ENZYMES AS INDICES OF GROWTH RATE AND NITRATE METABOLISM IN MARINE PHYTOPLANKTON

by

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ABSTRACT

Determining the in situ rates of growth and nitrogen incorporation of marine phytoplankton is critical to understanding energy transfer and nutrient and carbon cycling in the world's oceans. To overcome the limitations in current methods of estimating biological rates (i.e. incubations under unrealistic conditions, or inadequate estimates of spatial and temporal variability) the use of enzyme activity measurements was examined. Because enzymes are functional proteins that adapt to suit prevailing conditions, enzyme levels may provide an integrated index of in situ rates of phytoplankton metabolism.

Nucleoside diphosphate kinase (NDPK) an enzyme which directs cellular energy towards biosynthesis was examined as an index of specific growth rate (µ) in the diatom *Thalassiosira pseudonana* grown under light limitation. NDPK activity was significantly, but weakly correlated with µ. Activity per cell rose at high µ, but also increased at very low µ. Although of limited value as a predictive index by itself, NDPK may be useful in conjunction with measurements of ATP concentration, or adenylate turnover rates.

Nitrate reductase (NR), an enzyme specific for nitrate assimilation may be used in calculating rates of nitrate incorporation (µN) and thus new production, but previous measurements of NR have not matched µN. A new assay using bovine serum albumin to protect the enzyme from proteases was developed that gave close agreement with µN in light limited cultures of *T. pseudonana* and *Skeletonema costatum*. The relationship also held for *T. pseudonana* during transitions in irradiance, under nitrate limitation (although NR exceeded µN at low µ), during growth on light-dark cycles, different light spectra, in the presence of ammonium, and during nitrate starvation. In each case, NR accurately predicted µN. NR was closely related to nitrate incorporation rates in three additional diatom species, but for other taxa, particularly the Dinophyceae, NR underestimated µN. Preliminary field experiments were conducted in Monterey Bay, California during a diatom bloom. µN predicted from NR measurements always equalled or exceeded rates estimated by other methods, including 15N incorporation.
Appendices to the thesis compare and validate different protein assays in marine phytoplankton, provide details of a computer program to automate and collect enzyme kinetic data from a spectrophotometer, and compare methods of fitting rectangular hyperbolae to a variety of oceanographic data.
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protein assays. For clarity, only one out of six sets of bovine serum albumin (BSA, ●), bovine gamma globulin (BGG, ○) and casein (■) data are shown. Lines represent least squares regression fits to pooled data. Fits to algal protein data for high light grown (◇) and low light grown (□) cultures are not shown. Note scale changes for protein.

Figure A.2. Comparison of absorbance versus protein curves for A) Bradford, B) Lowry, and C) Smith protein assays for BSA samples with (●) and without (◇) additions of 0.1 mg chlorophyll a in 90% acetone. Points represent the means of two separately prepared standards. In all cases, associated error bars are smaller than the symbols. Lines represent least squares regression fits to the data.

Figure A.3. Comparison of protein content (expressed as pg cell⁻¹) for acetone-extracted (□) versus non-acetone-extracted (■) homogenates of Thalassiosira pseudonana (n = 6 for each treatment). Error bars represent standard errors of mean protein content.

Figure A.4. Comparison of protein content (expressed as pg cell⁻¹) for trichloroacetic acid (TCA) -precipitated (■) versus non-TCA-precipitated (□) homogenates of Thalassiosira pseudonana (n = 5 for each treatment). Error bars represent standard errors of mean protein content.

Figure B.1. Screen output of absorbance versus time progress curves from the enzyme kinetics program. From bottom to top, lines represent additions of 50, 20, 10, 5, 1, or 0 mU of lactate dehydrogenase, respectively. Other assay conditions are described in the text. Note that the screen image was taken only 6 min. into the reaction.

Figure B.2. Sample data file output from the enzyme kinetic program of cell (sample) number, time (min.) and absorbance. Cells 1 through 6 represent additions of 50, 20, 10, 5, 1, or 0 mU of lactate dehydrogenase, respectively. Other assay conditions are described in the text.

