rivkin and Swift 1979, Boni et al. 1989, González-Gil et al. 1998, Rengefors et al. 2001, Ho et al. 2002, Yamaguchi et al. 2005) including *P. minimum* (Sakshaug et al. 1984, Dyhrman and Palenik 1997, 1999, 2001).

Goals of this thesis

The extent to which DOM contributes to energy or nutritional demands for phytoplankton growth has not been broadly assessed. However, it appears clear that the key to understand the utilization of DOM by microbes including phytoplankton, is the study of ability to produce enzymes that are able to degrade HMW compounds and their regulation by biotic and abiotic factors.

Until this study, little is known about the ability (or lack of ability) of *P. minimum* or other dinoflagellates to express α and β-glucosidase activity. Although *P. minimum* is known to produce LAP and AP, the effect of nutrients, growth, and

environmental parameters on LAP activity was largely unknown, and the effects of physiological and environmental parameters on AP activity, besides P stress conditions, had not been assessed.

In this study I used *P. minimum* cultures to investigate ectocellular activity of α and β glucosidase (Chapter 2), leucine aminopeptidase (Chapter 2), and alkaline phosphatase (Chapter 3), in order to compare their relative activities and regulation by inorganic nutrients, irradiance, diel cellular rhythms, and growth phase. In field samples collected from tributaries of the Chesapeake Bay (MD, USA) during dinoflagellates blooms, I investigated the partitioning of the same enzyme activities into different size fractions to determine if the size fraction containing dinoflagellate was a significant contributor to bulk enzyme activities, particularly during blooms.

Fig. 1.1 Classification of enzymes.

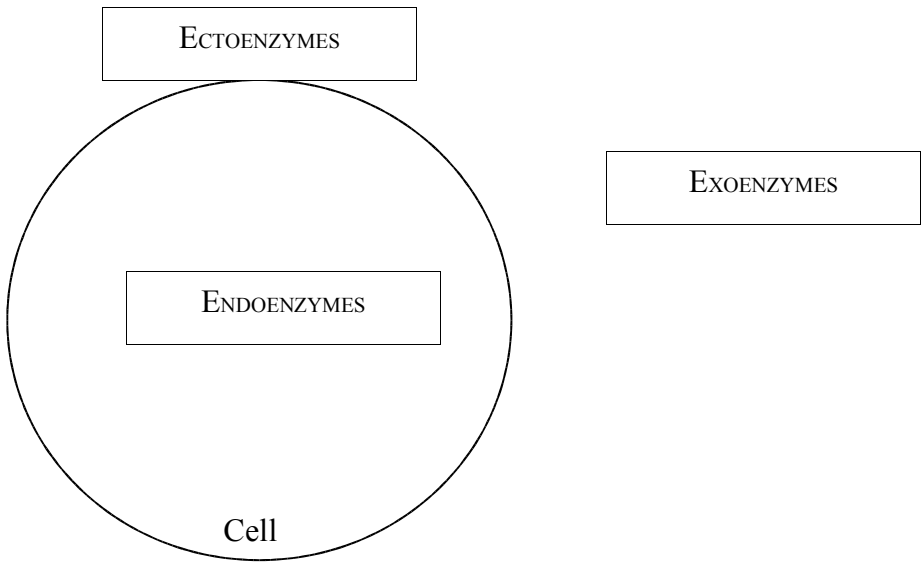


Fig. 1.2. Diagram of conceptual model of α and β-glucosidase (α/β Glc) reaction. Thick solid lines represent enzyme-mediated transformations of compounds (yellow boxes).

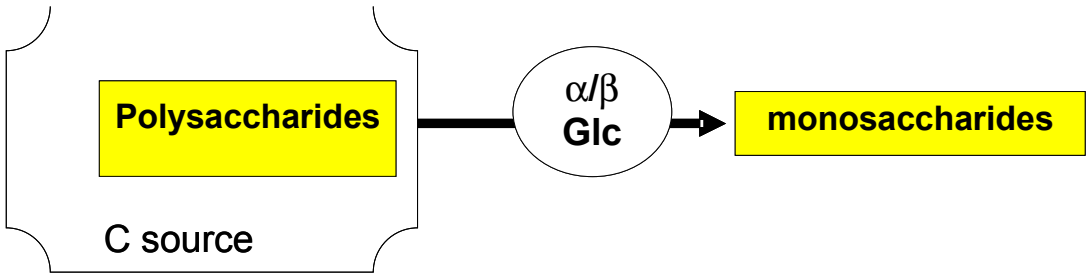


Fig. 1.3. Diagram of conceptual model of leucine aminopeptidase (LAP) reaction. Thick solid lines represent enzyme-mediated transformations of compounds (yellow boxes).

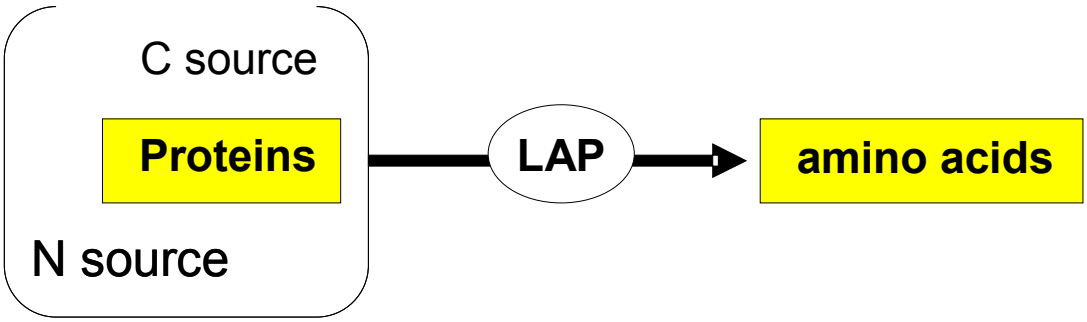
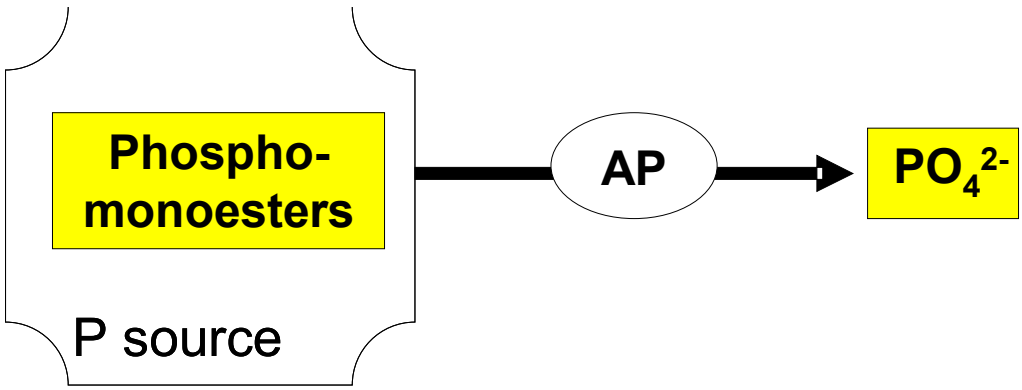


Fig. 1.4. Diagram of conceptual model of alkaline phosphatase (AP) reaction. Thick solid lines represent enzyme-mediated transformations of compounds (yellow boxes).



CHAPTER 2

Ectocellular α– and β–glucosidase and proteolytic activity of the mixotrophic dinoflagellate *Prorocentrum minimum* (Dinophyceae)

ABSTRACT

The activities of the enzymes α and β-glucosidases, and aminopeptidase were measured in cultures of the dinoflagellate *Prorocentrum minimum* (Pav.) J. Schiller, and in field samples collected during dinoflagellate blooms occurring in tributaries of the Chesapeake Bay. Activities were measured using fluorogenic artificial substrates and partitioned among the >5 μm size fraction containing dinoflagellates, small microbes fraction (0.1-5 μm) and dissolved phase (<0.1 μm). Little to no glucosidase activity was found associated with the dinoflagellate size fraction in either cultures nor in field samples, with most of the activity (67 to 92.7% in cultures, 54 to 100% in field samples) in the small microbes size fraction for both α and β glucosidase. In contrast, 67 to 90% of the total aminopeptidase (LAP) activity in cultures was found in the dinoflagellate size fraction indicating that *P. minimum* can contribute to protein and polypeptide degradation through enzyme-catalyzed hydrolysis. LAP activity in the dinoflagellate size fraction decreased in response to ammonia and urea additions, but not by nitrate. In field samples, LAP activity was positively correlated with dinoflagellate abundance and chl *a*, and negatively correlated with ammonium concentration. Dinoflagellate density explained 50% of the variability of LAP activity in the >5 μm fraction and ectoproteolytic activity associated with that fraction explained an important part (up to 52%) of variability of total *in situ* LAP activity. Based on these estimates I conclude that *P. minimum* can make a substantial contribution to ectocellular hydrolytic activity in the water column during blooms.

INTRODUCTION

The dissolved organic matter (DOM) in aquatic environments is a complex mixture of compounds that are largely uncharacterized (Hansell and Carlson 2002). While inorganic nutrients, when present, are immediately available for phytoplankton, only a very small part of DOM is readily available for organisms (Chróst et al. 1989). The major part of DOM is composed of polymeric high-molecular-weight compounds (Wheeler 1976) that can not be transported across the cytoplasm membrane (Nikaido and Vaara 1985, Antia et al. 1991). In order for polymeric substances to be biologically available, taken up and used by cells, they have to be broken down throughout enzymatic depolymerization and hydrolysis (Chróst 1991).

Exoenzymes are released by certain cells and can occur free in the water or adsorbed to surfaces other than those of their producers. Ectoenzymes are bound to the cell surface or are present in the periplasmic space (Chróst 1991). Both exo- and ecto-enzymes are important in degrading dissolved organic matter because they are able to catalyze the cleavage of covalent bonds (Azam et al. 1983, Chróst 1991, Martinez et al. 1996). Due to the location of ectoenzymes, monomers and oligomers released are concentrated near the cell surface and thus they may be preferentially taken up by cells. Exo- and ecto-enzyme activities (glucosidase, protease) are important in hydrolysis of specific dissolved organic compounds and decomposition of particulate organic matter in natural waters (Hollibaugh and Azam 1983, Hoppe 1983, Billen 1991, Boetius and Lochte 1994, 1996, Karner and Rassoulzadegan 1995, Martinez et al. 1996). Since dissolved organic nitrogen and phosphorus often comprise a significant fraction of the total dissolved nitrogen and phosphorus in marine systems (Antia et al. 1991, Karl and

Björkman 2002), their regeneration is a potentially important source of bioavailable organic and inorganic nutrients.

Several types of ecto- or exo-cellular enzymes have been measured in seawater including α and β-glucosidase (Hoppe 1983, Vrba et al. 1993, Karner et al. 1994, Bochdansky et al. 1995), and leucine aminopeptidase (Hoppe 1983, Hoppe et al. 1988, Karner and Rassoulzadegan 1995, Berges and Falkowski 1996, Lamy et al. 1999, Sala et al. 2001, Stoecker and Gustafson 2003). α and β glucosidases are broad-specificity enzymes that catalyze the hydrolysis of α- and β-glucosidic bonds. Carbohydrates constitute 10-25 % of the dissolved organic carbon (Benner 2002) in most marine systems. The activity of glucosidases is often used to describe the degradation of polymeric carbohydrates (Münster 1991, Vrba 1992, Hoppe et al. 1993). More than 95% of glucosidase activities have been found associated with the bacterial-size fraction both in seawater and freshwater (Hoppe 1983, Somville 1984, Chróst and Overbeck 1990). Even if glucosidase activities have been thought to be mainly bound to bacteria cell walls, dissolved enzymes are thought to play a significant role in hydrolysis of organic matter (Azam and Smith 1991, Smith et al. 1992). Although α and β glucosidases are commonly measured in water column, no information are available on their production (or lack of) by dinoflagellates.

Leucine amino peptidase (LAP) is thought to produce small peptides and amino acids from proteins and polypeptides in the ocean and coastal systems (Chróst 1991), acting at the termini of a broad spectrum of substrates with a free amino group with preference for N-terminal leucine and related amino acids (Mahler and Cordes 1966). Amino acids, peptides and proteins are a major potentially labile reservoir of organic

nitrogen in marine systems (Pantoja et al. 1997). Both in fresh and seawater ectoproteolytic activity has been attributed primarily to heterotrophic bacteria (Hoppe 1983, Rosso and Azam 1987, Hoppe et al. 1993, Sinsabaugh et al. 1997) but recently the contribution of phytoplankton to protease activity has been considered (Sala et al. 2001) and ectoproteolytic activity has been found in a number of dinoflagellate species (Sankievicz and Colepicolo 1999, Mulholland et al. 2002, Stoecker and Gustafson 2003). Although LAP activity has been reported in phytoplankton including *Prorocentrum minimum* (Pav.) J. Schiller, and other dinoflagellates, and has been demonstrated in axenic cultures of *P. minimum*, no data are available on regulation of its activity with respect to environmental variables.

Hydrolytic enzyme activity may play a role in nutrition of mixotrophic dinoflagellates because species able to use DOM, particularly as source of nitrogen or phosphorus, may have a competitive advantage in organically enriched environments where dissolved inorganic nitrogen or phosphorus are in short supply. Nitrogen is usually considered the major limiting nutrient for primary production and growth of phytoplankton in marine systems, even if recent studies demonstrated that phosphorus may be limiting not only in lakes, but also in marine systems including the subtropical Pacific, Sargasso Sea, western tropical and subtropical Atlantic, eastern Atlantic and Mediterranean Sea (Ammerman et al. 2003, Sundareshwar et al. 2003, Thingstad et al. 2005).

Degradation of proteins by peptidases may be important not only for the supply of nitrogen, but also to provide carbon (Nausch and Nausch 2000). During intense blooms, particularly in less saline waters or during summers with high insolation and calm weather that favor high pH conditions, dissolved inorganic carbon (DIC) can

become limiting to photosynthesis (Loftus et al. 1979, Hansen 2002). Numerous studies have shown stimulation of phytoplankton growth by low-molecular-weight organic compounds (Lewitus and Kana 1994) and inhibition of peptidase activity by glucose (Chróst 1991, Boetius and Lochte 1996).

Ectoenzyme activity associated with phytoplankton may enhance degradation of DOM and nutrient recycling during blooms. The capability of expressing enzyme activity could be an important factor controlling phytoplankton abundance and species composition in oligohaline waters. Ectocellular enzyme activities may be controlled not only by nutritional status of dinoflagellate cells but also by chemical or physical environmental parameters. However, since there is very limited information on the capability of phytoplankton to make ectoenzymes and their regulation, the potential importance of osmotrophy (uptake of DOM) for growth and nutrition of phytoplankton can not be evaluated.

It is also possible that extracellular hydrolysis of DOM has functions other than nutrition, such as defense against parasites, cell signaling, metabolic regulation or toxin degradation and production in phytoplankton (Berges and Falkowski 1996, Wolfe 2000, Ryan et al. 2002). Ecto- and exo-enzymes and their products may also result in competition or mutualistic interaction between phytoplankton and bacteria. These phenomena are not well investigated.

Most studies of ectoenzymes have focused on heterotrophic bacteria and not phytoplankton, consequently the contribution of autotrophic and mixotrophic phytoplankton to ectoenzyme activity is not well known. Moreover, although some phytoplankton species are known to have exo- or ecto-cellular hydrolytic activity, only few quantitative measurements of activity per cell are available (Berges and Falkowski

1996, Mulholland et al. 2003, Stoecker and Gustafson 2003). The capability of phytoplankton to hydrolyze polymeric compounds (carbohydrates, polypeptides and proteins, organic phosphate molecules) can be assessed through measurement of enzymatic activities. Ectocellular activity of three enzymes, α and β glucosidase, and leucine aminopeptidase was measured using cultured *Prorocentrum minimum*, in order to compare their relative activities and regulation by inorganic nutrients, irradiance, diel cycle, and growth phase. In field samples, the partitioning of enzyme activities into different size fractions was investigated to determine if the size fraction containing dinoflagellates made a significant contribution to bulk enzyme activities.

MATERIALS AND METHODS

Protist cultures. A non-axenic culture of *P. minimum* (D-5) isolated by Matthew D. Johnson from the Choptank River (Cambridge, MD, USA), a tributary of the Chesapeake Bay and maintained in culture for several years was used for all experiments. The culture was maintained in f/2-Si medium (Guillard 1975), at salinity 15, at a temperature of 15° C, with a 14:10-h light:dark photocycle under cool white fluorescent bulbs at 60 μmol photons·m^{-2}·s^{-1}. Media were prepared using water collected from Indian River filtered onto Whatman GF/F glass microfibre filters (Whatman International Ltd. Maidston, England) and autoclaved.

Parameters measured. For *P. minimum* cell enumeration, 1 to 5 mL samples, depending on cell densities, were fixed with glutaraldehyde (final concentration 1%) and filtered onto 2 μm pore size black polycarbonate filters (Porerics Corporation, Livermore, CA, USA). Filters were mounted on glass slides with immersion oil and

capped with coverslips to be ready for epifluorescence microscopy. Slides were stored frozen at -20° C and were subsequently examined with standard epifluorescence microscopy techniques (Gifford and Caron 2000) and enumerated at 400 x.

For enumeration of bacteria, samples were fixed in 2% glutaraldehyde (final concentration), stained with 4,6-diamidinophenylindole (DAPI, Sigma Inc., St. Louis, MO, USA), filtered onto 0.2 μm polycarbonate filters (Poretics Corporation, Livermore, CA, USA), and counted with epifluorescence microscopy (Nikon Eclipse E800, 1000 x) (Porter and Feig 1980). Since bacteria can be attached to particles, they were also enumerated on the 5 μm polycarbonate filters used for size fractionation. This was to ensure that attached bacteria were not common in the dinoflagellate size fraction.

Chlorophyll *a* content was determined by the standard fluorometric method (Parsons et al. 1984). Three replicates were used for each sample. 2.5 to 10 mL aliquots of cultures were collected by vacuum filtration onto Whatman GF/F glass microfibre filters (Whatman International Ltd. Maidston, England) and were immediately extracted or stored at -20° C for a later analysis. Extraction was performed in the dark in 90% acetone for 24 h at 4° C, and chlorophyll *a* concentration was determined using a Turner Design Model 10 fluorometer calibrated with pure Chl *a* against a Beckman model DK-22 spectrophotometer.

Dissolved inorganic nutrients (N, P) were measured following the standard procedure described by Grasshoff et al. (1983). To determine cellular nutrient ratios as indicators of N and P limitation, cells were collected at the beginning of the stationary phase and filtered onto precombusted Whatman GF/F glass microfibre filters (Whatman International Ltd. Maidston, England) and analyzed for CHN and particulate phosphorus. Particulate C, H, and N were determined by high temperature combustion

in an Exeter Analytical Model CE400 Elemental Analyzer and particulate phosphorus analysis was based on Andersen (1976).

Enzyme activities in different size-fractions were compared. To determine how the activities were distributed among different size fractions subsamples were divided into whole and filtered samples. Activities were measured in unfractioned samples (total activity), in samples gently filtered through a 5 μm pore size polycarbonate filters (Whatman Nuclepore®, Clifton, NJ, USA) and in samples gently filtered through 0.1 μm pore size polycarbonate filters (Whatman Nuclepore®, Clifton, NJ, USA). The 5 μm pore size was used so that the dinoflagellate cells would be retained but most bacteria, including small bacterial aggregates, would be in the filtrate. Activity associated with dinoflagellate fraction was calculated by difference between activity in unfiltered samples and activity in <5 μm. This filtration procedure was chosen because it was successfully used previously in a study demonstrating that dinoflagellate cell specific LAP activity in the >5 μm fraction of non-axenic cultures was similar to activity of axenic cultures (Stoecker and Gustafson 2003). Bacterial ectocellular enzyme activity was calculated as the difference between activity in the <5 μm and 0.1 μm fraction. Exocellular enzyme activity was calculated as the activity in the <0.1 μm fraction (cell free fraction). Herein, cell specific and chlorophyll *a* specific activities are calculated based on activity in dinoflagellate fraction (>5 μm).

Enzyme activities were measured following procedures based on Hoppe (1983) and Hoppe et al. (1988). The fluorogenic artificial substrate analogs, 4-methylumbelliferyl-α-D-glucoside (α-MUF), 4-methylumbelliferyl-β-D-glucopyranoside (β-MUF), and L-leucine 7-amido-4-methyl-coumarin (Leu-AMC)

(Sigma Chemical, St. Louis, MO, USA) were used for α-glucosidase (α-Glc), β-glucosidase (β-Glc), and leucine aminopeptidase (LAP) assays respectively. Substrate stock solutions were prepared dissolving α-MUF and β-MUF in 2-ethosyethanol (Methylcellosolve – Sigma Chemical, St. Louis, MO, USA) (1 mM final concentration) and Leu-AMC in 2-ethosyethanol (50%) and distilled water (50%) (5 mM final concentration). Substrate stock solutions were stored frozen until use.

Preliminary experiments were conducted to determine activity as a function of substrate concentrations. To verify that enzyme activities increase linearly with time a time course experiment was run: enzyme activity was measured every 1 h for 4 hours. Based on these data, the following protocols were adopted. Aliquots of substrate stock solutions, 20 μL of α-MUF, β-MUF, and Leu-AMC were added to 1 mL of culture in semi-UV cuvettes (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA), resulting in a final concentrations of 19.6 μM α-MUF and β-MUF, and 98 μM Leu-AMC, concentrations that were shown to be saturating. Michaelis-Menten uptake kinetics was assumed. Samples were incubated in the dark at 15° C for 30 min. At the end of the incubation sample fluorescence was immediately read on a Shimadzu Rf-5301PC spectrofluorometer (excitation 380 nm with a 10 nm slit, emission 440 nm with a 1.5 nm slit width for LAP, and excitation 455 nm, emission 365 nm for α-Glc, β-Glc). Increase in fluorescence is related to the release of the fluorescent part of the substrate analogs (MUF or MCA) via enzyme-catalyzed hydrolysis, therefore enzyme hydrolysis of substrates will be referred to as enzyme activities. Values of the half-saturation constant (K_s) and maximum rate of hydrolysis (V_m) were calculated as the mean of values estimated from linear fits to a Lineweaver-Burk and Eadie-Hofstee plots.

Two types of controls were used. The first type was samples with no added substrate, to control for natural fluorescence in the culture. The second control was autoclaved media with added substrate which was incubated along with the experimental samples in order to control for fluorescence due to the substrate or to bacterial contamination of the substrate that could result in product formation. For each assay, triplicate cuvettes were incubated.

Every time the enzyme assays were performed a standard curve was prepared in order to convert increases in fluorescence to hydrolysis rates. Autoclaved filtered (0.1 μm) media and concentration of products (7-amino-4-methyl- coumarin (AMC) (Sigma Chemical, St. Louis, MO, USA) for LAP and 4-methylumbelliferone (MUF) (Sigma Chemical, St. Louis, MO, USA) for glucosidases), ranging from 0 to 10 μM were used to prepare the standard curves.