Figure C.1. Data cases considered. Cases represent geometrically distributed data (Case 1), data where no points are lower than Kₘ (Case 2), data where no points are higher than Kₘ (Case 3), data where only points higher than 2 x Kₘ or lower than 0.5 x Kₘ are available (Case 4), and data where all points fall between 2 x Kₘ and 0.5 x Kₘ (Case 5). In each case, data sets of 10 points were generated with Vₘₐₓ = 10 and Kₘ = 2.295

Figure C.2. Examples of error levels (as percentages of V and S) assigned using Case 1 as an example. Constant error levels are set as percentages of 0.5 x Vₘₐₓ. Errors were assigned in a normal distribution.

Figure C.3. Examples of frequency distributions of Vₘₐₓ and Kₘ estimates for various fitting procedures for Case 1 data with 20% variable or constant error. Procedures are: O Lineweaver-Burk, ● Eadie Hofstee, □ Hanes-Woolf, ▼ Eisenthal and Cornish-Bowden, ○ Cleland-Wilkinson, and ■ Tseng-Hsu. Y-axis scale is relative percentage. True values of Vₘₐₓ and Kₘ are 10 and 2, respectively.

Figure C.4. Examples of real data sets fit to rectangular hyperbolae using different fitting methods: ———— Lineweaver-Burk, ——— Eadie Hofstee, ———— Hanes-Woolf, ——— Eisenthal and Cornish-Bowden, ——— Cleland-Wilkinson or Tseng-Hsu. A) Nitrate reductase activity versus nitrate concentration in extracts of the diatom Thalassiosira pseudonana, B) Phosphate uptake versus concentration in the marine macroalgae Fucus spiralis and C) growth rate versus prey concentration for the marine ciliate Strombidium sp. feeding on the marine alga Rhodomonas sp. Parameters for each fit are given in Table C.3.
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GENERAL INTRODUCTION

A principal goal of biological oceanography is to understand the flows of energy and materials through living systems and to understand their interaction with non-living systems (Parsons et al. 1984a). The first and probably most important interface between these systems occurs at the level of photoautotrophic organisms, primarily marine phytoplankton. Thus the ecology of these organisms has a central role in oceanic processes. Sakshaug (1980) summarized the major goals of phytoplankton ecology: to relate intrinsic algal properties quantitatively to growth rates and identify the factors which limit growth.

The importance of biological rates

Compared with terrestrial environments, biomass in marine ecosystems is very low, yet total primary production is equal or greater (Kelly 1989) because the growth rates of marine photoautotrophs are extremely high. While biomass measurements alone can provide important information about terrestrial systems, in the marine environment a precise knowledge of rates of biological processes is also critical (Longhurst 1984, Valiela 1984, Kelly 1989).

The rate of primary production, i.e. the rate of increase of biomass (often expressed as carbon) of the photoautotrophs is clearly an important quantity. Flynn (1988) has argued for the use of more precise terms such as "photoautotrophic production", "photosynthesis" or simply "carbon fixation", but "primary production" remains in common use. Primary production sets an upper limit on the potential production of commercially important fisheries, and may ultimately be the critical term in setting sustainable harvests (Mann 1984). Despite all the interactions and variation at points higher in the trophic food web, Iverson (1990) found that primary production was the single best indicator of fish production in a wide variety of marine systems. As well, concerns about global warming (the anthropogenic introduction of carbon into the atmosphere that is thought to lead to global temperature increases, see Broecker et al. 1979, Taylor and Lloyd 1992) have renewed efforts to estimate rates of primary production more precisely. As Broecker et al. (1979) pointed out, about 45% of the
carbon known to have been introduced to the atmosphere by human activity since the industrial revolution cannot be accounted for in any current carbon pool. Oceanographers think that this so-called "missing sink" for carbon can be accounted for in the oceans, due to physical processes that sequester dissolved CO$_2$ for long periods of time in deep ocean waters, or by increases in phytoplankton production that result in increased sedimentation of organic matter and thus increased carbon flux to the ocean floor (see Sarmiento and Siegenthaler 1992). However, terrestrial ecologists believe that carbon dioxide increases have caused a fertilization effect on terrestrial plants; the extra carbon may be tied up in increased production of tropical rainforests and boreal forests, either in biomass or in forest floor litter (Taylor and Lloyd 1992). Much uncertainty about the relative importance of phytoplankton in this the global carbon cycle remains. Based on a long-term data set of water clarity records, Falkowski and Wilson (1992) could not distinguish a systematic increase in ocean production caused by increased CO$_2$, but such measurements may reflect biomass and not necessarily production (see Welsh 1993). Good estimates of biological rate processes in the ocean are needed to resolve these questions.