To test if exposure to the substrate causes the dinoflagellates to lyse, releasing intracellular enzymes, samples of *P. minimum* were fixed with glutaraldehyde before and after 30 min exposure to substrate concentration equal to 1, 2 and 3 times the concentration used for enzyme assays, and checked with microscopy.

All statistical analyses were done with Analyse-it™ Version 1.71 (Analyse-it Software, Ltd.). Parametric tests were used unless data failed tests for homogeneity of variance and normality, in these cases, non-parametric tests were used.

Time course experiments. Time course experiments were run to determine whether enzyme activities depended on growth phase and nutrient concentrations. Cultures were grown at 15° C with a 14:10-h light: dark cycle under cool white fluorescent bulbs at 60 μmol photons·m^{-2}·s^{-1}. Cells in stationary phase were used as an inoculum to provide an initial cell concentration of 2000 cells·mL^{-1}. The four nutrient

concentration treatments consisted of f/2 (883 µM NO_3^{2-} and 36 µM PO_4^{2-}), f/4 (441 µM NO_3^{2-} and 18 µM PO_4^{3-}), f/2-lowN (36 µM PO_4^{3-} and 5.5 µM NO_3^{2-}), and f/2-lowP (883 µM NO_3^{2-} and 0.5µM PO_4^{3-}). Since local water was used to prepare media, Inorganic nutrient additions were on top of background inorganic concentrations (1.27 µM NO_3+NO_2, 2.88 µM NH_4, PO_4 not available). Triplicates were used for each treatment. Sampling occurred every other day for 3 to 4 weeks, until cells reached the stationary phase.

Nutrient addition experiments. A short-term experiment was run to measure the effect of nutrient additions on LAP activity. Treatments in triplicate consisted of additions of NO_3^{2-} (800 µM), NH_4^+ (100 µM), and urea (500 µM), and control without additions. Nutrients were added in a single input. Enzyme activities were measured at the beginning of experiment and 24 h after the additions. Experiment was carried out at 15° C with a 14:10-h light:dark cycle under cool white fluorescent bulbs at 60 µmol photons·m^{-2}·s^{-1}.

Effect of irradiance on LAP activity. To examine effects of light level on LAP activity triplicate *P. minimum* cultures were allowed to acclimate for 10 days to a 12:12-h light:dark cycle at the following irradiances: 4, 53 and 267 µmol photons·m^{-2}·s^{-1} in f/2 medium. The incubation temperature was 15° C. At the end of the acclimation period, LAP activity was measured.

Diurnal cycle experiment. Diurnal variations of LAP were assessed at 4-h intervals over two 24-h periods in *P. minimum* cultures growing in f/2, at 15° C, with a 12:12-h light:dark cycle under cool white fluorescent bulbs at 60 µmol photons·m^{-2}·s^{-1}.

Field experiments. Sampling occurred during dinoflagellate blooms in spring and fall 2003 in tributaries of the Chesapeake Bay. Samples were collected from the

Choptank River on March 19, April 1 and 14, and May 14; in Patuxent River on April 3, and in Corsica River on October 27. On the Corsica River, samples were collected from Gunston Day School dock (Station A) (Centreville, MD, USA) and at the Centreville public dock (Station B). Sampling stations are shown in Fig. 2.1. *In situ* temperature and salinity were measured with a portable YSI SCT meter. Samples were collected at surface and 1 m above the bottom (except on May and October when only surface samples were taken). Samples were filtered through a Nitex mesh to obtain the <200 μm fraction (containing phytoplankton, microzooplankton and bacteria), and another subsample was gently filtered through 5 μm and 0.1 μm pore size polycarbonate filters (Whatman Nuclopore®, Clifton, NJ, USA) to obtain small microbe fraction (containing bacteria and small protist species but not dinoflagellates) and free dissolved phase respectively. Total and fractionated LAP activities were measured for all samples, while α and β glucosidases were measured only in samples collected in Corsica River. Dinoflagellates were counted in samples fixed in glutaraldehyde (final concentration 1%). Chl *a* and nutrient analyses were also performed.

RESULTS

Laboratory experiments:

Time course experiments with inorganic nutrient treatments. Cell densities and Chl *a* during the *P. minimum* growth experiment are shown in Fig. 2.2 and values for stationary phase are summarized in Table 2.1. P-deficiency greatly affected the growth of *P. minimum*. Cell densities at stationary phase in f/4 and N-deficiency was not significantly different from that in f/2, while in the f/2-lowP treatment, cell density was

significantly different (reduction of 30.8%; 1-way ANOVA, $p<0.0001$). P deficiency did not affect chlorophyll *a* content while N deficiency resulted in a significant difference (reduction of 71%; 1-way ANOVA, $p<0.0001$). Content of C and N (Table 2.1) indicate that *P. minimum* cells are smaller in biomass in f/4 and f/2-lowN treatments. Bacterial densities are shown in Fig. 2.3.

α-glucosidase and β-glucosidase

Substrate saturation curve, Lineweaver-Burk plot, and Eadie-Hofstee plot for α-MUF and β-MUF hydrolysis by the *P. minimum* culture are shown in Fig. 2.4 and 2.5. The half-saturation constant (K_s) estimated was 10^{-8} μM and 7.8×10^{-9} for the hydrolysis α-MUF and β-MUF respectively, and the maximum rate of hydrolysis (V_m) was 10^{-3} $\mu M \cdot h^{-1}$ and 1.1×10^{-3} $\mu M \cdot h^{-1}$ for α-MUF and β-MUF respectively.

Alpha and β glucosidase activities for different size fractions were measured (Fig. 2.6 and Fig. 2.7). No activity was found associated with *P. minimum* fraction (>5 μm), 82.3% to 92.7% and 67.0% to 92.5% of α and β glucosidase respectively was associated with bacterial fraction (0.1 to 5 μm), and 7.3 to 17.7% and 7.5 to 33% of α-Glc and β-Glc respectively were found in dissolved phase. Thus, there is no evidence for ectocellular glucosidase activities in *P. minimum*, and therefore subsequent laboratory experiment did not include α and β glucosidase assays.

Leucine aminopeptidase

Substrate saturation curve, Lineweaver-Burk plot, and Eadie-Hofstee plot for Leu-MCA hydrolysis by the *P. minimum* culture are shown in Fig. 2.8. The half-saturation constant (K_s) estimated for the hydrolysis of Leu-MCA was 6.9 μM and the

maximum rate of hydrolysis (V_m) was 4.6 $\mu M \cdot h^{-1}$. Enzyme activity was linear over 4 h (Fig.2.8).

LAP activity was measured during the growth curve of *P. minimum* grown in media with four different nutrient concentrations. Since cultures used were not axenic, the experimental procedure was designed for the determination of the activity of the *P. minimum* fraction, bacteria fraction and free dissolved phase. Results of enzymatic activity measurements obtained from different size fractions at different nutrient concentration conditions are shown in Fig. 2.9, mean values and percentages for stationary phase are given in Table 2.2. Four each treatment, during the entire growth cycle the relative contributions of different size fractions were constant. In nutrient balanced conditions (f/2 and f/4) 85-90% of total LAP activity was associated with *P. minimum* fraction while bacteria contributed 8 to 12%, and the lowest activity was found in free dissolved phase, contributing only 2-3%. These results indicate that almost all LAP activity was ectocellular and most associated with the dinoflagellate cells in my cultures. In unbalanced nutrient concentration conditions (f/2-lowN and f/2-lowP) the contribution of *P. minimum* fraction to total activity was lower (67%) while that of bacteria fraction increased to 27%.

Cell specific and chlorophyll *a* specific activity in the *P. minimum* fraction (>5 μm) were calculated (Fig. 2.10 and Table 2.2). Lower cell-specific LAP activity was observed in cultures grown in low nitrate concentration medium (f/2-lowN). The mean cell-specific activity in f/2, f/4 and f/2-lowP at stationary phase was 7.81 ± 1.01 $x10^{-2}$ pmol $AMC \cdot cell^{-1} \cdot h^{-1}$, while in f/2-lowN the activity per cell was reduced by 67% (2.45 ± 0.32 x 10^{-2} pmol $AMC \cdot cell^{-1} \cdot h^{-1}$). The reduction of LAP activity per cell at stationary phase in f/2-lowN was highly significant (1-way ANOVA, $p<0.0001$). In f/2, f/4 and

f/2-lowP treatments the total LAP activity was strongly correlated with *P. minimum* density while the activity in low nitrogen concentration conditions (f/2-lowN) was not (Fig. 2.11).

Total LAP activity was also highly correlated with Chl *a* content in all treatments (r^2=0.90) (Fig. 2.12), including low nitrogen concentration conditions due to the lower Chl *a* content per cell (Fig. 2.2). When expressed on a per-chlorophyll *a* basis, LAP activity does not show any significant difference among treatments (1-way ANOVA, $p<0.005$). The mean activity per unit of Chl *a* in stationary phase for all treatments was 9.51 ± 1.76 nmol MCA·µg Chl $a^{-1}\cdot h^{-1}$.

Nutrient addition experiment. In the short-term single-pulse experiment high concentrations of NO_3^{2-}, NH_4^+ and urea were added to a culture of *P. minimum*. The results, shown in Fig. 2.13, indicated that addition of nitrate did not affect the LAP activity associated with *P. minimum* fraction (>5 µm) while statistically significant reductions of 56.4 and 65.7% were observed after 24 hours for urea and NH_4^+ addition treatments respectively.

Effect of irradiance on LAP activity. To determine if ectoproteolytic activity depended on light level, LAP activity associated with *P. minimum* fraction (>5 µm) grown at three light irradiances was measured. The cell-specific activities for each light level measured after 10 days of acclimation are shown in Fig. 2.14. LAP activity appeared to be inversely correlated to irradiance increasing from 2.88 x 10^{-2} pmol AMC ·$cell^{-1}\cdot h^{-1}$ at 267 µmol photons·$m^{-2}\cdot s^{-1}$ to 3.16 x 10^{-2} pmol AMC ·$cell^{-1}\cdot h^{-1}$ at 53 µmol photons·$m^{-2}\cdot s^{-1}$ to 4.98 x 10^{-2} pmol AMC ·$cell^{-1}\cdot h^{-1}$ at 4 µmol photons·$m^{-2}\cdot s^{-1}$. However the differences were not statistically significant.

Diurnal cycle experiment. No diurnal trend was found for LAP (data not shown).

Field experiments

Data collected during dinoflagellate blooms that occurred in spring and fall 2003 in tributaries of the Chesapeake Bay are summarized in Tables 2.3 and 2.4. In March and April the blooms in the Choptank River were dominated by *Heterocapsa rotundatum*, which reached densities of 88.9 x 10^3 cells·mL^{-1} in March and 67.7 x 10^3 cells·mL^{-1} in April. *Prorocentrum minimum* and *Karlodinium micrum* were dominant during the blooms that occurred in May. In the Patuxent River there was a bloom largely dominated by *H. rotundatum* (135.5 x 10^3 cells·mL^{-1}), while *Prorocentrum minimum* was the only dinoflagellate species present during a Corsica River bloom (65.7 x 10^3 cells·mL^{-1}).

Dinoflagellate abundance was correlated with chlorophyll *a* in the water (Fig. 2.15).

Alpha and β-glucosidase were measured during the *P. minimum* bloom in the Corsica River, but no activity was found associated with the fraction containing dinoflagellates (>5 μm) (Fig. 2.16).

In the Choptank and Patuxent Rivers total LAP activity ranged from 1.45 to 7.50 μM·h^{-1} with 2 to 34% of the total activity associated with the >5 μm fraction (Table 2.3). This percentage was higher (up to 79%) during the monospecific *P. minimum* bloom observed at the end of October in the Corsica River (Fig. 2.17). The combined field data showed that total LAP activity and ectoproteolytic activity associated with the fraction containing dinoflagellates (>5 μm) were correlated with dinoflagellate density (Fig. 2.18), and a significant correlation was also found between the percentage of LAP activity associated with the >5 μm fraction and the dinoflagellate abundance (Fig. 2.19),

but there was no significant correlation between the density of dinoflagellates and the activity in the <5 μm fraction (r^2=0.01, p=0.7170). LAP activity associated with dinoflagellates fraction explains up to 52% of the variability of total LAP activity in the water (Fig. 2.20).

Total LAP activity was correlated with chlorophyll *a* in the water (n=36, r^2=0.19, p=0.0086), and a closer correlation coefficient was found between LAP activity in the fraction containing dinoflagellates (>5 μm) and chlorophyll *a* (n=36, r^2=0.28, p=0.0009). The chlorophyll *a* specific LAP activity associated with the >5 μm fraction ranged between 4.32 and 264.87 nmoles AMC/mg Chl *a*/h, with 92% of the values less than 87.86 nmoles AMC/mg Chl *a*/h.

No correlation was found between salinity and dinoflagellate density or LAP activity (data not shown).

The LAP activity associated with the >5 μm fraction was negatively correlated with ammonium concentration (Fig. 2.21). No correlation was found between LAP activity in the water or in the dinoflagellate fraction and phosphate (n=22, r^2=0.10, p=0.1590; n=22, r^2=0.03, p=0.4660) or NO_3+NO_2 (n=23, r^2=0.07, p=0.2074; n=23, r^2=0.01, p=0.6551) concentration.

DISCUSSION

P. minimum is able to grow in a range of inorganic nutrient concentrations. Low phosphate concentration greatly affects the biomass maximum achieved at stationary phase. While low nitrogen concentration does not affect the cell density reached at the stationary phase, they largely affect the chlorophyll-*a* content. The fact that low nitrate concentration does not affect the cell density can be explained with the evidence that *P.*

minimum prefers ammonium rather than nitrate as nitrogen source (Fan *et al.* 2003) and in low nitrate treatment the ammonium concentration was equal to those of all other treatments. In fact while the nitrate concentration in f/2-lowN medium was 1/80 of the nitrate concentration in f/2, the reduction of cell content of nitrogen in low N treatment was only ½ compared to the reduction of carbon content. Another possible explanation is the reduction of cell size. In the N deficient treatment, C and N contents (biomass) of cells in stationary phase was reduced compared to the nutrient sufficient treatment (Table 2.1).

In marine environments ectoenzyme activities are thought to be primarily due to heterotrophic bacteria (Rego et al. 1985, Rosso and Azam 1987), therefore most studies of ectoenzyme activities have focused on heterotrophic bacteria and not phytoplankton, and the contribution of phytoplankton is not well known. Since enzyme activities may be distributed in the water (free exoenzymes) or associated with cell surfaces of different microorganisms, experiments were designed for the partitioning of the activity among dinoflagellate, bacterial, and free fractions. A number of studies have been conducted in order to investigate the relative importance of different size fractions by selective filtration (Somville and Billen 1983, Vives Rego et al. 1985, Karner et al. 1994, Bochdansky et al. 1995, Karner and Rassoulzadegan 1995, Mulholland et al. 2002, 2003, Stoecker and Gustafson 2003). In enzymatic studies size fractionation by filtration is a common approach but filtration may be a source of error that leads to over- or underestimations of enzyme activity. Possible problems include liberation of ectoenzymes from cells, retention of enzymes on filters, and inaccurate separation of different functional groups (Sala et al. 2001). Filtration-induced release or enzyme adsorption to the filters seems to play only a minor role as demonstrated by Karner and

Rassoulzadegan (1995). Moreover, Stoecker and Gustafson (2003) demonstrated that activity is not an artifact due to cell damage but rather that cell damage decreases LAP activity. In my study I would reject the occurrence of adsorption to filters, in fact if this occurred I should have found glucosidase activity in fraction >5 μm and this did not occur. Although size fractionation by filtration does not provide an absolute separation of dinoflagellates and bacteria, this procedure is sufficient to estimate with which fraction of the microbial community ectoenzymes are associated (Somville and Billen 1983).

More than 95% of glucosidase activities have been found associated with bacterial-size fraction both in seawater and freshwater (Hoppe 1983, Somville 1984, Chróst and Overbeck 1990). Glucosidase activities in dinoflagellates have not been reported. This study confirms that cultures of *P. minimum* do not express appreciable glucosidase activities, and no activity was found associated with the size fraction containing dinoflagellates during dinoflagellate blooms. Bacteria represent the major source of glucosidase activities, particularly during the stationary phase when the number of bacterial cells increases because *P. minimum* cells start dying. Glucosidase activity associated with bacteria is higher in low-N and low-P treatments where bacterial abundance is higher. The higher number of bacteria in low nitrate and even more in low phosphate treatments is probably due to the higher carbon availability because the unbalanced growth of *P. minimum* leads to an increase of carbon excretion. Although glucosidase activities have been thought to be mainly bound to bacteria cell walls, dissolved enzymes are thought to play a significant role in organic matter hydrolysis (Azam and Smith 1991, Smith et al. 1992, Bochdansky et al. 1995, Karner and Rassoulzadegan 1995). In this study the free dissolved phase comprised a

substantial portion of the total glucosidase activities, and the dissolved phase contribution is higher for β-glucosidase than for α-glucosidase. My results are consistent with those obtained by Karner et al. (1994) and Bochdansky et al. (1995), while the contribution of dissolved free phase that I estimated was lower than that calculated by Karner and Rassoulzadegan (1995) and higher than that reported by Vrba et al. (1993). I can not say if the presence of organic compounds and low light might induce glucosidase activity in *P. minimum*. During dense blooms light availability can be reduced by high cell density, in addition the increase of pH during the bloom might induce inorganic carbon limitation. These conditions might induce glucosidase activity in *P. minimum,* explaining its ability to maintain high cell density during dense blooms.

Ectoproteolytic activity, both in fresh and seawaters, has been attributed primarily to heterotrophic bacteria (Rosso and Azam 1987, Sinsabaugh et al. 1997). Only recently the protistan contribution to protease activity has been considered (Sala et al. 2001). Activity has been found in *Aureococcus anophagefferens* (Mulholland et al. 2002, Berg et al. 2002), heterotrophic nanoflagellates (Karner et al. 1994, Sala and Güde 1999) and in different species of dinoflagellates (Sankievicz and Colepicolo 1999, Mulholland et al. 2002, Stoecker and Gustafson 2003). Leucine aminopeptidase has also been found associated with the cell surface of intact *Chlamydomonas* cells (Langheinrich 1995). Using Leu-MCA, proteolytic activity was also found in axenic cultures of the dinoflagellates *Alexandrium tamarense*, *Heterocapsa triquetra* and *P. minimum*, and in non-axenic cultures of *Akashiwo sanguinea*, *Gonyaulax grindleyi*, *Gyrodinium uncatenum* and *Karlodinium micrum* (Stoecker and Gustafson 2003).

This study confirms that *P. minimum* can hydrolyze peptides. While the activity per cell shows high variability, with a significant reduction of activity per cell under low nitrate concentration, the chlorophyll *a* specific activity is less variable. The cell specific activity found in this study is very similar to that found by Stoecker and Gustafson (2003) in axenic cultures of *P. minimum*, and suggests that at bloom densities *P. minimum* may make an important contribution to total activity and may be responsible for degradation of proteins and polypeptides through enzyme-catalyzed hydrolysis. In fact during a monospecific *P. minimum* bloom a large fraction (79%) of LAP activity was likely due to dinoflagellates (>5 μm). During the dinoflagellate blooms investigated, the dinoflagellate size fraction made an important contribution to LAP activity: dinoflagellate density explained 30% and 50% of the variability of total activity and activity associated with the >5 μm fraction respectively. The ectoproteolytic activity associated with the dinoflagellate fraction explained an important part (up to 52%) of variability of total LAP activity in the water. *In situ* ectoproteolytic activity measured in this study is consistent with LAP activity measured by Stoecker and Gustafson (2003) in 2 to 200 μm fraction in samples collected in the Choptank River, with maximum values higher probably due to higher dinoflagellate density. In contrast with Stoecker and Gustafson (2003), but according to Mulholland et al. (2002), my results do not show any correlation between LAP activity and salinity. The range of values found for LAP activity is also consistent with Nausch and Nausch (2000) and Karner et al. (1994).

Even if the cell specific LAP activity is reduced in low nitrate treatment, N deficiency does not affect the chlorophyll *a* specific LAP activity. In fact in low nitrate treatment I observed the same reduction (30%) in chlorophyll *a* content and LAP activity. Since LAP activity might be a means to acquire nitrogen, the strong correlation

between LAP activity and chlorophyll *a*, a N containing molecule, might reflect higher requirements for nitrogen due to chlorophyll *a* synthesis. The reduction of cell specific LAP activity might be explained by the reduction of cell biomass (reduced C, N and P content) and chlorophyll *a* content and the subsequent reduction of N requirement or by lack of sufficient N for LAP synthesis.

. If enzymatic activity is used as a means to acquire nutrients by dinoflagellates, it may be important to bloom dynamics and physiological ecology. Although in the plankton it is generally thought that LAP activity may be partially inducible since high activity is often associated with low N:P ratio (Sala et al. 2001), this does not seem the case for *P. minimum*. Since ectocellular leucine aminopeptidase activity has been found in cultures grown in inorganic nutrient-rich media, this might suggest that the enzyme is constitutive, but organic nutrients inadvertently present in the seawater used to prepare media may have been concentrated enough to induce LAP activity in all treatments. However, nutrient availability leads to some changes in LAP activity. In my laboratory experiments nitrate deprivation caused a reduction of LAP activity per cell, even if the activity per unit of chlorophyll *a* remained constant, and addition of reduced forms of nitrogen, such as urea and ammonium, which have been shown to be preferred N sources for *P. minimum* (Fan et al. 2003), reduced the ectoproteolytic activity in cultures. I also observed that LAP activity was not inhibited by addition of nitrate, similarly to Nausch and Nausch (2000). I found evidence of the regulation of peptidase activity associated with dinoflagellate fraction by ammonium also in field samples, as reported by Chróst (1991) in eutrophic lakes. The increase of LAP activity with decreasing light, even if it's not statistically significant, might be related with the higher concentration of chlorophyll *a*. The higher content of chlorophyll *a* at low light leads to

higher nitrogen requirements that might be satisfied by increasing the uptake of N from amino acids released by LAP activity.

The free dissolved phase of LAP seems to play a minor role. The percentage of free proteolytic activity was lower than those of glucosidases, in accordance to Bochdansky et al. (1995), and consistent with that found in other studies (Karner and Rassoulzadegan 1995, Karner et al. 1994, Vives Rego et al. 1985, Hollibaugh and Azam 1983).

Proteolytic activity may play a role in nutrition of mixotrophic dinoflagellates and give them an advantage over the strictly phototrophic species. Amino acids released by LAP may be taken up directly by dinoflagellates as a source of N and C, or oxidized by amino acid oxidases on the surface of cells, to release ammonium, a preferred N source for many dinoflagellates including *P. minimum* (Mulholland et al. 1998, Fan et al. 2003). I suppose that leucine aminopeptidase, in combination with cell surface oxidase, is used by *P. minimum* to supply inorganic nitrogen, particularly ammonium. Therefore, inhibition by high ammonium concentration would represent end-product feedback regulation. Additional studies that measure simultaneously LAP activity and C or N uptake are needed in order to understand the role of LAP in nutrition of *P. minimum*.

It is generally thought that uptake of monomers is of little significance to phytoplankton nutrition in nature because of the relatively low *in situ* concentrations compared to the half-saturation constant (K_s) values of most phytoplankters for these labile substrates (reviewed in Antia et al. 1991, Granéli et al. 1999). However, this argument would not apply to monomers released by enzymes at the cell surface, since these monomers could be at much higher concentrations near cells than in bulk

seawater. The high affinity of leucine aminopeptidase for its substrate indicates that this enzyme could be efficient in supplying nutrients (nitrogen or carbon) as amino acids to *P. minimum*. The values I found for Michaelis-Menten parameters are consistent with the highest values measured by Rheinheimer et al. (1989) but higher than those measured by Nausch and Nausch (2000) in field samples collected from the Baltic Sea.

To better understand the role of proteolytic activity in nutrition of mixotrophic dinoflagellate more detailed studies comparing ectoenzyme activity and nutrient uptake are needed.

Fig. 2.1. Sampling stations in Choptank River (from EPA/NASA/NOAA CISNet), Corsica River (from Environmental Protection Agency US web site), and Patuxent River.

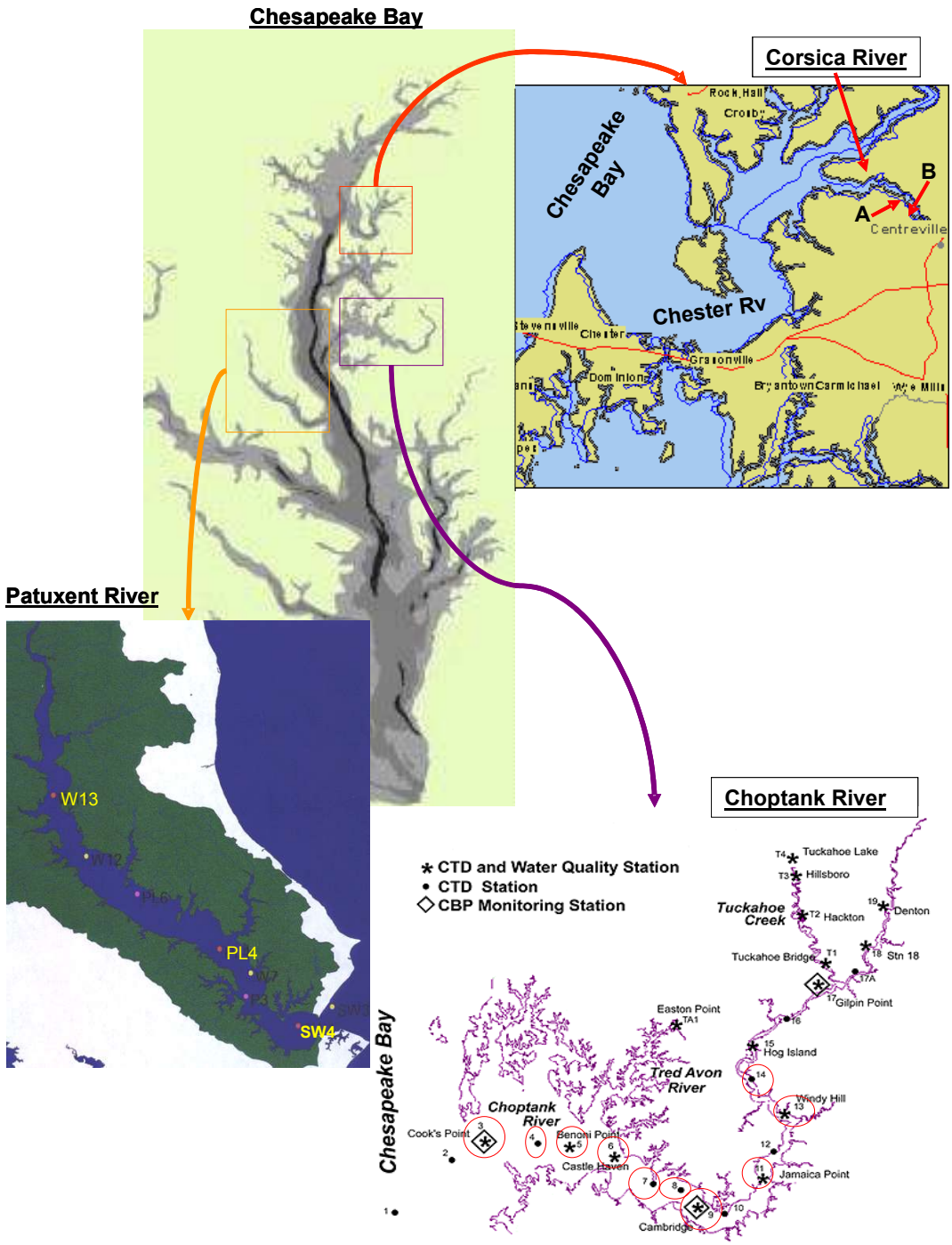


Fig. 2.2. (A) Abundance and (B) chlorophyll *a* content of *P. minimum* grown in different nutrient treatments.

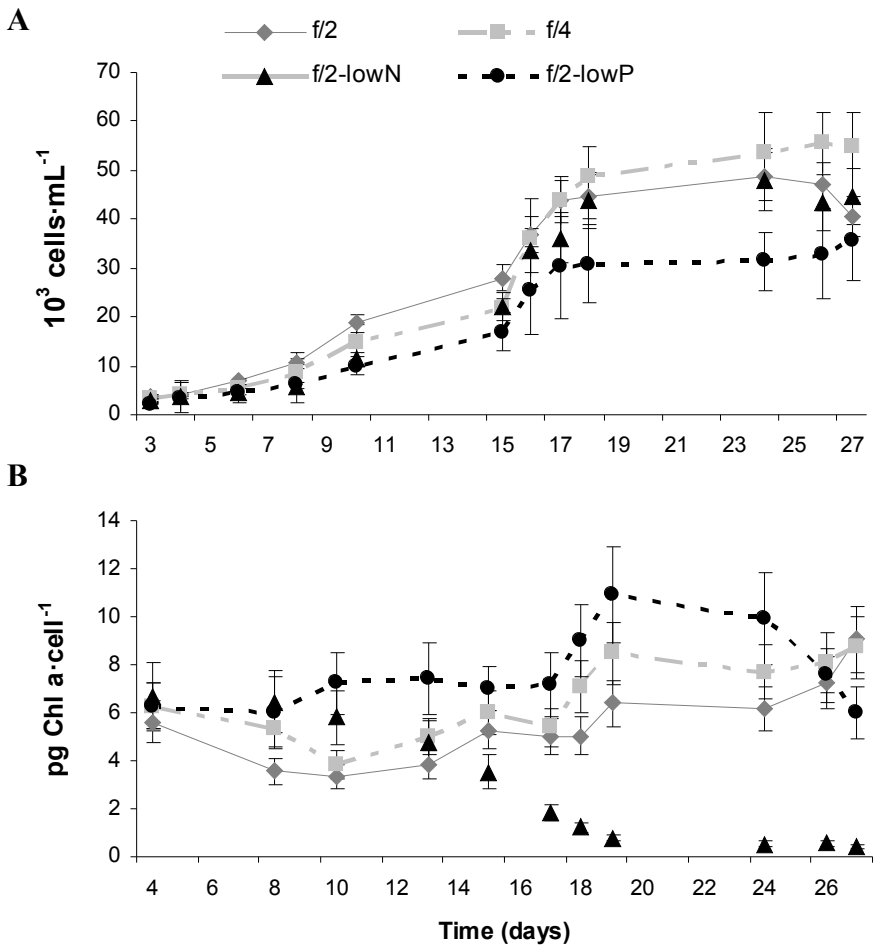


Fig. 2.3. Abundance of bacteria grown in different nutrient treatments.

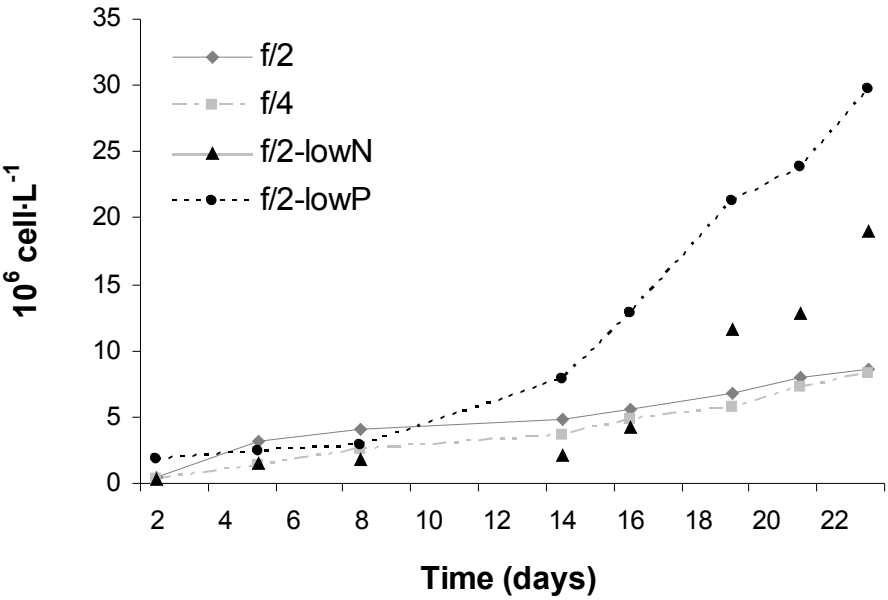


Fig. 2.4. (A) Substrate saturation curve, (B) Lineweaver-Burk plot and (C) Eadie-Hofstee plot of α-glucosidase activity.

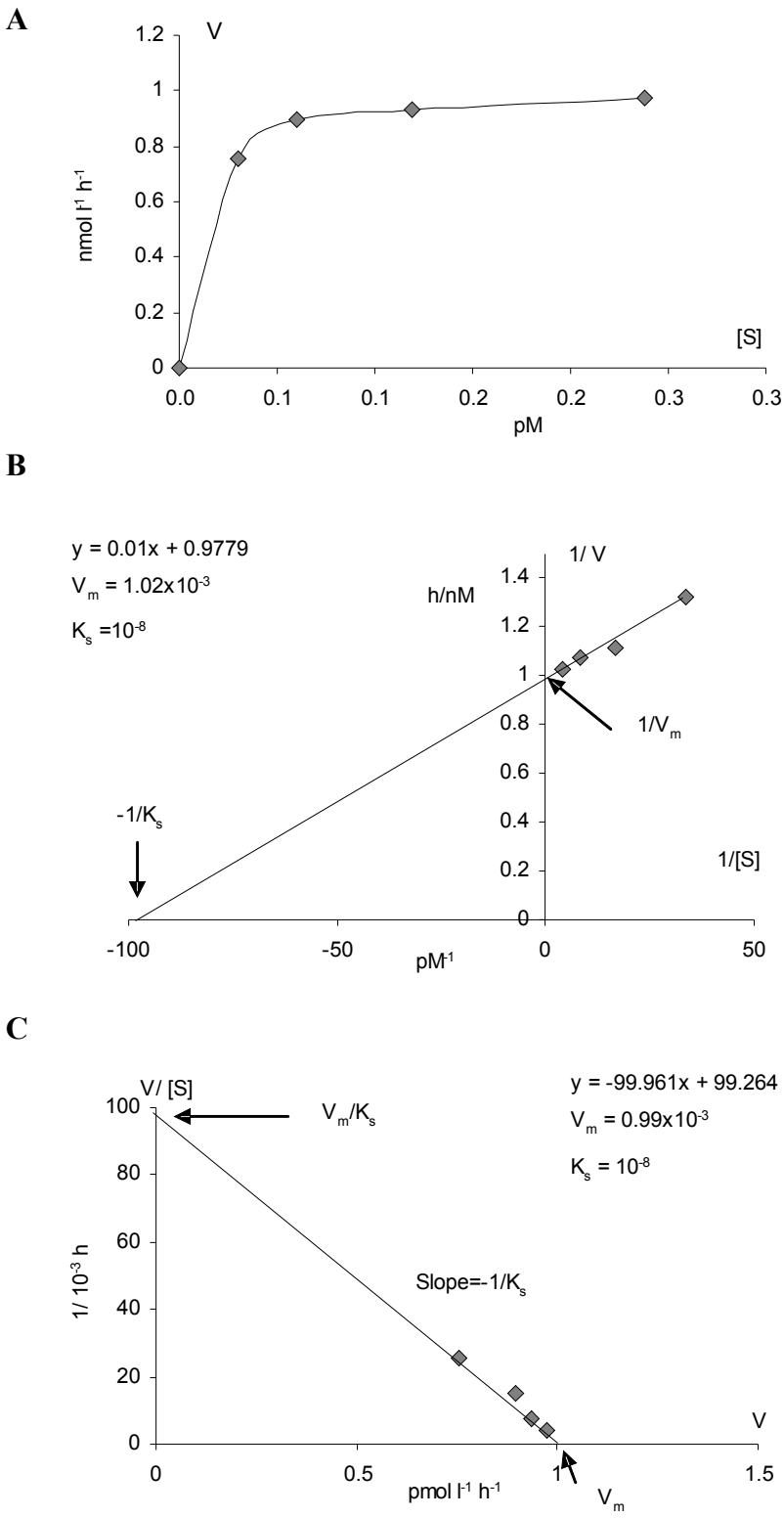


Fig. 2.5. (A) Substrate saturation curve, (B) Lineweaver-Burk plot and (C) Eadie-Hofstee plot of β-glucosidase activity.

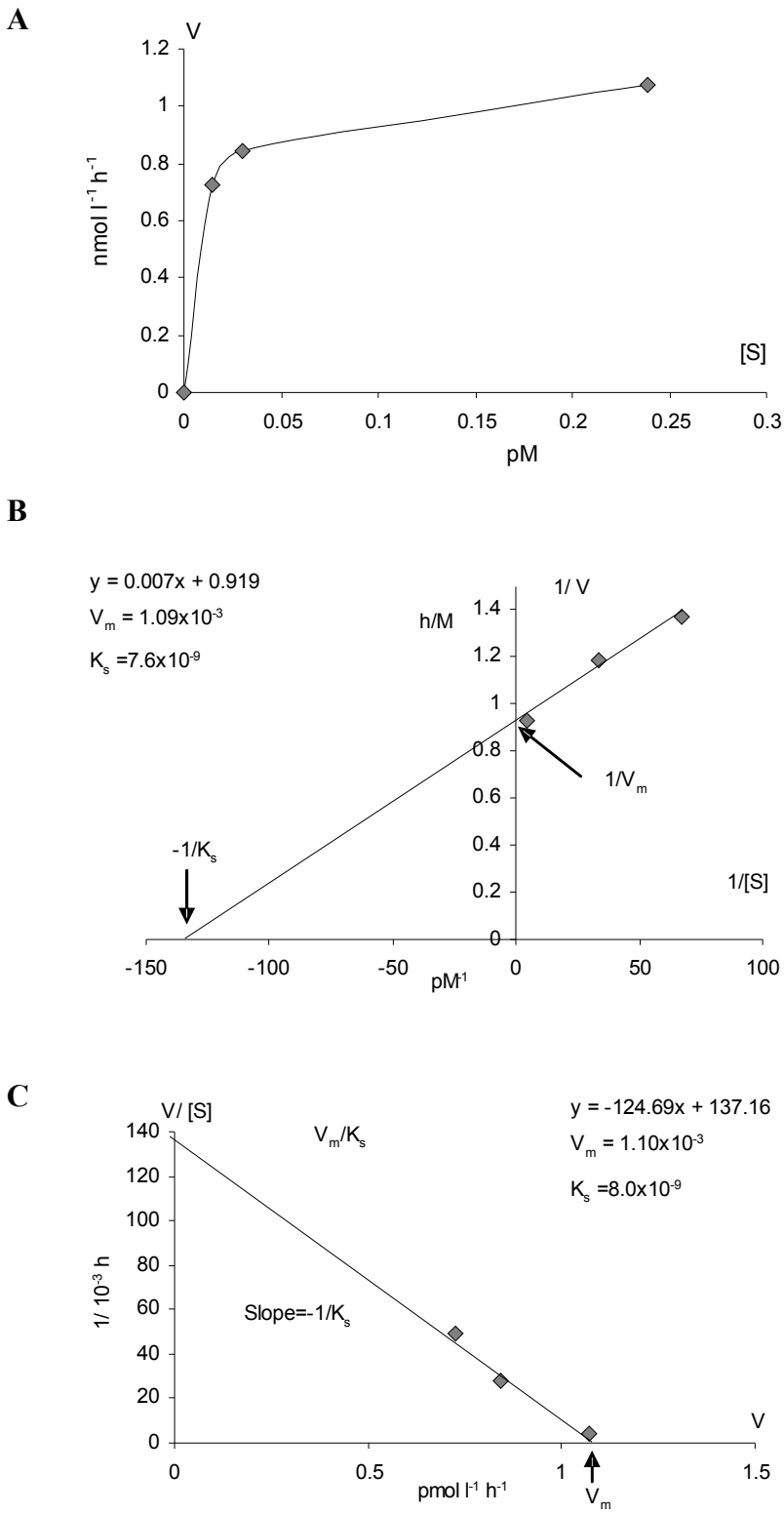
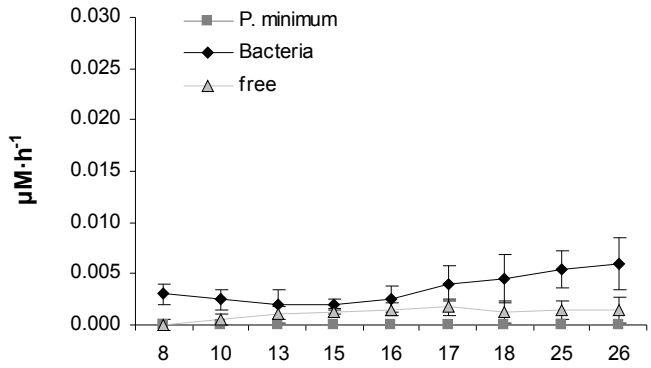


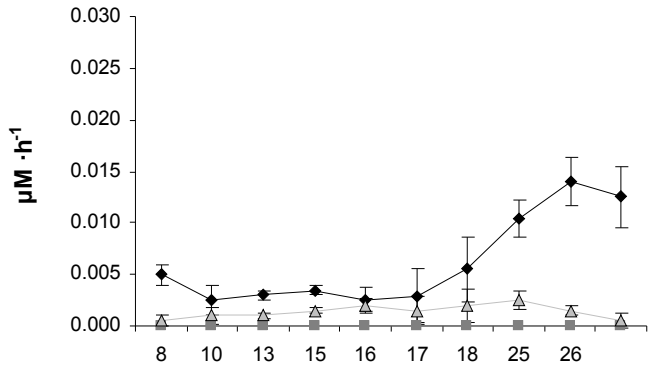
Fig. 2.6. α-glucosidase activity associated with different size fractions: >5 μm (*P.minimum*), 0.1-5 μm (bacteria) and <0.1 μm (free phase) in *P. minimum* cultures in four treatments (A: f/2, B: f/4, C: f/2-lowN, D: f/2-lowP).

A

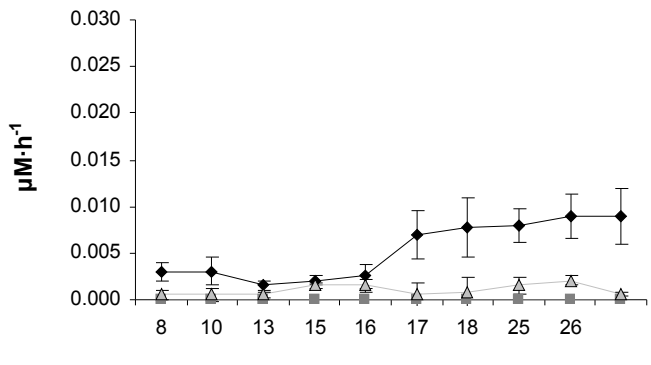
B


P. minimum
Bacteria
free
µM·h-1
0.030
0.025
0.020
0.015
0.010
0.005
0.000
8 10 13 15 16 17 18 25 26

C


µM ·h-1
0.030
0.025
0.020
0.015
0.010
0.005
0.000
8 10 13 15 16 17 18 25 26

D


µM·h-1
0.030
0.025
0.020
0.015
0.010
0.005
0.000
8 10 13 15 16 17 18 25 26

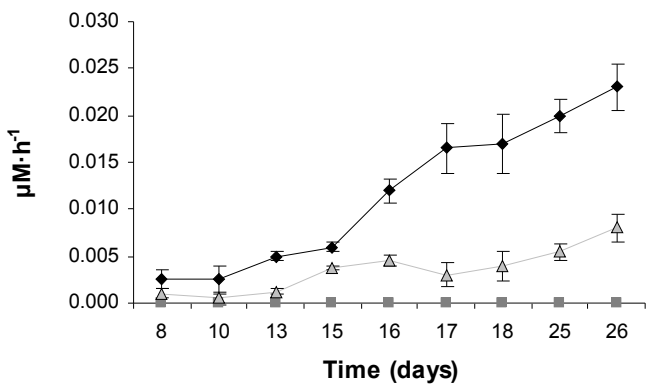

µM·h-1
0.030
0.025
0.020
0.015
0.010
0.005
0.000
8 10 13 15 16 17 18 25 26
Time (days)

Fig. 2.7. β-glucosidase activity associated with different size fractions: >5 μm (*P. minimum*), 0.1-5 μm (bacteria) and <0.1 μm (free phase) in *P. minimum* cultures in four treatments (A: f/2, B: f/4, C: f/2-lowN, D: f/2-lowP).