Since primary production is based largely on differences in growth rate rather than in biomass, growth rates of marine autotrophs are a key factor. Unfortunately, measuring growth rates in the ocean is difficult. Eppley (1981) pointed out that balanced growth (i.e. matching increases of carbon, nitrogen and other elements) is rarely, if ever, seen in phytoplankton in the ocean; temporal and spatial variations in irradiance, nutrient, and biomass lead to mismatches between cell division rates and nutrient uptake rates. Furthermore, the recycling of nutrients (see Harrison 1992), and the presence of grazers (see Banse 1992) complicate things further. Often the problem is not one of quantity or quality of data, but of interpreting data within existing and potentially constraining concepts (see Eppley 1981). There are several recent examples of this phenomenon. Traditionally, there has been little doubt that large species of diatoms are very important in marine production. Using routine sampling methods, these species have been shown to comprise up to 25% of total net global primary production (a quantity comparable to the production of pine species in temperate and boreal forests, or of
grasses in savannah and cultivated areas), and to be capable of very high growth rates, and, thus, to dominate oceanic production (see Guillard and Kilham 1977, Thomas et al. 1978, Willen 1991). Since diatoms are most abundant in coastal regions, the open ocean areas have traditionally been considered relative "deserts", owing to their low diatom biomass and very low nutrient concentrations (see Ryther 1969). However, there has been a growing awareness that small species (so called "picoplankton", defined as organisms between 0.2 and 2.0 μm in diameter) are abundant and may account for up to 80-90% of production in certain freshwater and marine systems (see Stockner 1988). Previously, due to the concept that large diatoms were important, these picoplanktonic organisms had been under sampled and their true importance underestimated by the sampling techniques used. To some degree, concepts have now shifted to the other extreme; phytoplankton ecologists are now focusing on the very smallest organisms, the picoplankton (e.g. Li 1986, Stockner 1988). Goldman (1989) has demonstrated that, even if they exist in substantial numbers, these small cells can represent substantially less production than a few large diatoms. Sampling designed with the importance of picoplankton in mind would probably result in under sampling the large, rarer diatom species.

A second example of the problem caused by unchallenged preconceptions concerns ideas about the growth rates of oceanic phytoplankton species. Low nutrient concentrations were previously thought to indicate nutrient limitation and thus low growth rates (e.g. Ryther 1969). Work on bacteria and protozoa has shown that there is an active recycling of materials at rates that are very high (see Pomeroy 1974, Azam et al. 1983). This so-called "microbial loop" has led to the view that nutrients are so rapidly recycled that a "spinning wheel" develops and high regeneration rates support high growth rates. Traditional methods of assessing growth rate are poorly suited to these situations and may have perpetuated the idea of slow rates of growth (see Leftley et al. 1983). Goldman et al. (1979) have suggested that based on composition, cells in the open ocean are, in fact, growing at near maximal rates. This is based on the observation that there is a highly conserved ratio of elements in most of the ocean; C:N:P by atoms is usually 106:16:1, the so-called Redfield ratio (Redfield 1958,
but see also Takahashi et al. 1985). In laboratory culture, organisms appear only to achieve this ratio when they are growing at their maximal (i.e. non-nutrient-limited) rates (McCarthy and Goldman 1979, Goldman 1980, but see also Tett et al. 1985 and Goldman 1986 for different interpretations of the ratio in the case of light versus nutrient limitation). These examples perhaps illustrate a general feature of science; while paradigms are helpful in framing questions and directing research, they also bias efforts in particular ways (see Kuhn 1970). The controversies mentioned above cannot be easily resolved because of the inadequacy of current methods of determining biological rates.