A

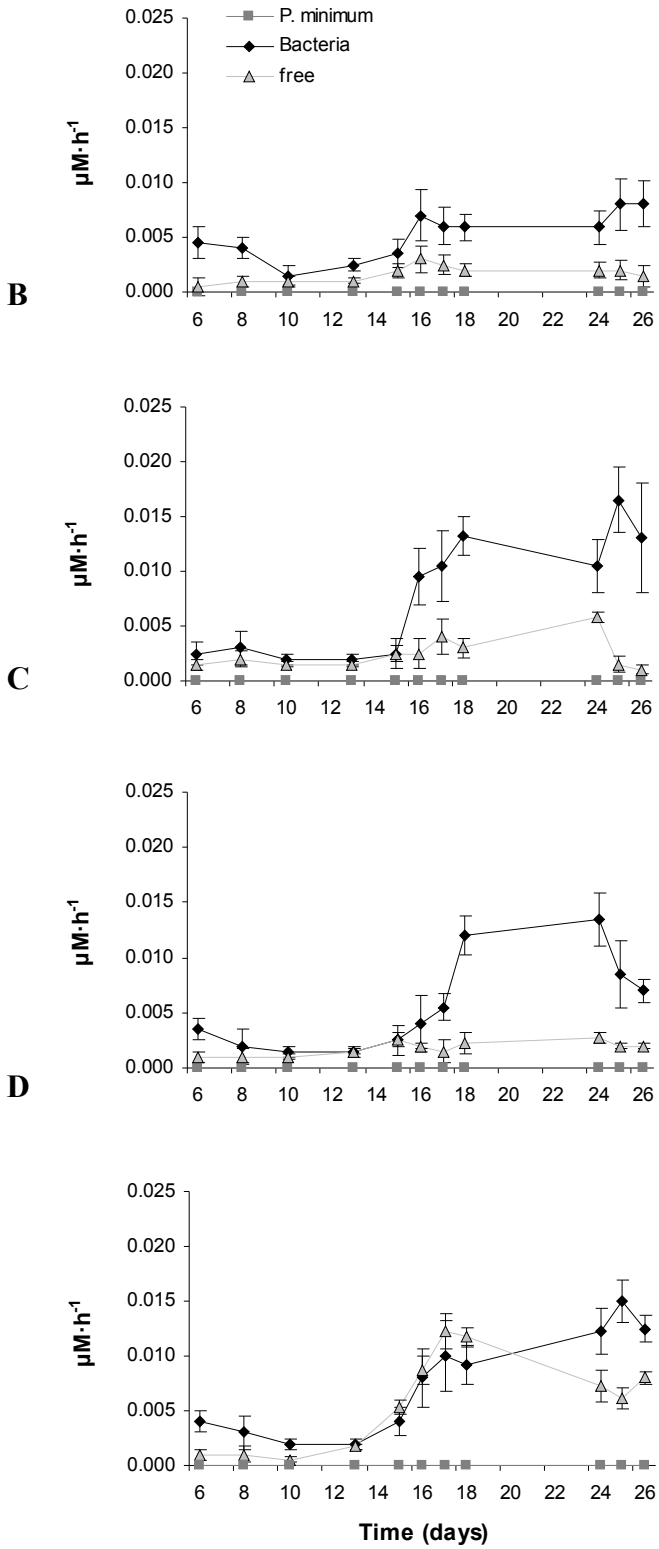
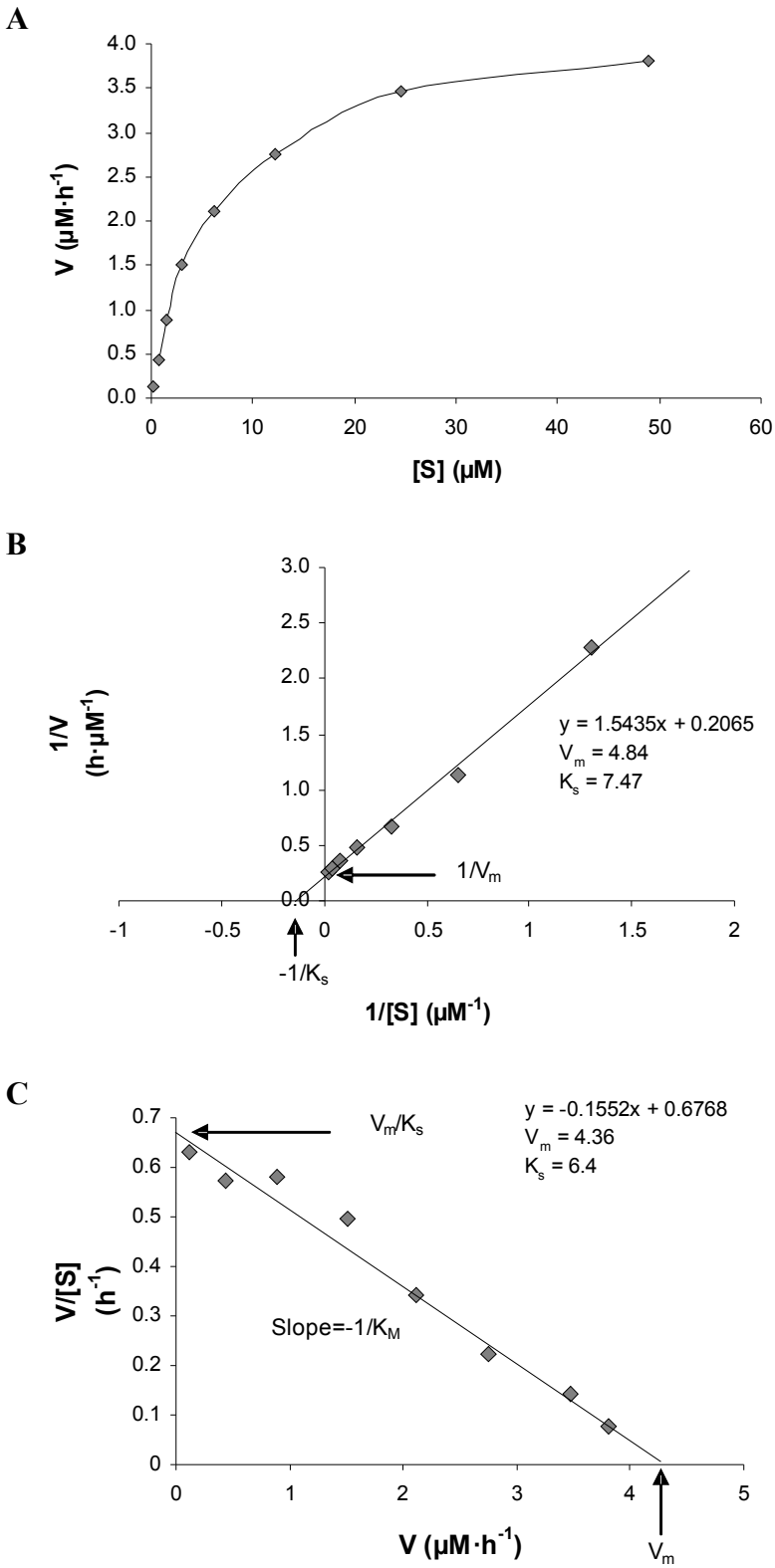

P. minimum
Bacteria
free
µM·h-1
0.025
0.020
0.015
0.010
0.005
0.000
6 8 10 12 14 16 18 20 22 24 26
B
C
D
Time (days)

Fig. 2.8. (A) Substrate saturation curve, (B) Lineweaver-Burk plot, (C) Eadie-Hofstee plot and (D) time course measurements of LAP activity.



D

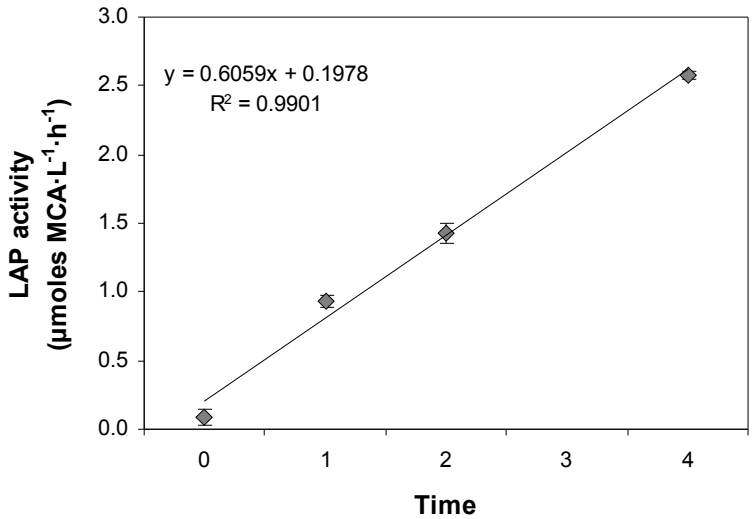
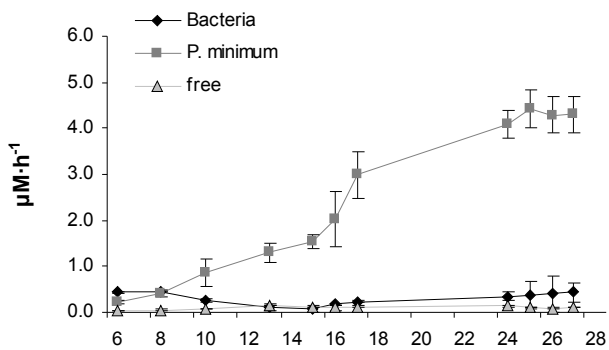

y = 0.6059x + 0.1978
R² = 0.9901
LAP activity
(µmoles MCA·L-1·h-1)
Time
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4

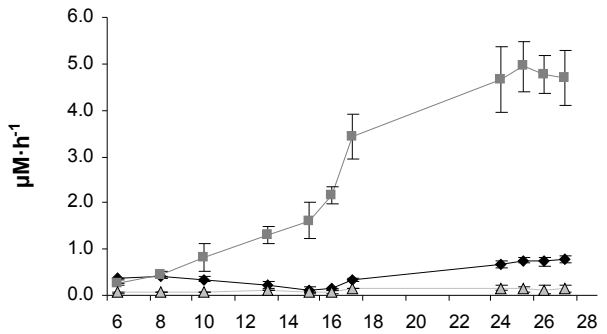
Fig. 2.9. Leucine aminopeptidase activity associated with different size fractions: >5 μm (*P. minimum*), 0.1-5 μm (bacteria) and <0.1 μm (free phase) in *P. minimum* cultures in four treatments (A: f/2, B: f/4, C: f/2-lowN, D: f/2-lowP).

A

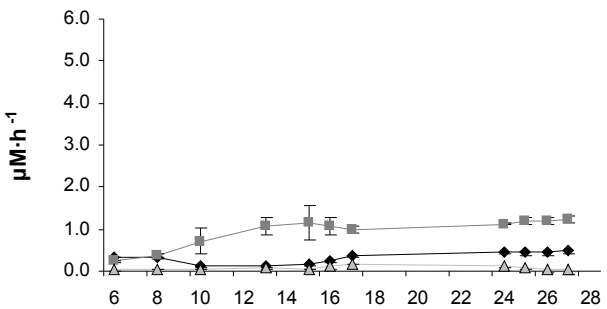
B


Bacteria
P. minimum
free
µM·h-1
6.0
5.0
4.0
3.0
2.0
1.0
0.0
6 8 10 12 14 16 18 20 22 24 26 28

C


µM·h-1
6.0
5.0
4.0
3.0
2.0
1.0
0.0
6 8 10 12 14 16 18 20 22 24 26 28

D


µM·h-1
6.0
5.0
4.0
3.0
2.0
1.0
0.0
6 8 10 12 14 16 18 20 22 24 26 28

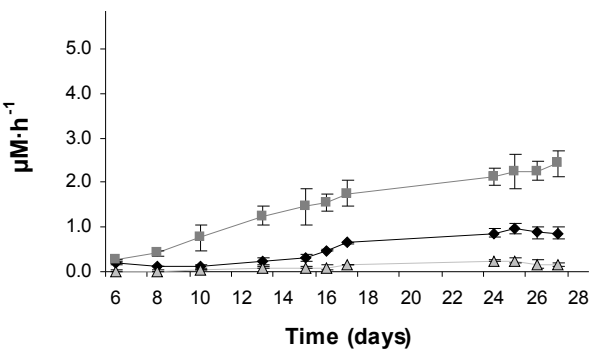

µM·h-1
5.0
4.0
3.0
2.0
1.0
0.0
6 8 10 12 14 16 18 20 22 24 26 28
Time (days)

Fig. 2.10. (A) Cell-specific LAP and (B) chlorophyll *a* specific LAP activity of *P. minimum* grown in four nutrient media (f/2, f/4, f/2-lowN, f/2-lowP).

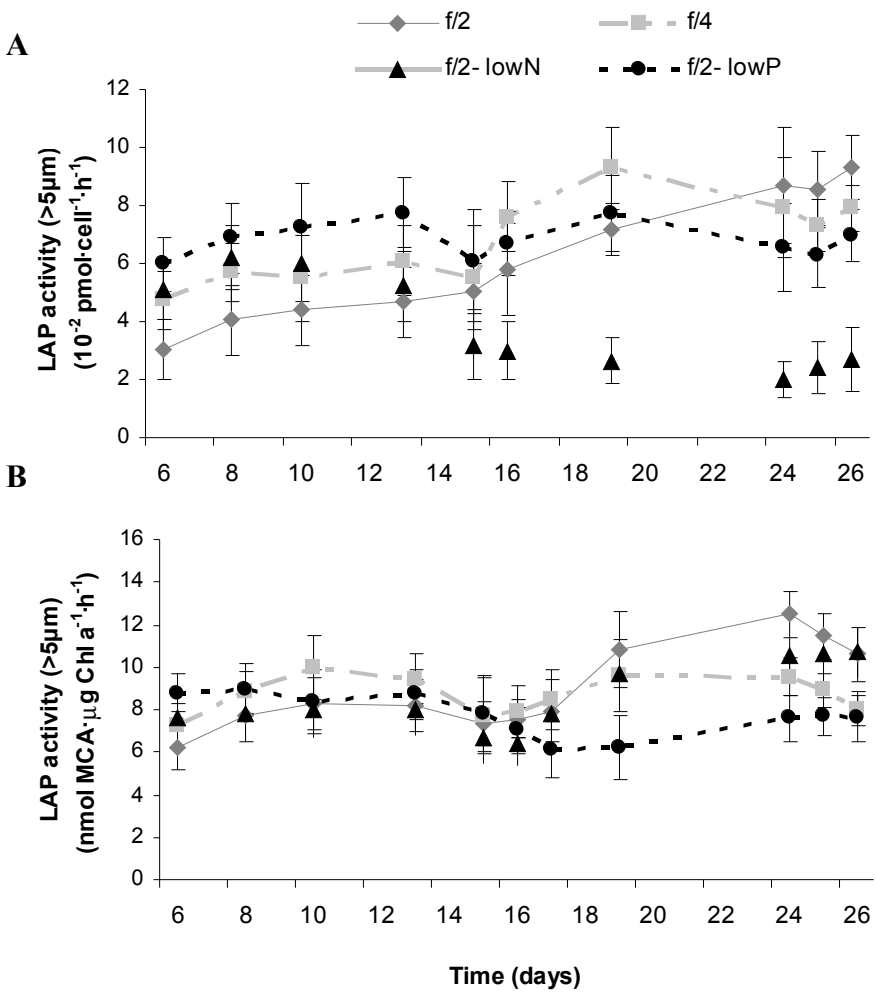


Fig. 2.11. Total LAP activity as a function of *P. minimum* density. Black circles refer to f/2, f/4 and f/2-lowP (n=27, $p<0.0001$, SE of estimate=0.4155, SE of constant=0.1601, SE of x coefficient=0.0000), gray diamonds refer to f/2-lowN. Total LAP activity was not correlated with *P. minimum* density for the f/2-lowN treatment.

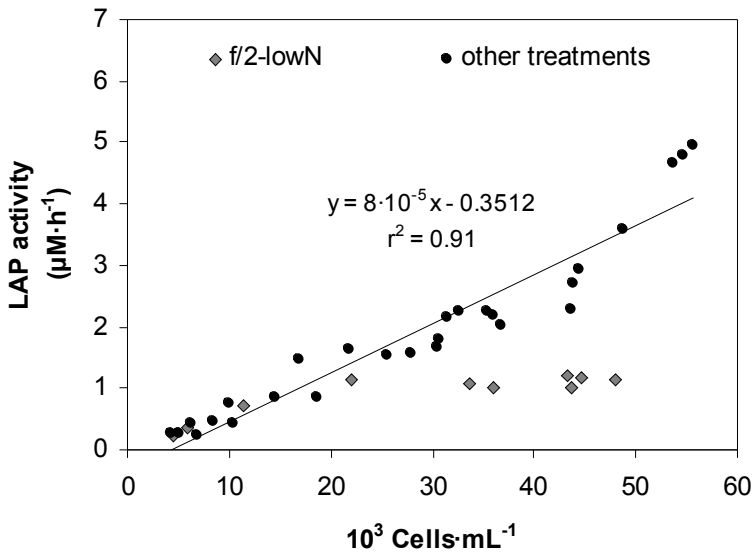


Fig. 2.12. Total LAP activity as a function of chlorophyll *a* content for all treatments (f/2, f/4, f/2-lowN and f/2-lowP). n=48, p<0.0001, SE of estimate=0.4351, SE of constant=0.1043, SE of x coefficient=0.0004.

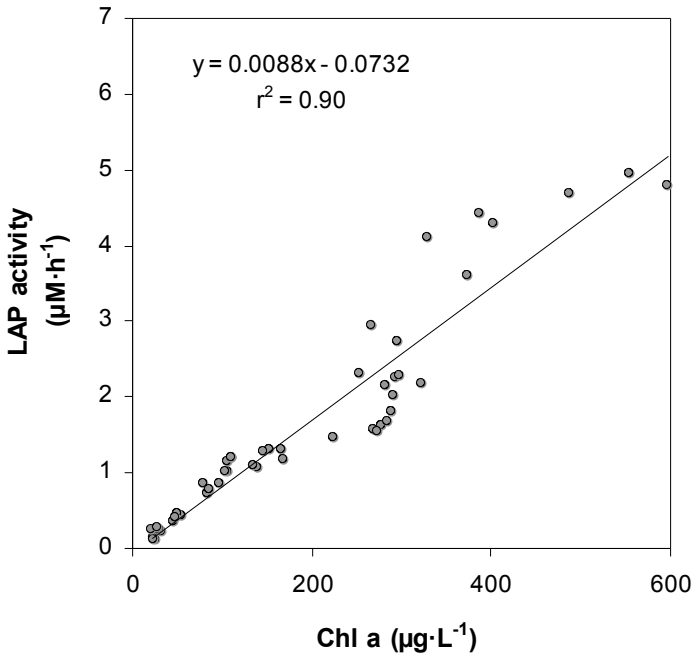


Fig. 2.13. Effects of addition of inorganic and organic N sources on LAP activity of *P. minimum*. Treatments were designed as control (unamended), +NO3 (addition of 800 μM nitrate), +urea (addition of 500 μM), and +NH4 (addition of 100 μM of ammonium). * and ** indicate that LAP activity after 24 h in the treatment and control are significantly different (* p=0.0002; ** p<0.0001), ns indicates that LAP activity in the treatment and control are not significantly different (p=0.3097) (ANOVA 1-way). Error bars denote 95% confidence intervals.

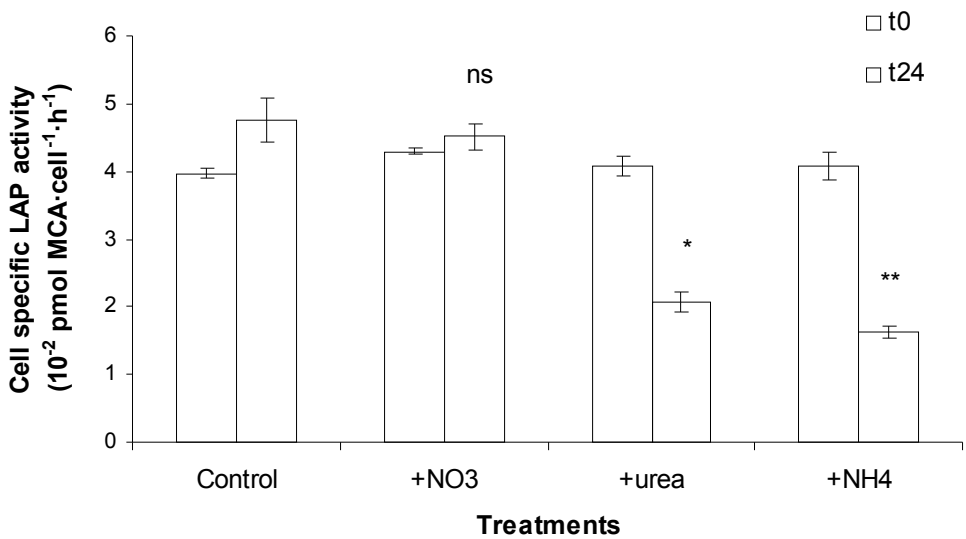


Fig. 2.14. Cell-specific LAP activity of *P. minimum* at three light irradiances (error bars denote 95% confidence intervals; $p>0.05$).

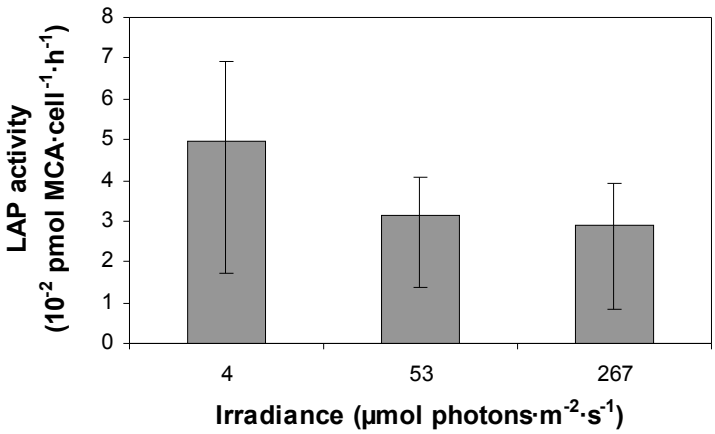


Fig. 2.15. Chlorophyll *a* and dinoflagellate density in field samples. n=36, p<0.0001, SE of estimate=23.5832, SE of constant=4.8104, SE of x coefficient=0.1225.

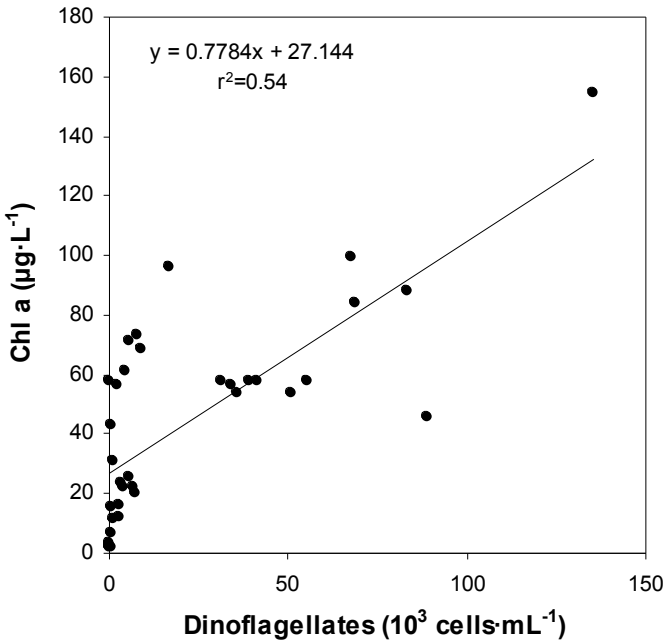


Fig. 2.16. α and β-glucosidase activities associated with dinoflagellate fraction (>5 μm), bacterial fraction (0.1-5 μm) and free dissolved phase (<0.1 μm) in field samples collected in Corsica River.

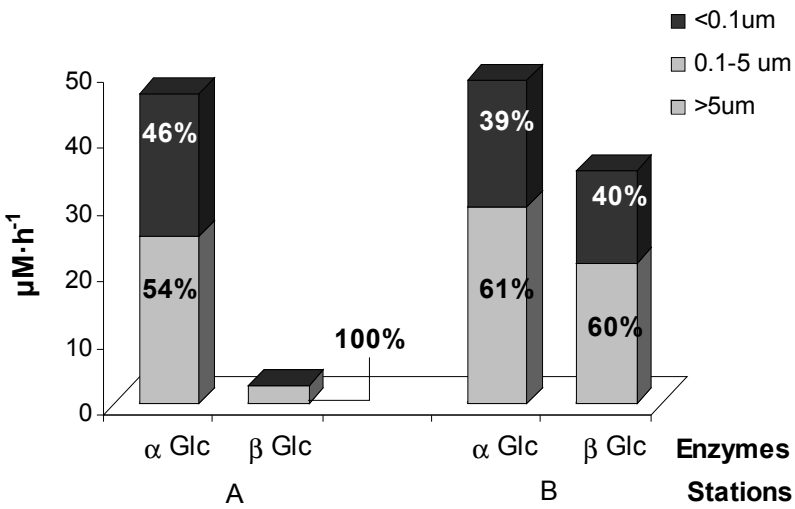


Fig. 2.17. LAP activity associated with dinoflagellate fraction (>5 μm), bacterial fraction (0.1-5 μm) and free dissolved phase (<0.1 μm) in field samples collected in Corsica River.

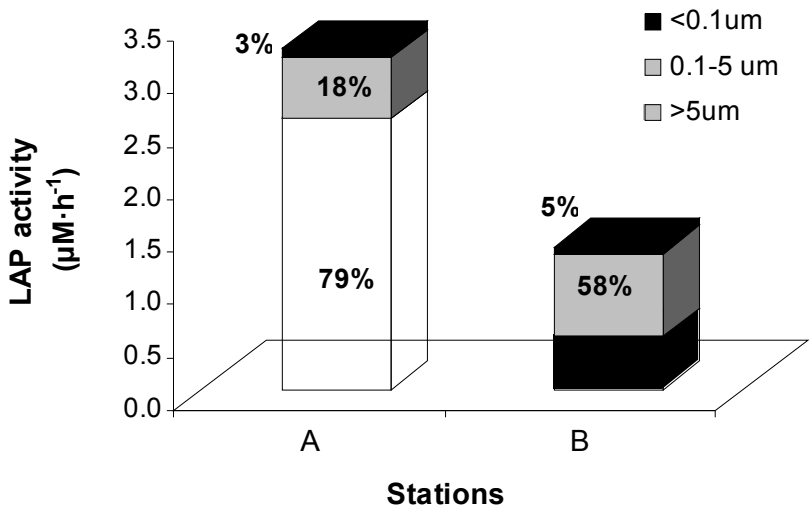


Fig. 2.18. Total LAP activity (A) and LAP activity associated with dinoflagellate fraction (>5µm) (B) in field samples as a function of dinoflagellate density. A: n=43, p=0.0001, SE of estimate=1.4441, SE of constant=0.2714, SE of x coefficient=0.0069; B: n=43, p<0.0001, SE of estimate=0.4445, SE of constant=0.0836, SE of x coefficient=0.0021.

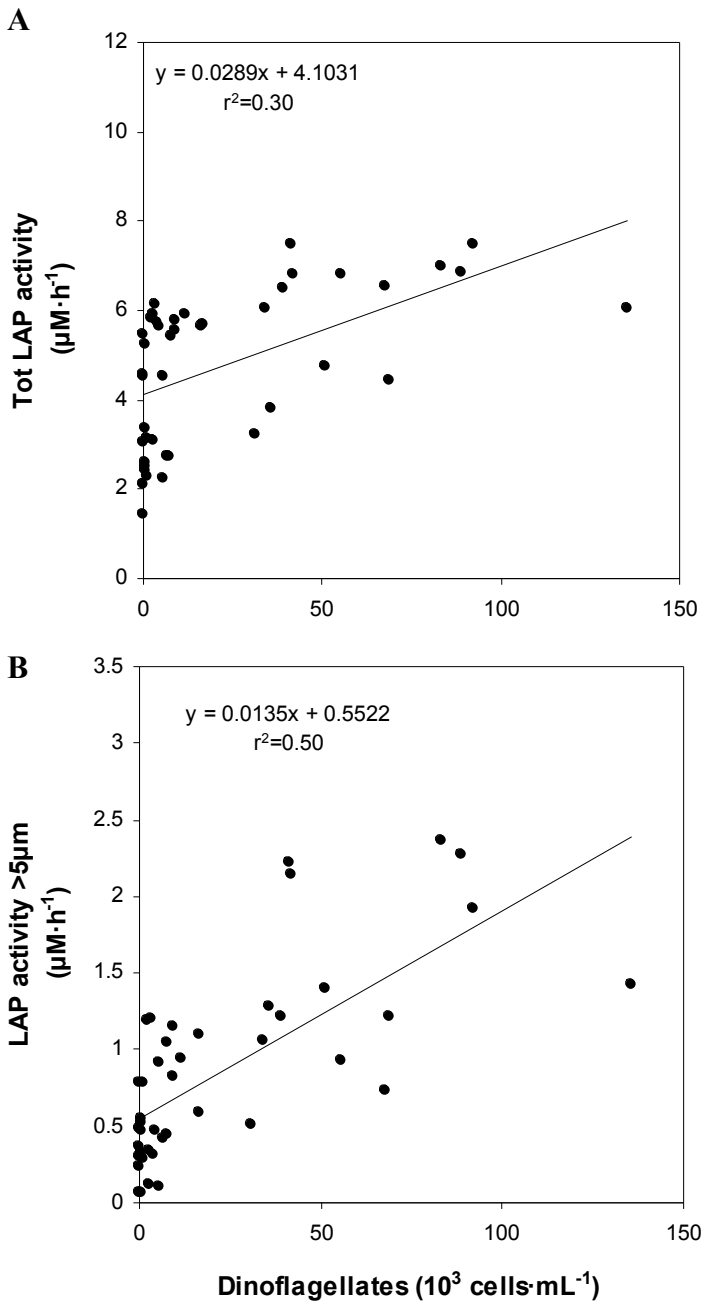


Fig. 2.19. Percentage of LAP activity associated with the >5 μm fraction and dinoflagellate density in field samples. n=43, $p<0.0001$, SE of estimate=7.2044, SE of constant=1.3542, SE of x coefficient=0.0345.

Treatment	Cell density (10^3 cell·mL^{-1})	Chl *a* (pmol·cell^{-1})	C (pg·cell^{-1})	N (pg·cell^{-1})	C:N (molar)	N:P (molar)
f/2	45.16 ± 3.58	7.21 ± 1.31	1.25	0.17	7.2	11.6
f/4	53.22 ± 3.07	8.24 ± 0.44	0.58	0.08	7.3	8.6
f/2-lowN	44.95 ± 2.13	0.57 ± 0.17	0.96	0.06	16.4	5.3
f/2-lowP	32.56 ± 2.07	8.59 ± 2.72	1.07	0.09	12.3	28.5

Fig. 2.20. Total LAP activity as a function of activity associated with the fraction containing dinoflagellates (>5 µm). n=44, p<0.0001, SE of estimate=1.2150, SE of constant=0.3155, SE of x coefficient=0.3013.

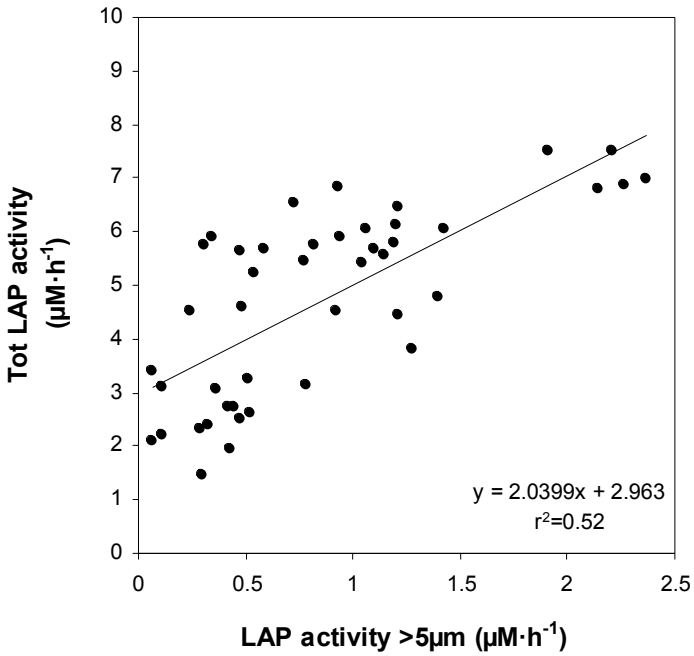


Fig. 2.21. Total LAP activity as a function of ammonium concentration in field samples. n=23, p=0.0242, SE of estimate=0.4810, SE of constant=0.1229, SE of x coefficient=0.0088.

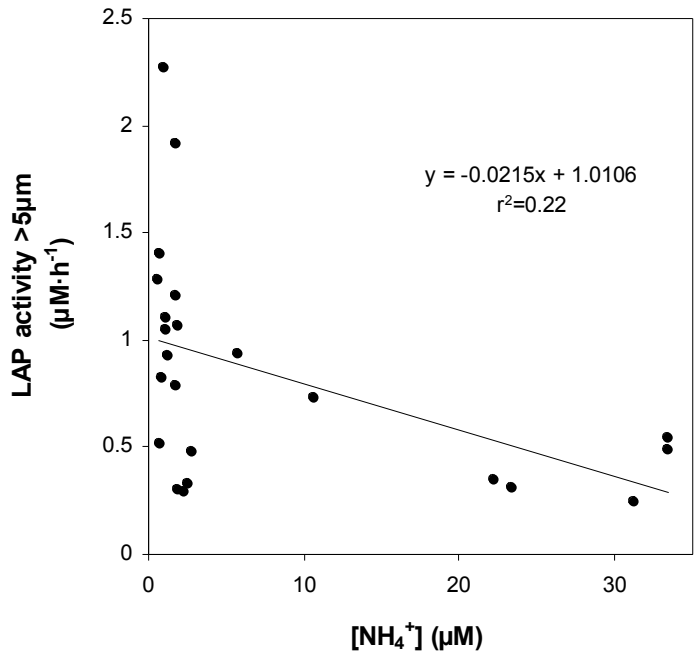


Table 2.1. Cell density, cell-specific chlorophyll *a*, C and N content, C:N and N:P rations of *P. minimum* cultures (mean ± st.dev.), time course experiments in different media. Averages have been calculated over the stationary phase.

Table 2.2. Average of LAP activity associated with different size fractions: *P. minimum* fraction (>5 μm), bacterial fraction (0.1-5 μm), and free phase (<0.1 μm) (mean ± st.dev.). Averages have been calculated over the stationary phase, relative percentages are shown in parentheses. For >5 μm fraction LAP activity is expressed also as activity per cell and per unit of chlorophyll *a*.

Table 2.3. Dinoflagellate abundance, environmental parameters and LAP activity in Choptank, Patuxent and Corsica Rivers, 2003. s = surface water sample, d = 1 m above the bottom.

Table 2.4. Major dinoflagellate species in Choptank, Patuxent and Corsica Rivers, 2003 (s = surface

Sampling station/date	*Heterocapsa rotundata*	*Heterocapsa triquetra*	*Karlodinium micrum*	*Prorocentrum minimum*	Small naked spp.	Small armored spp.
Choptank Rv						
19 March						
5s	6589					
5d	5682		1802			
7s	35757	167				
7d	30768					333
9s	67855	333			333	
9d	68687	333			499	
11s	50868				337	
11d	88261				674	
13s	41772					
1 April						
3s	3774					
3d	3458					
5s	4118	69		23	69	
5d	2237			23		
7s	68				68	
7d	34024					
9s	38751				166	
9d	41245					
11s	67711					
11d	55548					
14 April						
3s		10	91	19	185	183
3d	1088	90	180		1450	
5s			52	22	465	
5d				46	251	
7s	90		544			
7d	604		181			
9s			22	8		
9d	23		8		15	
11s			134		77	
11d					19	

Sampling station/date	*Heterocapsa rotundata*	*Heterocapsa triquetra*	*Karlodinium micrum*	*Prorocentrum minimum*	Small naked spp.	Small armored spp.
14 May						
4s			1088	15330		75
5s			1472	7756		
6s			1813	9045		725
7s			576	7237		
8s			1451	7615		57
9s			731	16045		
11s			94	5368		
13s			24	198		
14s				405		
Patuxent Rv						
3 April						
SW4s	181		497			46
SW4d	19		19	2610	209	114
PL4s	549	23	343	23		
PL4d	1469		367	152		
W13s	91900	23	316	23		
W13d	126000				38	

50

Corsica Rv	
27 October	
A	65700
B	7280

CHAPTER 3

Alkaline phosphatase activity

of the mixotrophic dinoflagellate *Prorocentrum minimum* (Dinophyceae)

ABSTRACT

Alkaline phosphatase (AP) activity in marine and freshwater communities has been associated with phosphorus (P) limitation. AP activity was measured using a fluorogenic artificial substrate in cultures of *Prorocentrum minimum* (Pav.) J. Schillerand in samples collected during a monospecific *P. minimum* bloom in Corsica River (MD, USA). The effect of growth phase, inorganic nutrients, irradiance and diel cycle on AP activity were tested. Activity was partitioned among the dinoflagellate (>5 μm), small microbe (0.1-5 μm) and dissolved free (<0.1 μm) size fractions. Alkaline phosphatase in cultures exhibited a strong response to P-deficiency, increasing up to 330 times in P-depleted cells compared to P-replete conditions. After addition of phosphate, AP activity showed a rapid decrease to 54 and 36% of the initial value after 12 and 24 h respectively, In laboratory cultures AP activity showed a temporal pattern related to light-dark changes. Although AP activity is thought to be primarily due to algae, my results indicate that in natural assemblages, even during a dense *P. minimum* bloom, the contribution of this dinoflagellate to alkaline phosphatase activity in water column can be low (5 to 8% of total) if inorganic phosphate concentration is relatively high. *In situ* cell specific AP activity and chlorophyll *a* specific activity were similar to that observed in *P. minimum* cultures grown in phosphate-replete media, indicating that *P. minimum* cells were not P-limited during the bloom.

INTRODUCTION

In aquatic ecosystems nutrient deficiency is often studied as it is generally believed that variation in nutrient limitation may control growth and collapse of blooms (Sala et al. 2001). While in freshwaters phosphate is the primary nutrient limiting phytoplankton growth (Ryther and Dunstan 1971, Schindler 1977), there is a recent debate about which is the more likely the limiting nutrient for primary production in marine systems (Downing 1997, Tyrrell and Law 1997, Tyrrell 1999, Guildford and Hecky 2000, Karl et al. 2001). Nitrogen is generally considered the major limiting nutrient for primary production and growth of phytoplankton in marine systems, but recent studies demonstrate that phosphorus in addition to being the major limiting factor in lakes, also can be a significant limiting factor in some oceanic regions including the subtropical Pacific, Sargasso Sea, western tropical and subtropical Atlantic, northern and eastern Atlantic (Gruber and Sarmiento 1997, Guildford and Hecky 2000, Wu et al. 2000, Karl et al. 2001, Dyhrman et al. 2002, Ammerman et al. 2003, Sundareshwar et al. 2003), northeastern margin of the Gulf of Mexico (Smith 1984), Scandinavian fjords (Myklestad and Sakshaug 1983), and the Mediterranean Sea (Krom et al 1991, Thingstad et al. 1998, 2005, Zohary and Robarts 1998). A seasonal phosphorus limitation of phytoplankton growth rate has been demonstrated in various coastal regions including Chinese coastal waters (Harrison et al. 1990), and Chesapeake Bay (Fisher et al. 1992).

It is important to understand the factors controlling phytoplankton growth particularly for species that may form harmful algal blooms (HABs). A number of studies suggested that phosphorus may be the limiting nutrient in some phytoplankton blooms (Granéli et al. 1993, Dahl and Tangen 1993, Dyhrman and Palenik 1997).

Phosphate stress may play a role in inducing toxin production in some harmful algae (Anderson et al. 1990, Edvardsen and Paasche 1992, Carmelo and Baden 1993, Hallegraeff 1993, Meldahl et al. 1993, Pan et al. 1996). In addition, P-limitation may induce encystment in some dinoflagellate species (Anderson and Lindquist 1985).

Nutrient limitation may induce cells of some species to synthesize ectoenzymes that allow the acquisition of the nutrients in low supply from alternative sources such as dissolved organic compounds (Hoppe 1983). From this perspective ectoenzyme activities may serve as a proxy for nutrient limitation, by describing the physiological state of the cell or the total microbial community (Sala et al. 2001). Nutrient concentrations by themselves are not adequate indicators of nutrient limitation of individual species because different species are likely to be differently sensitive to nutrient concentrations due to different physiologies (e.g. different nutrient requirements and uptake capabilities). Some algae can store inorganic nutrients and thus nutritional history is also important; therefore, nutrient cycling rates may not be reflected by one-time assessments of nutrient concentration (Dyhrman and Palenik 1999). Ectoenzymes known to be induced by nutrient stress can indicate the nutritional state of phytoplankton. In addition, ectoenzymes can have an important impact not only on ecology of species by which they are produced, but also on fluxes and distribution of nutrients and organic matter in the water column.

Ectoenzyme activity associated with phytoplankton may enhance degradation of DOM and nutrient recycling during blooms. Among dissolved organic phosphorus (DOP) compounds, phosphomonoesters seem to be selectively remineralized (Clark et al. 1998), therefore phosphomoesterases, such as alkaline phosphatase, are important in DOP cycling (Karl and Björkman 2002). Alkaline phosphatase (AP) is a cell-surface

enzyme that hydrolyzes organic phosphorus compounds at alkaline pH, typically 7.2-9.8 (Kuenzler and Perras 1965). This enzyme acts on phosphate esters such as sugar phosphates, phospholipids, and other phosphomonoesters, releasing orthophosphate groups that may be taken up by cells. Distribution and seasonal variation of AP activity in coastal waters have been investigated previously, and most AP activity in coastal surface waters is thought to be produced by algae (Ammerman 1990, Yamaguchi et al. 2004b).

A putative AP protein has been identified and purified from cell surface of the dinoflagellate *Prorocentrum minimum* (Pav.) J. Schiller (Dyhrman and Palenik 1997). This ectoenzyme is a membrane associated multimeric metalloprotein. Both AP activity and the presence of the AP protein have been shown to be induced in response to P stress in late-log phase cultures (Dyhrman and Palenik 1997, 1999) and lost after phosphate addition (Dyhrman and Palenik 2003). Regulation of alkaline phosphatase by phosphate supply in *P. minimum* has also been demonstrated *in situ*. In field samples collected in Narragansett Bay, Dyhrman and Palenik (2001) found a negative correlation between number of cells expressing AP and phosphate concentration in the water, and observed that the number of cells with active alkaline phosphatase decreased after phosphate addition (Dyhrman and Palenik 1999).

Ectocellular enzyme activities may be controlled not only by nutritional status of cells but also by chemical or physical environmental parameters. A number of biotic and abiotic factors may control the synthesis and expression of ecto- and exo-enzymes including pH, the presence of humic substances, oxygen conditions, hydrogen sulphide, UV-B radiation, heavy metals and herbicides (Nausch and Nausch 2000). The regulation of alkaline phosphatase in aquatic environments by phosphate availability has

been extensively described (Halemejko and Chróst 1984, Gage and Gorham 1985, Paasche and Erga 1988, Hernández et al. 1993, Sala et al. 2001). It appears that production and regulation of alkaline phosphatase is different in different phytoplankton groups (Rivkin and Swift 1979, 1980, Cembella et al. 1984a, Dyhrman and Palenik 1997, 1999). Generally AP activity is thought to be produced under phosphate-deficient conditions and repressed by the presence of inorganic P (Kuenzler 1965, Cembella et al. 1984a, 1984b, González-Gil et al. 1998, Yamaguchi et al. 2004a). Due to phosphate-regulation, alkaline phosphatase is often used as a biochemical indicator of P stress or P deficiency (Perry 1972, Pettersson 1980, Smith and Kalff 1981, Gage and Gorham 1985, Ammerman 1990, 1993, Vrba et al. 1995, Rose and Axler 1998). Although the synthesis of AP generally is induced by low phosphate concentration in the water column (Cembella et al. 1984a), the threshold concentration inducing AP activity varies among phytoplankton species. For dinoflagellates, particularly red tide forming species, the threshold concentration typically ranges between 0.2 and 0.4 μM (Dyhrman and Palenik 1999, Yamaguchi et al. 2004a).However, the threshold phosphate concentrations for AP activity in the dinoflagellate *Gymnodinium catenatum* is many times higher in the other dinoflagellates in which AP induction has been investigated (Ho et al. 2002). Yamaguchi et al. (2005) proposed the classification of marine phytoplankton into at least three groups on the basis of AP activity: species that are not able to produce AP such as *Heterosigma akashiwo* and *Fibrocapsa japonica* (Yamaguchi et al. 2004a, Yamaguchi et al. 2005), species that synthesize AP under phosphate stress such as *Prorocentrum minimum* (Dyhrman and Palenik 1999), *Gymnodinium catenatum* (Ho et al. 2002), *Cheaetoceros ceratosporum* (Tamaguchi et al. 2005), *Karenia mikimotoi* and *Skeletonema costatum* (Yamaguchi et al. 2004a), and

species that can produce AP only when phosphate concentration is low and organic phosphorus compounds are present such as *Heterocapsa circularisquama* (Yamaguchi et al. 2005). These observations indicate that different species have different capability to utilize organic phosphorus compounds (Yamaguchi et al. 2005). For this reason it is important to investigate AP production and regulation in different phytoplankton species.

Although many studies have been carried out in order to demonstrate the expression of alkaline phosphatase in phosphate stressed cultures of phytoplankton species (González-Gil et al. 1998, Dyhrman and Palenik 1997, 1999, 2001, Rengefors et al. 2001), cell specific and chlorophyll specific hydrolytic rates have been reported only for few species. The effects of many environmental parameters on AP activity have not been quantified. Moreover, 30 to 50% of pelagic AP activity has been found in the dissolved phase indicating that it is not always associated with phytoplankton cell surfaces (Wetzel 1981).