**Limitations of primary production**

Before examining the methods and the problems with the determination of rates of growth, it is first necessary to understand how growth rates are limited in the ocean. Marine primary producers are ultimately constrained by the quantity of light energy available and this is particularly limiting in polar regions during dark periods, in winter temperate regions where mixing processes drive phytoplankton deep into the water column, and for phytoplankton populations deep in the water column or in turbid waters such as estuaries (see Parsons et al. 1984b). However, the quantity of mineral nutrients is more often likely to cause limitation of production (see review by Platt et al. 1992a). This has been expressed as Liebig's "law of the minimum", which states that the material in lowest concentration relative to the requirement will be limiting (Liebig, 1840). Therefore, identifying the limiting nutrient becomes an important problem to solve (see e.g. Dugdale 1967, Droop 1973).

Thingstad and Sakshaug (1990) have pointed out that there is a problem with terminology between descriptions of so-called "controlling" and "limiting" factors. For example, in a chemostat culture where cell growth is regulated by the addition of a single nutrient (see Rhee 1979), it is the particular nutrient that is "limiting", but it is actually the rate of supply of that nutrient that is "controlling". In most cases, it is experimentally difficult to separate limitation from control. In this thesis, mindful of the distinction, the term "limiting" will be used. To add another complication, as pointed out by Dugdale (1967), and
re-emphasized by Falkowski et al. (1992), the limiting nutrient may limit growth rate or the ultimate biomass achieved by a phytoplankton community, or its effects may only alter species composition. For example, a deficiency of silicate, a nutrient required by diatoms, may not change the primary production of a system, but it may result in the decline of diatoms, and their succession by other species. Alternatively, if only the biomass is limited by a nutrient, primary production can be calculated from a knowledge of light alone, while if growth rate itself is nutrient-limited the case becomes much more complex (see Falkowski et al. 1992).

In marine systems, primary production is probably most often limited by nitrogen, phosphate or silicate. It is worth noting that although there are many nitrogen forms, P and Si are available to phytoplankton in seawater predominately as phosphate and silicate, respectively) (see Raymont 1980, Parsons and Harrison 1983). The classical view of the marine ecosystem held that nitrogen was the limiting nutrient (e.g. Ryther and Dunstan 1971), and indeed there is clear evidence that this is true in many cases (McCarthy 1980, Collos and Slawyk 1980, Wheeler 1983). However, there is also evidence that phosphate may be limiting in some locations such as estuaries and coastal regions (e.g. Harrison et al. 1990a, Peeters and Peperzak 1990, Fisher et al. 1992). In the view of many geochemists, phosphate should be the limiting nutrient, based on the results of modeling phosphorus budgets and flux rates (see Smith 1984), although the turnover rates of phosphate are also very high (see Lean and Cuhel 1987). Silicate limitation has also been reported in coastal and polar regions (e.g. Nelson and Treguer 1992, Egge and Asknes 1992, Brzezinski 1992), although its effects are more likely to involve shifts in species composition than changes in production (see Dortch and Whitledge 1992). At least part of the problem lies in the inadequacy of methods used to determine whether a particular nutrient is limiting (see discussion in Peeters and Peperzak 1990).