In this study *P. minimum* was used as the model organism. This species blooms in a number of natural environments that experience seasonal or periodic phosphate stress, including Chesapeake Bay. Using cultured *P. minimum*, the regulation of alkaline phosphatase activity by inorganic nutrients, irradiance, diel cycle, and growth phase was investigated. Additionally the partitioning of enzyme activity into different size fractions was also studied to determine if the size fraction containing *P. minimum* made a significant contribution to bulk enzyme activity during a bloom of this species.

MATERIALS AND METHODS

Protist cultures. A non-axenic culture of *P. minimum* (D-5) isolated by Matthew D. Johnson from the Choptank River (Cambridge, MD, USA), a tributary of the Chesapeake Bay and maintained in the Horn Point culture collection for several years was used for all experiments. The culture was maintained in f/2-Si medium (Guillard 1975), at salinity 15, at a temperature of 15° C and with a 14:10-h light:dark photocycle under cool white fluorescent bulbs at 60 μmol photons·m^{-2}·s^{-1}. Media were prepared using water collected from Indian River filtered onto Whatman GF/F glass microfibre filters (Whatman International Ltd. Maidston, England) and autoclaved.

Parameters measured. For *P. minimum* cell enumeration, 1 to 5 mL samples, depending on cell densities, were fixed with glutaraldehyde (final concentration 1%) and filtered onto 2 μm pore size black membrane filters (Porerics Corporation, Livermore, CA, USA). Filters were mounted on glass slides with immersion oil and capped with coverslips to be ready for epifluorescence microscopy. Slides were stored frozen at -20° C and were subsequently examined with standard epifluorescence microscopy techniques (Gifford and Caron 2000) and enumerated at 400 x.

For enumeration of bacteria, samples were fixed in 2% glutaraldehyde (final concentration), stained with 4,6-diamidinophenylindole (DAPI, Sigma Inc., St. Louis, MO, USA), filtered onto 0.2 μm polycarbonate filters (Poretics Corporation, Livermore, CA, USA), and counted with epifluorescence microscopy (Nikon Eclipse E800, 1000 x) (Porter and Feig 1980). The presence of bacteria was also checked on the 5 μm polycarbonate filters used for fractionation in enzyme measurements, since this fractionation was designed to retain *P. minimum* cells but not bacteria.

Chlorophyll *a* content was determined by standard fluorometric method (Parsons et al. 1984). Three replicates were used for each sample. 2.5 to 10 mL aliquots of

cultures were collected by vacuum filtration onto Whatman GF/F glass microfibre filters (Whatman International Ltd. Maidston, England) that were immediately extracted or stored at -20° C for a later analysis. Extraction was performed in the dark in 90% acetone for 24 h at 4° C, and chlorophyll *a* concentration was determined using a Turner Design Model 10 fluorometer calibrated with pure Chl *a* against a Beckman model DK-22 spectrophotometer.

Dissolved inorganic nutrients (N, P) were measured following standard procedures (Grasshoff et al. 1983). To determine intracellular nutrient ratios as indicators of N and P limitation, cells were collected at the beginning of the stationary phase and filtered onto precombusted Whatman GF/F glass microfibre filters (Whatman International Ltd. Maidston, England) and analyzed for CHN and particulate phosphorus. Particulate C, H, and N were determined by high temperature combustion in an Exeter Analytical Model CE400 Elemental Analyzer and particulate phosphorus analysis (Andersen 1976).

AP activities in different size-fractions were compared. To determine how the activities were distributed among different size fractions subsamples were divided into untreated and filtered samples. Activities were measured in unfractioned samples (total activity), in samples gently filtered through a 5 µm pore size polycarbonate filters (Whatman Nuclepore®, Clifton, NJ, USA) and in samples gently filtered through 0.1 µ m pore size polycarbonate filters (Whatman Nuclepore®, Clifton, NJ, USA). The 5 µm porosity was used so that the dinoflagellate cells would be retained but most bacteria, including small bacterial aggregates, would be in the filtrate. Activity associated with the dinoflagellate fraction was calculated by the difference between activity in unfiltered samples and activity in <5 µm fraction. Bacterial ectocellular enzyme activity was

calculated in cultures as the difference between activity in the <5 μm and 0.1 μm fraction. Exocellular enzyme activity was calculated as the activity in the <0.1 μm fraction (cell free fraction). Herein, cell specific and chlorophyll *a* specific activities are calculated based on activity in dinoflagellate fraction (>5μm).

Alkaline phosphatase activity was measured using the fluorogenic artificial substrate analog 4-methylumbelliferyl phosphate (P-MUF) (Sigma Chemical, St. Louis, MO, USA) (Hoppe 1983, Hoppe et al. 1988). The substrate stock solution was prepared by dissolving P-MUF in 2-ethosyethanol (50%) and distilled water (50%) (5 mM final concentration) and stored frozen until use. For the assay 30 μL aliquots of substrate stock solution were added to 1 mL of culture in semi-UV cuvettes (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA), resulting in a final concentration of 145 μM P-MUF. Preliminary experiments have shown this concentration to be saturating. Michaelis-Menten kinetics was assumed. Samples were incubated in the dark at 15° C for 30 min. At the end of the incubation, sample fluorescence was immediately read with a Shimadzu Rf-5301PC spectrofluorometer (excitation 455 nm, emission 365 nm). Increases in fluorescence reflected the release of the fluorescent part of the substrate analogs (MUF) via enzyme-catalyzed hydrolysis, therefore enzyme hydrolysis of substrate will be referred to as enzyme activity. Half-saturation constant (K_s) and maximum rate of hydrolysis (V_{max}) were calculated as the mean of values estimated from linear fits to Lineweaver-Burk and Eadie-Hofstee plots.

Two types of controls were used. The first type was the samples with no added substrate, to control for natural fluorescence in the culture. The second control was autoclaved media with added substrate which was incubated along with the experimental samples in order to control for fluorescence due to the substrate or to

bacterial contamination of the substrate that could result in product formation. For each essay triplicate cuvettes were used.

Everytime the enzyme assays were performed a standard curve was prepared in order to convert increases in fluorescence to hydrolysis rates. To prepare the standard curve, autoclaved filtered (0.1 μm) media and concentrations of the product [4-methylumbelliferone (MUF) (Sigma Chemical, St. Louis, MO, USA)], ranging from 0 to 10 μM were used.

To test if exposure to the substrate causes the dinoflagellates to lyse, releasing intracellular enzymes, samples of *P. minimum* were fixed with glutaraldehyde before and after 30 min exposure to substrate concentration equal to 1, 2 and 3 times the concentration used for enzyme assays, and checked with microscopy for evidence of cell lysis.

All statistical analyses were done with Analyse-it™ Version 1.71 (Analyse-it Software, Ltd.). Parametric tests were used unless data failed tests for homogeneity of variance and normality, in these cases, non-parametric tests were used.

Time course experiments. Time course experiments were run to determine whether enzyme activities depend on growth phase and nutrient concentrations. Cultures were grown at 15° C with a 14:10-h light:dark cycle under cool white fluorescent bulbs at 60 μmol photons·m^{-2}·s^{-1}. Cells in stationary phase were used as an inoculum to provide an initial cell concentration of 2000 cells·mL^{-1}. The four nutrient concentration treatments consisted of f/2 (883 μM NO_3^{2-} and 36 μM PO_4^{2-}), f/4 (441 μM NO_3^{2-} and 18 μM PO_4^{3-}), f/2-lowN (36 μM PO_4^{3-} and 5.5 μM NO_3^{2-}), and f/2-lowP (883 μM NO_3^{2-} and 0.5μM PO_4^{3-}). Since local water was used to prepare media, inorganic nutrient additions were on top of background inorganic concentrations (1.27 μM

NO_3+NO_2, 2.88 µM NH_4, PO_4 not available). Triplicates were used for each treatment. Sampling occurred every other day until cells reached the stationary phase.

Nutrient addition experiments. A second short-term experiment was run to determine the effect of phosphate on AP. Treatments in triplicate consisted of additions of PO_4^{3-} (50 µM), and control without the addition. Nutrients were added in a single input. AP activity was measured at time 0, 12, 48 h. The experiment was carried out at 15° C with a 14:10-h light:dark cycle under cool white fluorescent bulbs at 60 µmol photons·m^{-2}·s^{-1}.

Effect of irradiance on AP activity. To examine effects of light level on AP activity, triplicate bottles with *P. minimum* culture in f/2 medium were allowed to acclimated for 10 days on a 12:12-h light:dark cycle at the following irradiances: 4, 53 and 267 µmol photons·m^{-2}·s^{-1} . The incubation temperature was 15° C. At the end of the acclimation period, AP activity was measured.

Diel cycle experiment. Temporal variations of AP were assessed at 4-h intervals over two 24-h periods in *P. minimum* cultures growing in f/2-lowP, at 15° C, with a 12:12-h light:dark cycle under cool white fluorescent bulbs at 60 µmol photons·m^{-2}·s^{-1}. The light was turned on at 6 h and turned off at 18 h. AP assays were performed at 2, 6, 10, 14, 18, 22 h on two consecutive days. At 6 and 8h, the AP assays were done immediately after the lights had been turned on or off.

Field experiments. Sampling occurred during a monospecific bloom of *P. minimum* Corsica River on October 27 2003. Samples were collected at two locations: Station A is located at Gunston Day School dock (Centreville, MD, USA) and Station B at Centreville public dock. *In situ* temperature and salinity were measured with a portable YSI SCT meter. Samples were collected at the water surface, filtered through a

Nitex mesh to obtain the <200 μm fraction (containing phytoplankton, microzooplankton and bacteria), and another subsample was gently filtered through a 5 μm polycarbonate filters (Whatman Nuclopore®, Clifton, NJ, USA) to obtain a <5 μm fraction (containing bacteria and small protists but not *Prorocentrum*). Total and fractionated AP activities were measured. Dinoflagellates were counted in samples fixed in glutaraldehyde (final concentration 1%). Chl *a* and nutrient analyses were also performed.

RESULTS

Time course experiments with inorganic nutrient treatments.

Cell densities and Chl *a* during *P. minimum* growth are shown in Fig. 3.1 and values for stationary phase are summarized in Table 3.1. P-deficiency greatly affected the number of *P. minimum* cells. Cell densities at stationary phase in f/4 and the low N treatment were not significantly different from that in f/2, while in the f/2-lowP treatment all densities were significantly different and lower (reduction: 30.8%; 1-way ANOVA, $p<0.0001$). P deficiency did not affect chlorophyll *a* content while N deficiency resulted in a significant reduction of 71% in chlorophyll *a* per cell (1-way ANOVA, $p<0.0001$). Content of C and N (Table 2.1) indicate that *P. minimum* cells are smaller in biomass in f/4 and f/2-lowN treatments. P content and N:P ratio indicate that in low P treatment cells are P limited, low phosphate availability leads to the reduction of P content and also N content.

Substrate saturation curve, Lineweaver-Burk plot and Eadie-Hofstee plot are shown in Fig. 3.2. The half-saturation constant (K_s) estimated for the hydrolysis of P-MUF was 32.83 μM, and the maximum rate of hydrolysis (V_m) was 0.60 $\mu M \cdot h^{-1}$. This

high value for the half-saturation constant was calculated for *P. minimum* growing in f/2 where the concentration of phosphate was 36 μM.

AP activity was measured during the growth curve of *P. minimum* grown in four different media. Since cultures were not axenic, the experiment was designed for the partitioning of the activity among the *P. minimum* fraction (>5 μm), bacterial fraction (0.1 to 5 μm), and free fraction (<0.1 μm). AP activity associated with different size fractions are shown in Fig. 3.3 and mean values calculated over late exponential and stationary phase are given in Table 3.2. In media with high phosphate concentration (f/2, f/4, f/2-lowN), AP activity was distributed among different fractions with similar pattern: 9 to 22% of AP activity was associated with the *P. minimum* fraction while the bacterial fraction contributed 37 to 41%, and the free dissolved phase appeared to be the most important component representing 38 to 54% of total activity. The smallest contribution from the *P. minimum* fraction was observed in the low nitrate treatment. In the low phosphate treatment, the contribution of *P. minimum* fraction to total activity increased to up to 64% while that of bacteria fraction slightly decreased (21%) and the free dissolved phase strongly decreased to 15% of total activity.

Total activity associated with the *P. minimum* fraction was negligible in nutrient-replete cells, but was significant during P-deficiency. In the low phosphate treatment, AP activity was up to 250 times higher than that in the other treatments (Table 3.2). Statistical analysis for total AP activity associated with >5 μm fraction in different treatments are reported in Table 3.3, showing that the difference between phosphate-replete treatments and phosphate stress treatment is statistically significant, while small differences among phosphate-replete treatments are not.

Cell specific alkaline phosphatase activity in *P. minimum* fraction was calculated over the growth curve; mean values for stationary phase are reported in Table 3.2. In the high phosphate treatments cell specific AP activities during lag and exponential phase were one order of magnitude higher than in stationary phase (data not shown). In the low P treatment, cell specific AP activity sharply increased during exponential phase (Fig. 3.4) reaching values 61-fold higher than that in lag phase and 330-fold higher than that in other treatments. Cell specific AP activities in phosphate-replete treatments were similar, while in the low phosphate treatment AP activity was significantly higher (Table 3.3). With respect to cell abundance, the cell specific AP activity measured in high phosphate treatments (f/2, f/4, and f/2-lowN) was inversely correlated with *P. minimum* cell density while it was positively correlated with cell density in the low phosphate treatment (Fig. 3.5).

Chl *a* specific AP activity during the *P. minimum* growth curve was calculated. Mean values for stationary phase are reported in Table 3.2. As with cell specific AP activity, chlorophyll *a* specific activities in high phosphate treatments during lag and exponential phase were one order of magnitude higher than in stationary phase (data not shown), while chlorophyll *a* specific AP activity sharply increased during exponential phase in the low phosphate treatment (Fig. 3.6). During stationary phase the chlorophyll *a* specific alkaline phosphatase activity was more than 100 times higher in the low phosphate treatment than in phosphate-replete media (f/2, f/4, and f/2-lowN). Chlorophyll *a* specific AP activities in all treatments with high phosphate concentration were similar, while activity was significantly higher in the phosphate stress treatment (Table 3.3). The correlation between chlorophyll *a* specific AP activity and chlorophyll

content of *P. minimum* cells was negative in high phosphate treatments, but positive in the phosphate stress treatment (Fig. 3.7).

Nutrient addition experiment. In a short-term single nutrient pulse experiment, phosphate was added to a culture of *P. minimum*. The results, shown in Fig. 3.8, indicate that addition of phosphate affected the cell specific AP activity associated with *P. minimum* fraction (>5 μm), leading to a significant reduction in AP activity of 46% after 12 h and 64% after 48 h.

Effect of irradiance on AP activity. To assess if irradiance have any effect on AP activity in nutrient replete cells, I measured AP activity associated with *P. minimum* fraction at three light levels. The cell specific activity measured after 10 days of acclimation are shown in Fig. 3.9. No significant difference was found between activity at 4 and 53 μmol photons·m^{-2}·s^{-1} (1-way ANOVA, p=0.5033), while AP activity at 267 μmol photons·m^{-2}·s^{-1} was significantly different (1-way ANOVA, p=0.0053) and lower.

Diel cycle experiment. A clear temporal variation was found for cell specific AP activity as shown in Fig. 3.10. A sharp decrease in AP activity was observed everytime the light was turned on or turned off. After decreasing, AP activity slowly increased reaching the maximum values at 6 h and 18 h. Minimum values were registered at the middle of the day and the night.

Field experiment. Samples were collected at two stations in the Corsica River during a monospecific bloom of *P. minimum*. Dinoflagellate density, AP activity associated with >5 μm fraction and environmental parameters are reported in Table 3.4. Since *P. minimum* was the only >5 μm phytoplankton species present in the water, cell specific and chlorophyll *a* specific AP activity were also calculated (Table 3.4). *P. minimum* density and chlorophyll *a* concentration were one order of magnitude higher

in station A than in station B. The water was discolored (Plate 3.1) particularly in Station A. Blooms of *P. minimum* are often referred to as "mahogany tides" due to this discoloration. Phosphate concentration was lower at Station A where total AP activity in the water was higher. Also total AP activity associated with dinoflagellate fraction (>5 μm) was higher at Station A than Station B, but this was due to higher cell density at Station A. In fact, I found a lower cell specific and chlorophyll *a* specific AP activity at Station A. I calculated the AP activity associated with the dinoflagellate fraction (>5 μm), small microbe containing bacteria (0.1 to 5 μm) and in the free dissolved phase (<0.1 μm) and results are shown in Fig. 3.11. Only a small portion of total AP activity was associated with dinoflagellate fraction, while the small microbe fraction accounted for most of the activity.

DISCUSSION

Changes in extracellular enzyme activity can be the initial response of microorganisms to environmental changes (Karner and Rassoulzadegan 1995) and have been demonstrated to be a good indicator of nutritional status of a biological system (Sala et al. 2001). For many enzymes, stimulation and repression by substrates and end-products are assumed to be the major regulatory mechanism (Chróst 1990). This appears to be true for alkaline phosphatase. Alkaline phosphatase on the outer surface of cells may cleave a variety of phosphate monoesters allowing phytoplankton to utilize organic phosphate when inorganic phosphate is no longer available (Rengefors et al. 2001).

In surface coastal waters the concentration of dissolved organic phosphorus (DOP) is often higher than the concentration of dissolved inorganic phosphate (DIN)

(Kobori and Taga 1979, Hernández et al. 2000, Yamamoto et al. 2002, Yamaguchi et al. 2004a). In coastal waters, DIP typically ranges from 0.2 to 0.4 μM with values below 0.2 μM during summer (Yamaguchi et al. 2005), while DOP seems to reach values one order of magnitude higher. In Chesapeake Bay, Fisher et al. (1988) reported values ranging from 0.1 to 1 μM (0.4 ± 0.2 μM) for DIP concentration, and from 0.5 to 1.4 (1 ± 0.4 μM) for DOP concentration, with DOP representing 67-73 % of total P. Therefore, organic phosphorus can represent a very significant portion of algal-available phosphorus and may be an important alternative source of phosphorus for phytoplankton including *P. minimum* (Malone et al. 1996).

AP in *P. minimum* exhibits a strong response to P-deficiency. The ability of *P. minimum* to increase its alkaline phosphatase activity when growing in phosphorus-limiting media has been clearly demonstrated in my study and confirms previous results of other researches (Sakshaug et al. 1984, Dyrman and Palenik 1997, 1999, 2001, Gonzáles-Gil et al. 1998). The fact that AP activity was initially low during the growth curve in the low P treatment may be due to the presence of background inorganic phosphate in local water used for preparing the medium, or may be attributable to reserves of internal phosphorus. It is also possible that the low activity was due to the fact the inoculum was from a stationary phase culture and that the cells were still in lag phase and had not yet synthesized new enzymes in response to the change in their culture conditions. In contrast, a rapid response of alkaline phosphatase to increased inorganic phosphate availability was demonstrated in this study, with AP activity rapidly decreasing after addition of phosphate to growing cultures. This is similar to observations made by Sala et al. (2001). Reduction of cell specific activity after phosphate addition was faster than the reduction in number of cells showing alkaline

phosphatase activity obtained using the ERF-97 fluorometric method (Dyhrman and Palenik 1999). Moreover, the method used in this study seems to be more sensitive to changes in cell physiology than measurements of AP positive cells using the ERF-97 fluorometric method. Dyhrman and Palenik (1999) observed AP activity only in the late exponential phase and stationary phase whereas in my study AP had already reached high values by late exponential phase. The fact that some AP activity was observed even in nutrient replete cells, demonstrates that alkaline phosphatase may be partially constitutive. However, induction of AP activity by DOP in the seawater used to make the media can not be excluded. AP activity in phosphate-replete media has also been reported by Sakshaug et al. (1984) in *P. minimum* and other dinoflagellate species. Nicholson et al. (in prep.) found AP activity in dinoflagellate taxa despite replete P conditions.

AP activity has been measured in many different phytoplankton species. In Table 3.5 I compare reported cell specific alkaline phosphatase activity in *P. minimum* with that reported in other dinoflagellates under phosphate stressed conditions. The range of AP activity measured varied by 6 order of magnitude among different species. For *P. minimum,* the AP activity measured in this study using P-MUF is 72 times higher than the activity measured by Sakshaug et al. (1984) using 3-*o*-Methylfluorescein phosphate (MFP) as the substrate. As suggested by Dyhrman (2005), one has to be careful when comparing AP activities measured in different studies using different AP substrates. Furthermore, AP activity can be strongly dependent on culture conditions and phosphate-limited growth rate (Riegman et al. 2000). It is also possible that there are large differences in AP activity among different isolates or strains of *P. minimum*.

From the Lineweaver-Burk and Eadie-Hofstee plots, a K_M of 32.82 μM was calculated. This value is lower than that reported for alkaline phosphatase from cultures of *Progonyaulax tamarensis* (50 μM) by Boni et al. (1989) and *Peridinium cinctum* by Carpené and Wynne (1986), indicating a stronger affinity for organic phosphorus by *P. minimum* than by the other dinoflagellates.

Diurnal fluctuation in AP activity has been previously noted for dinoflagellates from natural populations (Rivkin and Smith 1979, Wynne 1981, Boni et al. 1989). Highest AP activity was reported during the day and lowest during night. My results confirm a temporal cycle in AP activity related to light-dark changes, but the cycle appears to be semi-diurnal, with two maxima during the 24 h period. More frequent sampling would be necessary to resolve the pattern, however sharp reduction in AP activity seems to be associated with changes in irradiance. On average, higher AP activity was only observed during the day during the second 24-h period. However, in the light acclimation experiment, AP activity appeared to be reduced by high irradiance. Both cell specific and chlorophyll *a* specific AP activity increased with increasing chlorophyll *a* and decreasing light. This behavior may be due to the fact that at low light cells require more chlorophyll *a* and more nitrogen to build chlorophyll molecules, therefore to balance the increased uptake of nitrogen more phosphorus has to be taken up by cells.

Ecto-enzyme activities may be used as an indicator of nutritional status of the community, but since they can be produced by a diversity of organisms, it is necessary to identify the organisms that synthesize the ectoenzymes. Many studies have investigated the relative importance of bacterial, algal and free enzymes by selective filtration. Size fractionation, although it has some limitations, provides information on

distribution of enzyme activities among different types organisms (Jansson 1976, Pettersson et al. 1993, Hantke et al. 1996a, b). Alkaline phosphatase has been reported to be produced both by algae and bacteria, but the algal contribution is usually thought to be more important than the bacterial contribution. Dissolved activity is variable and may be due to either bacteria, algae or other organisms (Francko 1983).

AP activity was determined during a monospecific *P. minimum* bloom in Corsica River. The total AP activity was higher at the station characterized by lower inorganic phosphate, particularly for AP activity associated with dinoflagellate fraction (>5 μm). The same range was observed for the cell specific AP activity *in situ* and in cultures grown in phosphate-replete media. The chlorophyll *a* specific AP activities in field samples and in phosphate-replete cultures were also consistent. Values of *in situ* AP activity compared to those observed in laboratory experiment indicate that *P. minimum* cells were not P-limited during the bloom in Corsica River. Inorganic phosphate concentration was high during the dinoflagellate bloom in the Corsica River,compared to typical values reported for Chesapeake Bay (Fisher et al. 1988, 1992, 1999). N concentration was fairly low, with the N:P ratio less than the Redfield ratio, suggesting N stress rather that P stress during the bloom. The AP activity per chlorophyll *a* in my samples is 6 to 7-fold higher than the . chlorophyll *a* specific AP activity in samples from the eutrophic Lake Erken (Sweden), as reported by Rengefors et al. (2001). The cell specific AP activity calculated in this study during the monospecific bloom in the Corsica River is 6 orders of magnitude lower than that measured at stations in the Caribbean and Sargasso Seas during *Pyrocystis noctiluca* blooms by Rivkin and Swift (1979) where the ambient phosphate concentration is much lower (3-18-fold) than in Corsica River. Compared to the cell specific AP activity

measured by Wynne (1981) in samples collected from Lake Kinneret (Israel) during *Peridinium cinctum* bloom, the values reported in this study are 3 times lower.

In the field samples that I analyzed, the most important contribution to bulk alkaline phosphatase came from small microbe fraction (0.1 to 5 μm) , which was probably dominated by bacteria. The contribution from the dinoflagellates was less than 8% of the total activity, the same percentage I observed in *P. minimum* cultures grown in low nitrate medium. The AP activity associated with the bacterial fraction measured in this study (0.128-0.205 $\mu M \cdot h^{-1}$) is similar to that reported by Nausch and Nausch (2004) for bacterial samples from the Baltic Sea (0.002 to 0.164 $\mu M \cdot h^{-1}$).

The free dissolved phase made an important contribution to bulk alkaline phosphatase activity in the Corsica River, contributing 28 to 36% of total activity. This result is similar to that found by Wetzel (1981), who observed that 30 to 50% of pelagic alkaline phosphatase activity can be in dissolved phase. However, my values are lower than those calculated by Münster et al (1992) who found 50 to 70% of total AP activity free in the water column.

Although alkaline phosphatase activity in the water column is thought to be primarily due to algae, my results show that in Corsica River, even during a dense *P. minimum* bloom, the bulk AP activity was not due to the dinoflagellate fraction. It is interesting that although *P. minimum* density was high, AP activity was not elevated and cells did not appear P-limited during the bloom. However, *P. minimum* may make an important contribution to total AP activity during blooms when inorganic P concentrations are lower. Partitioning of AP activity between algal and smaller microbial fractions can not be predicted from the species composition and abundance of

phytoplankton alone, nor from inorganic nutrient availability, but is a complex response of organisms to their nutritional history and environmental conditions.

Fig. 3.1. (A) Abundance and (B) chlorophyll *a* content of *P. minimum* grown in different nutrient media.

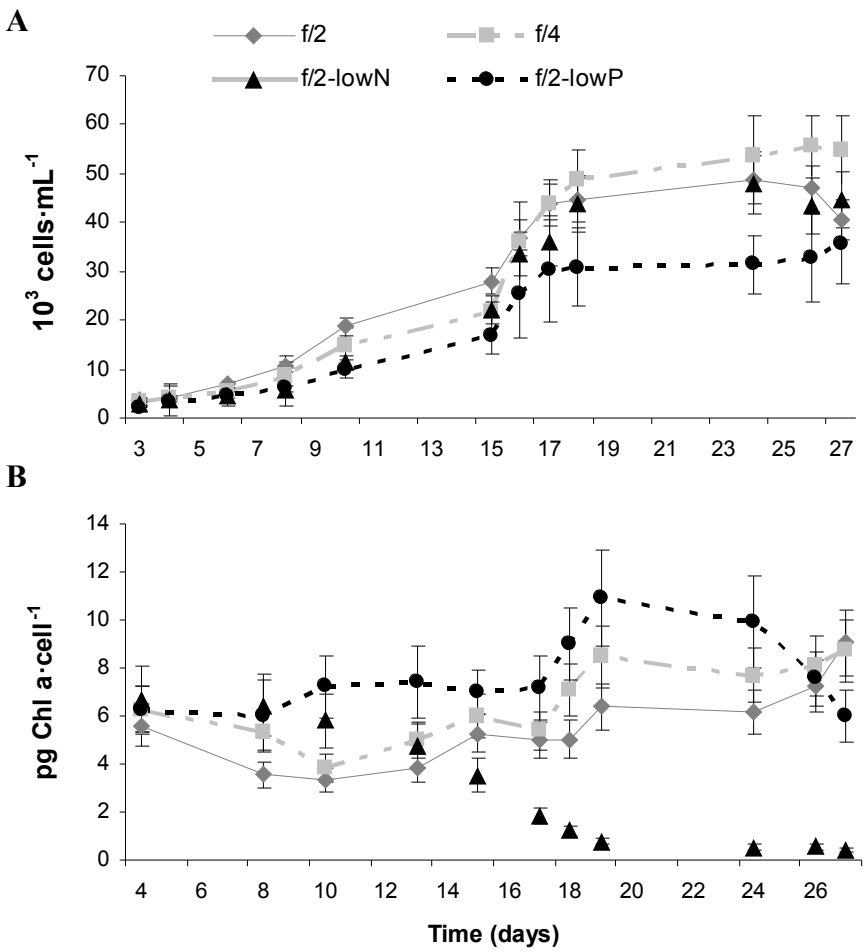


Fig. 3.2. (A) Substrate saturation curve, (B) Lineweaver-Burk plot and (C) Eadie-Hofstee plot of AP activity.

A

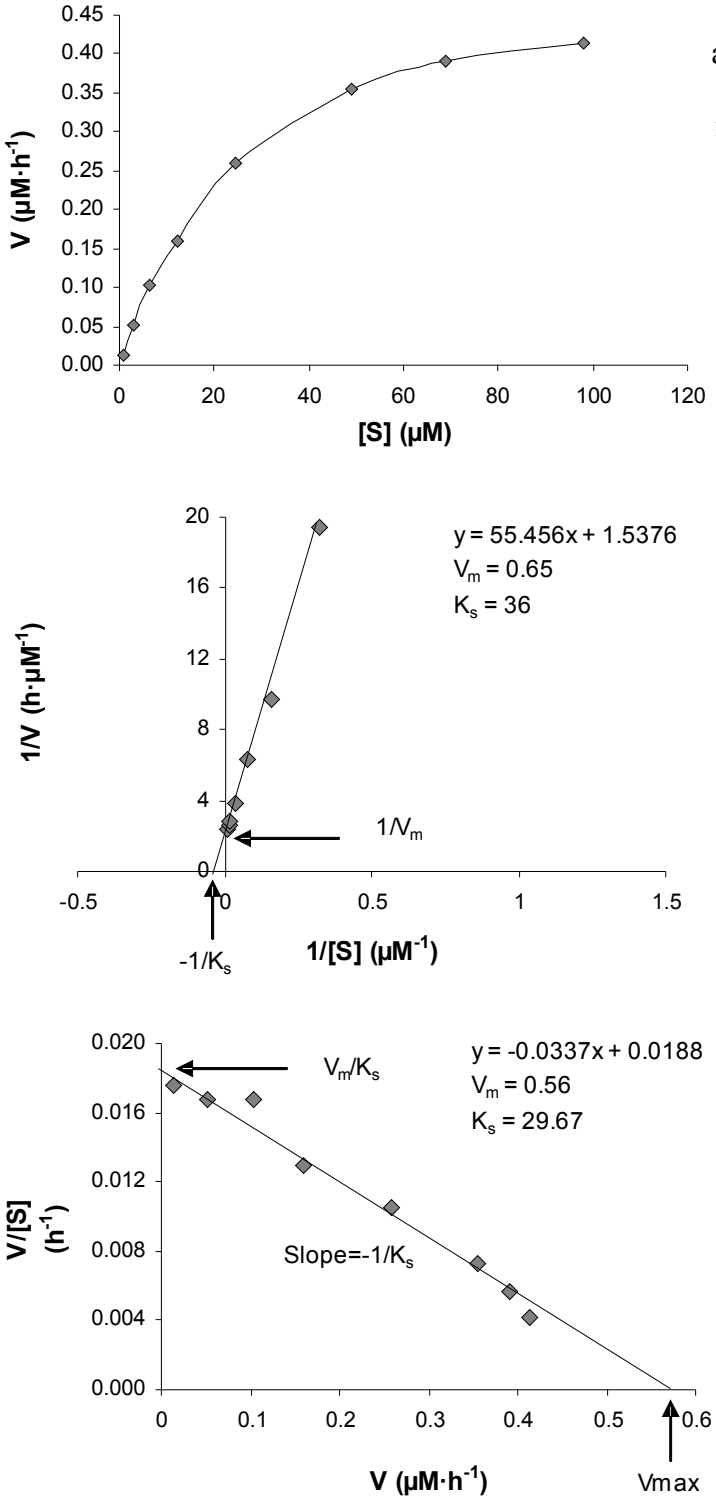
B

C

Fig. 3.3. A[illegible] ze fractions: >5 µm

(*P. minim*[illegible] ase) in four nutrient

treatments [illegible] in scale in panel D.



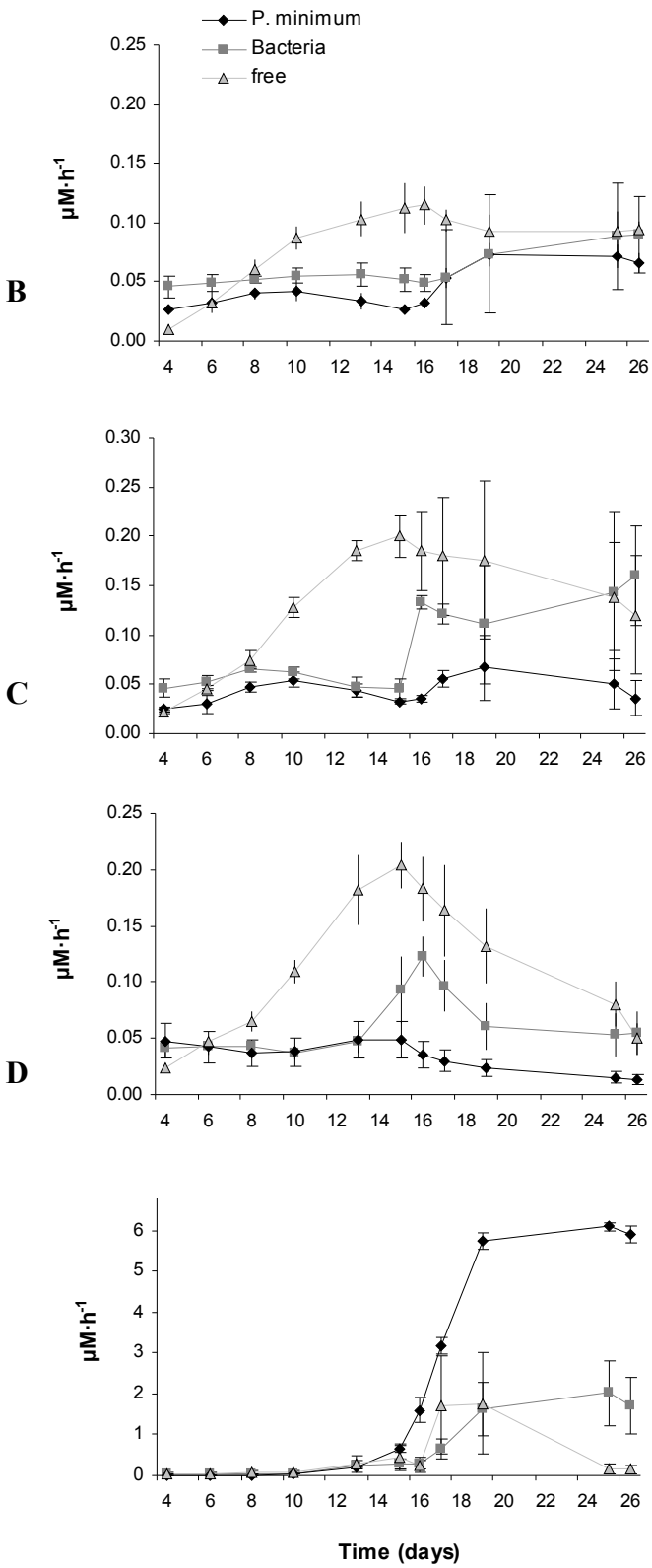

P. minimum
Bacteria
free
B
C
D
µM·h-1
Time (days)

Fig. 3.4. Cell-specific alkaline phosphatase activity and *P. minimum* cell density during growth in low phosphate treatment (f/2-lowP).

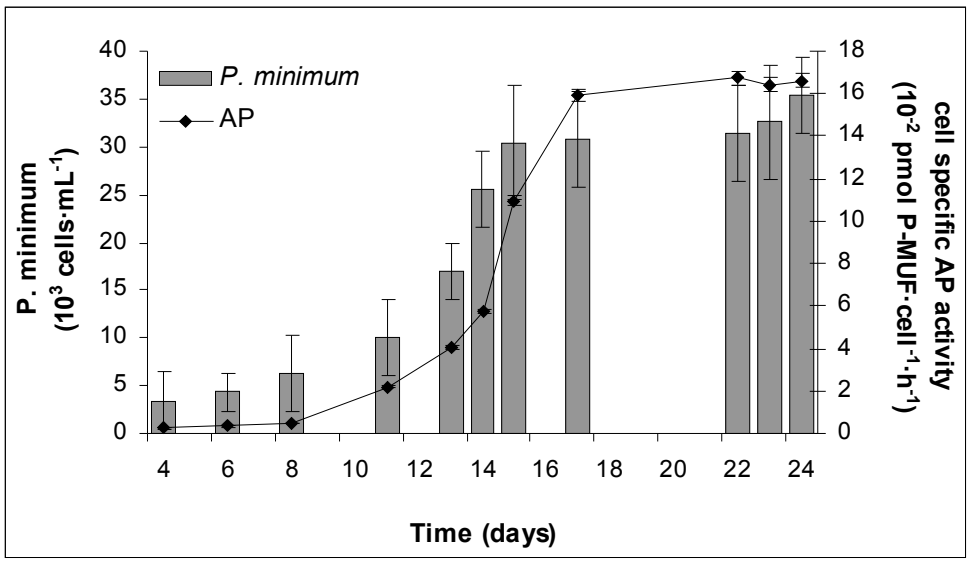


Fig. 3.5. Cell specific alkaline phosphatase activity as a function of *P. minimum* density for phosphate-replete treatments (A) (n=31, $p<0.0001$) and phosphate stress treatment (B) (n=10, $p<0.0001$).

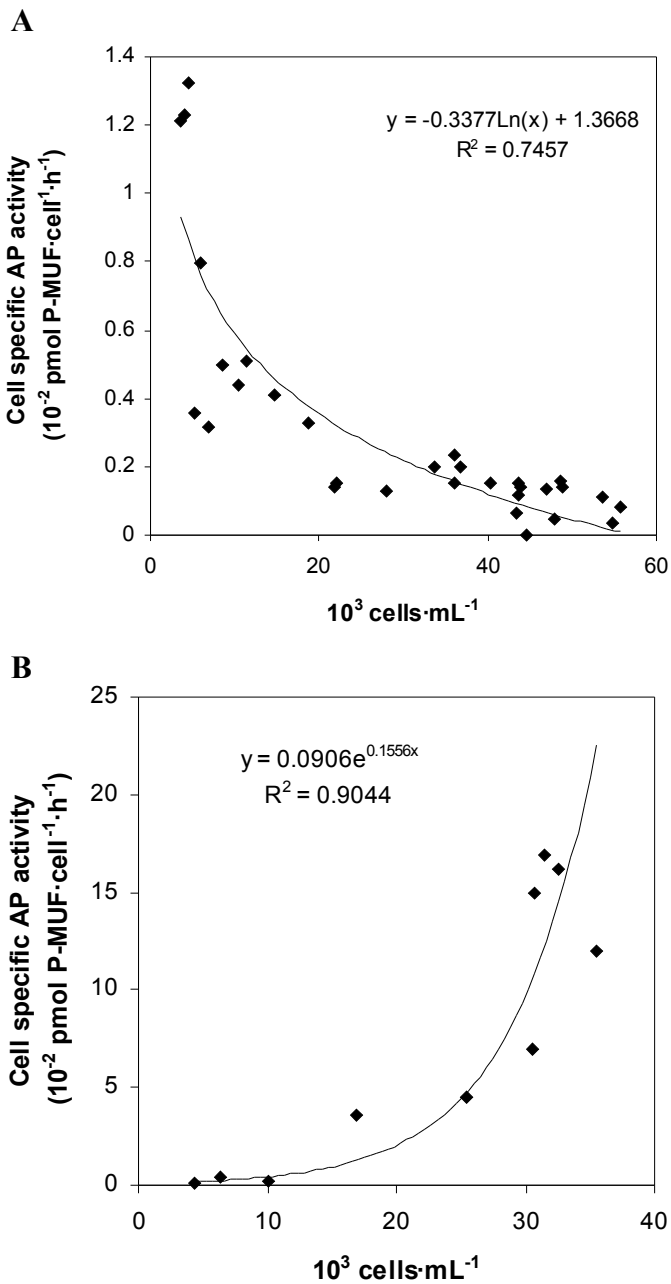


Fig. 3.6. Chlorophyll *a* specific alkaline phosphatase activity and *P. minimum* cell density during growth in low phosphate treatment (f/2-lowP).

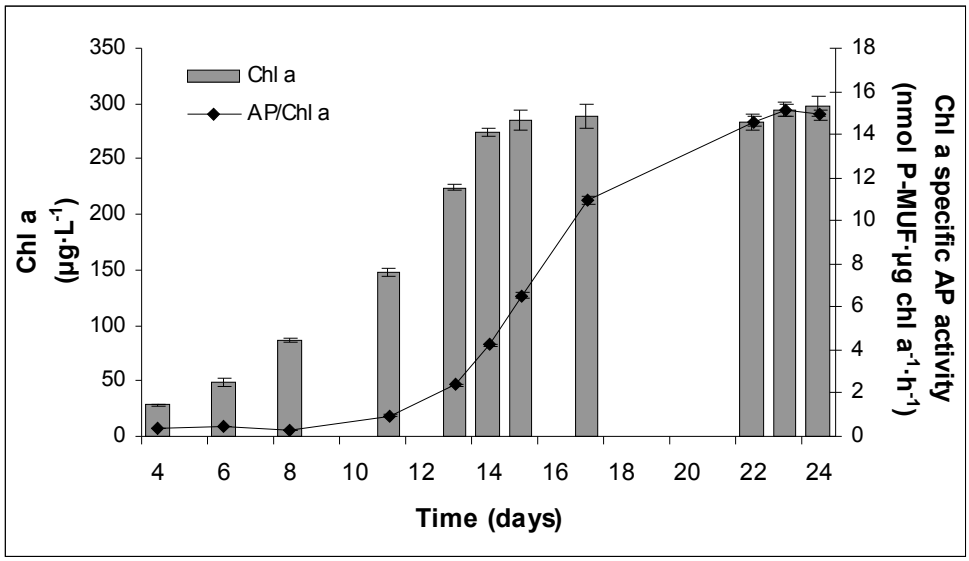


Fig. 3.7. Chlorophyll *a* specific alkaline phosphatase activity as a function of chlorophyll *a* content of *P. minimum* cells for phosphate-replete treatments (A) (n=38, $p<0.0001$) and low phosphate treatment (B) (n=11, $p<0.0001$).

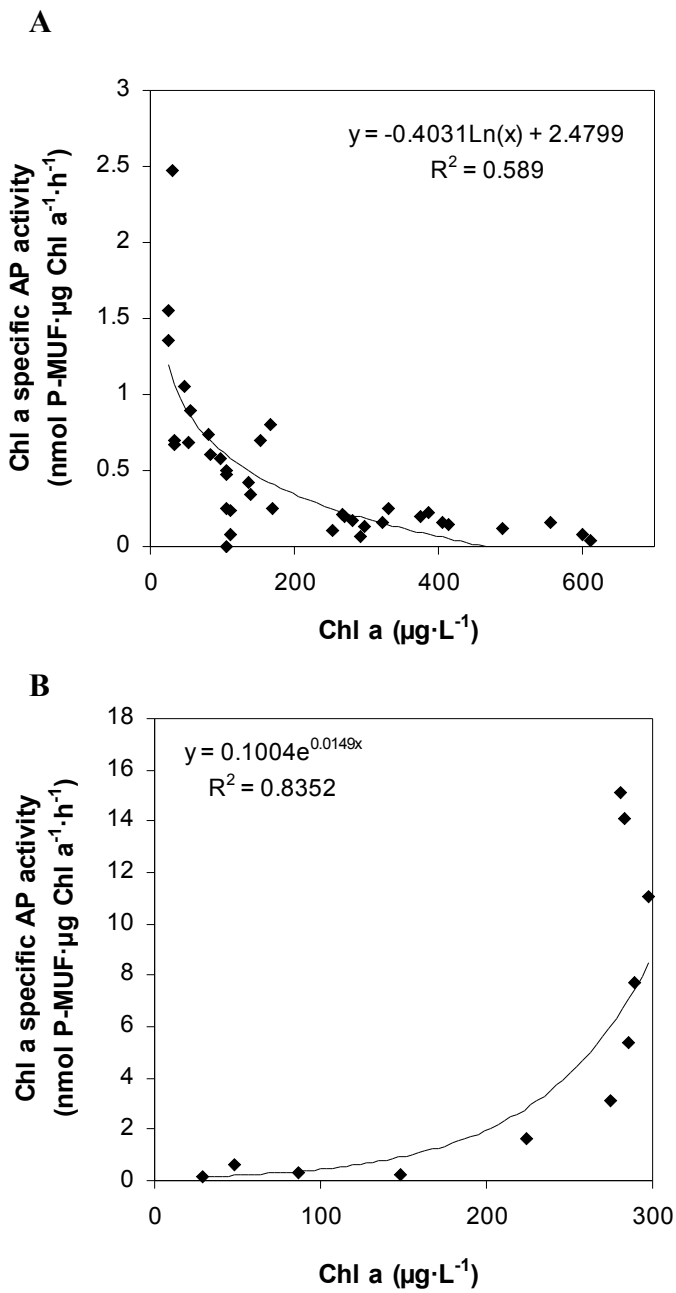


Fig. 3.8. Effects of addition of phosphate on AP activity of *P. minimum*. Treatments were designed as control (unamended), +PO3 (addition of 50 μM phosphate). * and ** indicate that AP activity in the treatment and control are significantly different (* p=0.0060; ** p=0.0005), ns indicates that AP activity in the treatment and control are not significantly different (p=0.2929) (ANOVA 1-way). Error bars denote 95% confidence intervals.

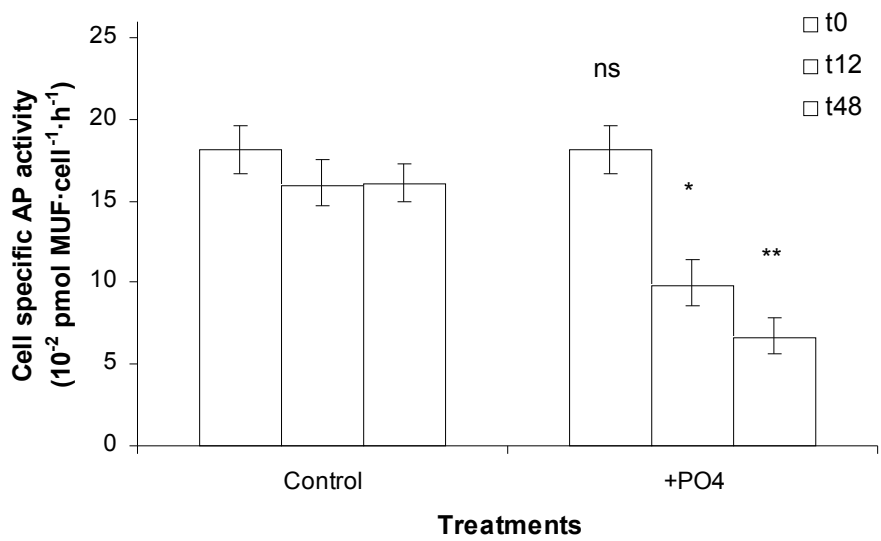


Fig. 3.9. Cell-specific AP activity of *P. minimum* at three light irradiances. Error bars denote 95% confidence intervals.

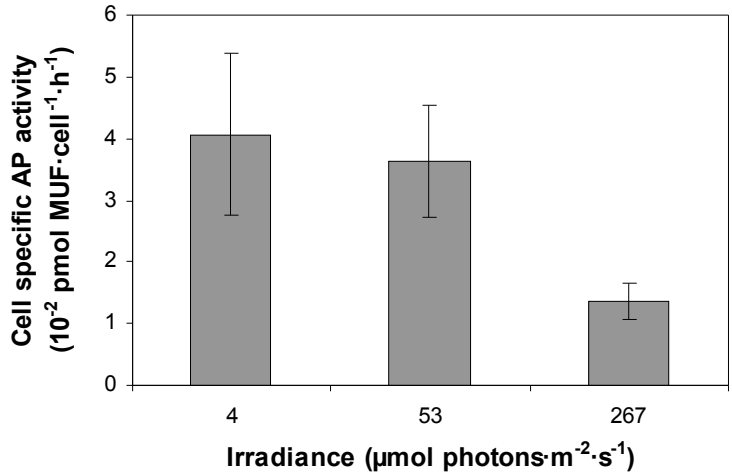


Fig. 3.10. Diel cycle of cell specific alkaline phosphatase associated with fraction >5 µm in *P. minimum* culture (f/2-lowP). Shaded gray areas correspond to dark periods.

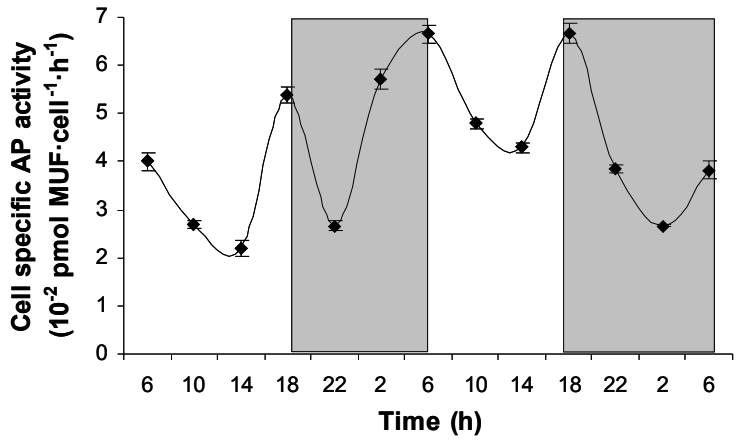


Fig. 3.11. AP activity associated with dinoflagellate fraction (>5 μm), small microbe fraction (0.1-5 μm) and free dissolved phase (<0.1 μm) in field samples collected in Corsica River.

Treatment	Cell density (10^3 cell·mL^{-1})	Chl *a* (pmol·cell^{-1})	C (pg·cell^{-1})	N (pg·cell^{-1})	P (pg·cell^{-1})	N:P (mola
f/2	45.16 ± 3.58	7.21 ± 1.31	1.25	0.17	0.015	11.3
f/4	53.22 ± 3.07	8.24 ± 0.44	0.58	0.08	0.009	8.9
f/2-lowN	44.95 ± 2.13	0.57 ± 0.17	0.96	0.06	0.011	5.4
f/2-lowP	32.56 ± 2.07	8.59 ± 2.72	1.07	0.09	0.003	28.5

Table 3.1. Cell density, cell-specific chlorophyll *a*, C, N, and P content, C:N and N:P rations of *P. minimum* cultures (mean ± st.dev.), time course experiments in different media. Averages have been calculated over the stationary phase.

Table 3.2. Average of AP activity associated with different size fractions: *P. minimum* fraction (>5 µm), bacterial fraction (0.1-5 µm), and free phase (<0.1 µm) (mean ± st.dev.). Averages have been calculated over the stationary phase, relative percentages are shown in parentheses. For >5 µm fraction AP activity is expressed also as activity per cell and per unit of chlorophyll *a*.

	>5 µm			0.1-5 µm	<0.1 µm
Treatment	µM h^{-1}	10^{-2} pmol P-MUF	nmol P-MUF	µM h^{-1}	µM h^{-1}
	(%)	·$cell^{-1}$·h^{-1}	·µg Chl a^{-1}·h^{-1}	(%)	(%)
f/2	0.07 ± 0.01	0.13 ± 0.04	0.19 ± 0.04	0.10 ± 0.07	0.10 ± 0.02
	(22.3)			(39.3)	(38.4)
f/4	0.06 ± 0.02	0.10 ± 0.03	0.11 ± 0.04	0.14 ± 0.05	0.15 ± 0.04
	(13.9)			(41.4)	(44.7)
f/2-lowN	0.02 ± 0.01	0.05 ± 0.03	0.14 ± 0.02	0.08 ± 0.05	0.12 ± 0.07
	(8.9)			(36.7)	(54.4)
f/2-lowP	5.91 ± 0.14	16.43 ± 0.36	14.90 ± 0.25	1.33 ± 0.59	0.95 ± 0.92
	(63.8)			(21.2)	(15.1)

Table 3.3. Results from 1-way ANOVA test on total AP activity associated with *P. minimum* fraction (>5 µm), cell specific and chlorophyll *a* specific AP activity. High P treatments comprise f/2, f/4, and f/2-lowN, low P represents f/2-lowP. *** = <0.0001.

Source of variation	*SS*	*df*	*MS*	*Fs*	
Total AP activity >5 µm					
Treatments					
High P vs. low P	36.427	1	36.427	24.27	***
Among high P	0.001	2	0.000	0.61	ns
Within	67.529	43	1.570		
Total	103.957	46			
Cell specific AP activity					
Treatments					
High P vs. low P	501.334	1	501.334	39.99	***
Among high P	0.101	2	0.050	0.79	ns
Within	551.455	42	13.130		
Total	1052.890	45			
Chlorophyll *a* specific AP activity					
Treatments					
High P vs. low P	409.805	1	409.805	24.25	***
Among high P	0.541	2	0.270	1.37	ns
Within	759.970	43	17.674		
Total	1170.316	46			

Table 3.4. Dinoflagellate abundance, environmental parameters and AP activity in Corsica River, 27 October 2003.

Parameter	Station A	Station B
Temperature (° C)	15.5	16.1
Salinity	7.3	6.2
NH_4^+ (µM)	1.11	7.98
PO_4^{2-} (µM)	0.36	0.92
Prorocentrum minimum (cells·mL^{-1})	65700	7280
Chlorophyll *a* (µg·L^{-1})	259	34
Chlorophyll *a* content in *P. minimum* cells	4.7	3.9
(pg·cell^{-1})		
Total AP activity (µM·h^{-1})	0.228 (0.023)	0.139 (0.032)
AP activity in >5 µm fraction (µM·h^{-1})	0.023 (0.013)	0.011 (0.010)
Cell specific AP activity	0.035 (0.017)	0.151 (0.146)
(10^{-2} pmol P-MUF·cell^{-1}·h^{-1})		
Chlorophyll *a* specific AP activity	0.089 (0.044)	0.329 (0.312)
(nmol P-MUF·µg chl *a*$^{-1}$·h^{-1})		

Table 3.5. Cell specific alkaline phosphatase activity in *P. minimum* and other dinoflagellates from low phosphate conditions. Substrates used to detect AP activity are reported. (MUF-P: 4-Methylumbelliferyl phosphate; MFP: 3-*o*-Methylfluorescein phosphate; *p*-NPP: *p*-Nitrophenyl phosphate).

Plate 3.1. Pictures of "mahogany tide" due to *P. minimum* bloom at Gunston Day School dock, in Corsica River, 27 October 2005.

Organism	Activity (fmol P·cell^{-1}·h^{-1})	Reference	Substrate
Prorocentrum minimum	164	Present study	MUF-P
Prorocentrum minimum	2.28	Sakshaug et al. 1984	MFP
Amphidinium carterae	0.49	Sakshaug et al. 1984	MFP
Ceratium tripos	19-30	Sakshaug et al. 1984	MFP
Scrippsiella trochoidea	7.23	Sakshaug et al. 1984	MFP
Pyrocistis noctiluca	360 x 10^3	Rivkin and Swift 1979	MFP
Protogonyaulax tamarensis	2-8 x 10^3	Boni et al. 1989	*p*-NPP
Heterocapsa circularisquama	3.44	Yamaguchi et al. 2005	MUF-P
Peridinium cinctum	1.13 x 10^3	Wynne 1981	*p*-NPP

CHAPTER 4

Discussion and Conclusions

The physiological flexibility of *Prorocentrum minimum* in response to changes in environmental parameters and its ability to use both inorganic and organic nutrient sources has been recognized (reviewed in Heil et al. 2005). At the beginning of this research no information was available on the ability (or lack of ability) of *P. minimum* to express α and β-glucosidase. Only one study had assessed the leucine amino peptidase (LAP) activity of *P. minimum* (Stoecker and Gustafson 2003), but the effect of nutrients, growth, and environmental parameters on LAP activity of *P. minimum* was unknown. A number of studies had described the capability of *P. minimum* to synthesize alkaline phosphatase (AP) in response of phosphorus (P) limitation (Sakshaug et al. 1984, Dyhrman and Palenik, 1997, 1999, 2001, 2003). In spite of the number of investigations, only one study (Sakshaug et al. 1984) reported cell specific AP activity, and the effects of physiological and environmental parameters on AP expression, besides P stress conditions, had not been assessed. A number of questions had arisen regarding the role of enzymes in nutritional physiology of *P. minimum*, their regulation, and their importance in bloom dynamics.

P. minimum is known to use urea as a source of carbon as well as nitrogen, although the contribution of urea to carbon uptake seems to be small (Fan and Glibert 2005; Heil et al. 2005). There is evidence that humic acids play a role in *P. minimum* nutrition, stimulating its growth and accumulation (Kondo et al. 1990; Stonik 1995; Heil 1996, 2005; Carlsson et al. 1999; Granéli et al. 1999). This study demonstrated that *P. minimum* is not able to express appreciable glucosidase activities in inorganic nutrient-replete media nor in low nitrate and low phosphate media. Experiments in which organic matter was added were not conducted in this study, so it can not be

determined whether the presence of added organic compounds would induce the synthesis of glucosidases. However, samples collected during a monospecific bloom of *P. minimum* were in accord with the laboratory result in that no activity was found associated with the size fraction containing *P. minimum although activity was detected in smaller size fractions.*. Besides the significance for *P. minimum* physiology, these results confirm that the methodology used is able to detect enzyme activity that is genuinely associated with *P. minimum* cells, and that overestimation due to filtration, as discussed in chapter 2 ("Discussion" section), is not an issue in this case.

P. minimum is known to be able to grow in culture on a variety of nitrogen sources including NO_3^-, NH_4^+, dissolved free amino acids (DFAA) and urea (Fan et al. 2003). Factors affecting the extent to which a specific nitrogen substrate is used include the relative proportion of different nitrogen sources available, the temperature, and the nutritional state of the cells (Lomas and Glibert 1999; Fan et al. 2003). Fan et al. (2003), studying the affinity of *P. minimum* for different nitrogen substrates, demonstrated that the highest uptake was observed for NH_4^+, followed by DFAA, urea and nitrate. However, in nitrogen dynamics it is also important to consider the potential ability to use dissolved combined amino acids, such as proteins and peptides, since they may represent up to 20% of dissolved organic nitrogen (Mulholland et al. 2003). Proteases, including LAP, can be involved in nitrogen metabolism because they are able to hydrolyze DCAA and liberate non-polar amino acids such as leucine (Stoecker and Gustafson 2003). This current research explored this aspect which had not been well understood.

This study confirmed that *P. minimum* can significantly express LAP activity. Since LAP activity has been found in cultures grown in inorganic nutrient-rich media,

this suggests that the enzyme may be constitutive. However, it possible that low levels of polypeptides or proteins are necessary to induce enzyme activity. This can not be ruled out since the seawater used to make the culture media undoubtedly contained some dissolved organic matter. The concentration of nitrate and phosphate do not seem to affect LAP activity, while the addition of reduced forms of nitrogen, such as urea and ammonium, which have been shown to be preferred N sources (Fan et al. 2003), reduces the ectoproteolytic activity of *P. minimum* cultures. Evidence for the regulation of dinoflagellate peptidase activity by ammonium was also found in field samples.

The cell specific activity suggests that at bloom densities *P. minimum* may make an important contribution to bulk LAP activity, unless ammonium concentration is high, and may be responsible for degradation of proteins and polypeptides in the water column through enzyme-catalyzed hydrolysis. This has been confirmed during a monospecific *P. minimum*. During the bloom LAP activity was elevated and a large portion (79%) of LAP activity was due to dinoflagellate fraction, demonstrating that the dinoflagellate size fraction may make an important contribution to LAP activity. In fact, the ectoproteolytic activity associated with the dinoflagellate fraction explained an important part (up to 51%) of the variability in total LAP activity in the water.

The mechanism with which LAP is used for nutrition of *P. minimum* cells is proposed in Fig. 4.1. Amino acids released by LAP may be taken up directly by dinoflagellates as a source of N and C, or oxidized by amino acid oxidases on the surface of cells, to release ammonium, a preferred N source for *P. minimum* (Mulholland et al. 1998, Fan et al. 2003). I propose that leucine aminopeptidase is used preferentially in combination with cell surface oxidase, to supply inorganic nitrogen, particularly ammonium. Therefore, inhibition by high ammonium concentration would

represent end-product feedback regulation. Additional studies that measure simultaneously LAP activity and uptake of C or N from amino acids and ammonium are necessary in order to better understand the role of LAP in nutrition of *P. minimum*.

P. minimum grows well with inorganic phosphorus sources, but is able to utilize organic phosphorus by means of alkaline phosphatase (AP) enzyme activity in both cultures (Dyhrman and Palenik 1997) and field populations (Dyhrman and Palenik 1999). AP, localized on the surface of *P. minimum*, has been identified and purified previously (Dyhrman and Palenik 1997), and is induced by phosphorus starvation. P limitation can occur in coastal and oceanic regions, and the concentration of dissolved organic phosphorus (DOP) in surface coastal waters is often higher than the concentration of dissolved inorganic phosphate (DIP). Thus DOP can represent a significant portion of algal-available phosphorus (Kobori and Taga 1979, Fisher et al. 1988, Hernández et al. 2000, Yamamoto et al. 2002, Yamaguchi et al. 2004a). Therefore, AP activity plays a role in utilizing DOP as alternative source of phosphorus for *P. minimum* (Malone et al. 1996).

This research confirms that AP activity in *P. minimum* is greatly enhanced by P-deficiency, but since AP activity has been detected also in nutrient replete cells, this enzyme may be partially constitutive. However, the possibility that low levels of DOP are necessary to induce AP activity cannot be excluded since the seawater used to prepare the media may have contained organic P. AP activity in P-limited cells is more than two orders of magnitude higher than in P-replete *P. minimum*. The response of alkaline phosphatase to phosphate variability is very rapid, with inhibition occurring within 12 hours of exposure to inorganic phosphate. I also found a temporal cycle in AP activity related to light-dark changes.

The end-product feedback regulation of AP is shown in Fig. 4.2. Due to its regulation mechanism, AP activity seems a good indicator of P limitation. Since the bloom studied was monospecific, cell specific AP activity and chlorophyll *a* specific AP activity in field samples could be calculated and compared to those observed in laboratory experiments.. This comparison indicated that *P. minimum* cells were not P-limited during the bloom in Corsica River. In the Corsica River during the bloom, inorganic phosphate concentration was high compared to typical values for Chesapeake Bay (Fisher et al. 1988, 1992, 1999). However N concentration was fairly low, with the N:P ratio less than the Redfield ratio, suggesting N stress rather that P stress. The contribution from the dinoflagellates fraction to bulk AP activity was less than 8%, the same percentage we observed in non-axenic *P. minimum* cultures grown in low nitrate medium.

It is interesting to consider that although alkaline phosphatase activity in the water column is thought to be primarily due to algae (Ammerman 1990, Yamaguchi et al. 2004b), results from this study show that in the Corsica River, even during a dense *P. minimum* bloom, the bulk AP activity was not due to the dinoflagellate fraction. However, during dinoflagellate blooms, unless ammonium concentrations are very high, LAP activity associated with dinoflagellate fraction is expected to be high, because LAP appears to be constitutive. Dinoflagellate AP activity is not expected to be high in natural assemblages, unless orthophosphate concentration is very low.

Osmotrophic mixotrophy is potentially important to the physiological ecology and bloom dynamics of dinoflagellates. Ectocellular enzyme activities may play a role in nutrition of mixotrophic dinoflagellates, and may give them an advantage over the strictly phototrophic species in organically enriched environments. Due to the diversity

of phytoplankton, more species-specific studies are needed in order to understand the extent to which ectocellular hydrolysis coupled with osmotrophy is potentially used among phytoplankton. However, simultaneous measurement of enzyme activity and uptake rate are necessary to clarify the real importance of organic compounds for nutrient supply.

Fig. 4.1. Conceptual diagram of LAP regulation in *P. minimum* and its role in nutrition. LAP = leucine amino peptidase; aa = amino acids; aa ox = amino acid oxidase. Thick solid lines represent enzyme-mediated transformations of compounds (yellow boxes); thin solid lines represent enzyme regulation (- = inhibition, ? = unknown); thick dotted lines represent possible processes not investigated in this study.

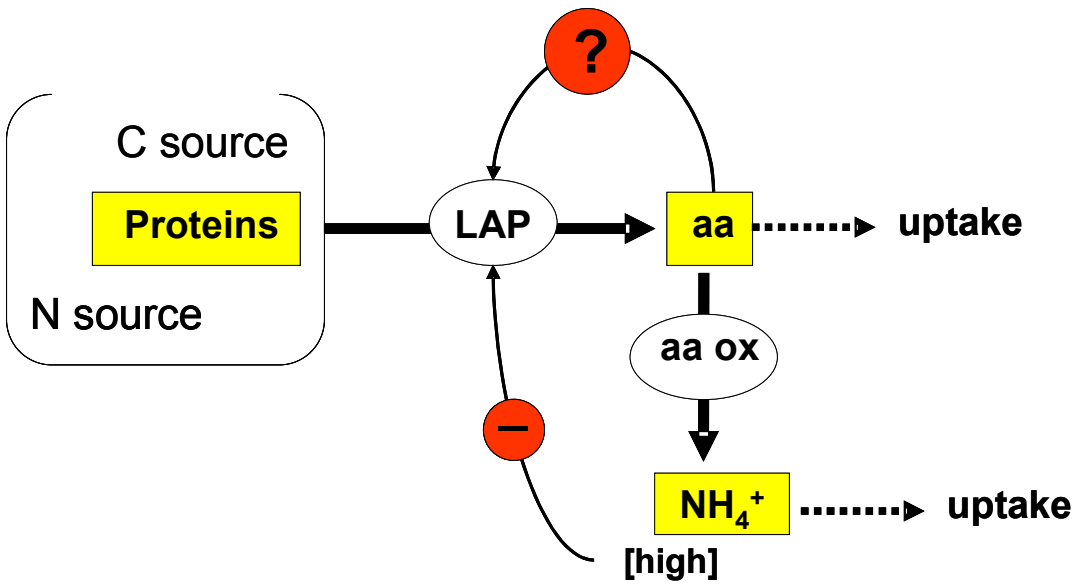
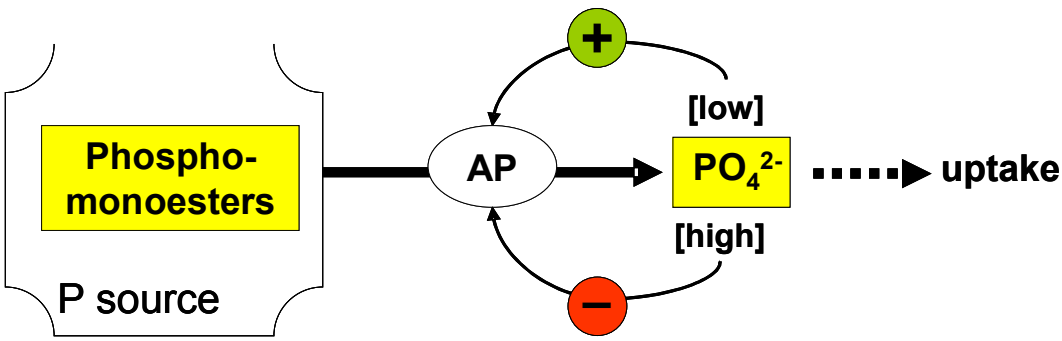


Fig. 4.2. Diagram of conceptual model of AP regulation in *P. minimum* and its role in nutrition. Thick solid lines represent enzyme-mediated transformations of compounds (yellow boxes); thin solid lines represent enzyme regulation (- = inhibition, + = stimulation); dotted lines represent possible processes not investigated in this study.



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