Recently, it has been suggested that iron (or other trace metals, see Morel et al. 1991) may limit primary production in large areas of the ocean (see review by Martin 1992, but see also Cullen 1991). Iron limitation has been tested in previous enrichment experiments (e.g. Menzel and Ryther 1961, Tranter and Newell 1963, Menzel et al. 1963, but note that Menzel and Spaeth 1962 found different results), but has more recently become a topic of controversy,
brought on by the proposal that iron could be used to fertilize tracts of the ocean and thereby increase primary production and draw down elevated atmospheric carbon dioxide levels (see Chisholm and Morel 1991). It is unclear whether fertilization results in a change in production, or in a shift in species (see Banse 1990, Wells et al. 1991, DiTullio et al. 1993), or changes in preference for nitrogen forms (see Price et al. 1991). There is also disagreement over the method of calculating growth rates in iron enrichment experiments (compare Martin et al. 1991 and Banse 1990). There is little doubt that the poor ability to determine growth rates is a central problem here.

Nutrient limitation can prove to be complicated. Howarth and Cole (1985) demonstrated the potential for interactions between nutrients. In seawater, there is evidence that high sulphate concentrations result in an impaired ability of phytoplankton to take up molybdenum. Since molybdenum is required for activity of nitrogenase during N₂ fixation, and in enzymes involved in nitrate and nitrite reduction, this may result in additional costs to marine species to perform these functions, but may manifest itself as nitrogen limitation. The same sort of interaction may occur between nitrogen and iron (see Price et al. 1991).

Finally, in a radical departure from the N, P, Si, or Fe limiting nutrient paradigm, Riebesell et al. (1993) recently suggested that carbon may be limiting to marine phytoplankton. Because the pH of seawater is usually close to 8.2 (Riley and Chester 1971), and can rise further during blooms, this means that while the total concentration of carbon in seawater is usually adequate to support production, the actual concentration of CO₂ is low due to the chemical equilibrium favoring HCO₃⁻ over CO₂. Riebesell et al. (1993) argue that many species are unable to use HCO₃⁻ effectively; thus, in some cases carbon may be limiting. To date, this observation has been confined to laboratory cultures, and there has been criticism (see Turpin 1993) of the experiments performed by Riebesell et al. (1993).

**Phytoplankton nitrogen metabolism and the concept of new production**

Nitrogen is considered the principle limiting nutrient in the oceans. Marine phytoplankton cells obtain inorganic nitrogen principally as nitrate and ammonium
reviews by McCarthy 1980, Syrett 1981, Collos and Slawyk 1983, Wheeler 1983, Syrett 1989). There are also cyanobacterial species that can fix atmospheric nitrogen gas (N₂), and although this represents less than 1% of oceanic nitrogen globally, it can represent up to 20% in areas such as the Baltic Sea (Howarth et al. 1988). Uptake of forms of dissolved organic nitrogen is poorly understood due to analytical difficulties; urea and amino acids may be important at times, but peptides, proteins, ureides, amino sugars, and pyrimidines and purines also contribute (see Antia et al. 1991 for an extensive review). These organic compounds are also used by bacteria, and thus may compete with phytoplankton for these nutrients. Palenik et al. (1989) and Palenik and Morel (1990) have reported that amino nitrogen may be made available for uptake as ammonium by the action of cell surface amino acid oxidases that occur in many marine phytoplankton species.

An important division exists between production based on different nitrogen forms; the source of the nitrogen is related to the ultimate fate and importance of the production. Dugdale and Goering (1967) first drew attention to the concepts of "new" and "regenerated" production. As illustrated in Fig. 1, the euphotic zone in many areas of the ocean can be treated in isolation due to density stratification of the water column resulting in a thermocline or pycnocline. Nitrogen can enter from upwelling of deep water (principally as nitrate), from atmospheric deposition (e.g. rain, as nitrate and ammonium, see Paerl 1985, Duce 1986) and from the atmosphere as N₂ gas for nitrogen fixation. These forms are termed new since they have entered the system for the first time. Regenerated nitrogen arises from that incorporated into organisms (particulate organic nitrogen, PON) that is released by death or by cell lysis, grazing, or viral attack (see Suttle et al. 1990) principally as ammonium, urea and amino acids (see Harrison 1992). The distinction between these forms lies in the fact that, if systems are assumed to be in steady state, and if they do not "run down", only an amount of material equal to the new production can be exported from the system via sedimentation, fish production, etc. (see Longhurst 1991, Platt et al. 1992b). This sets practical upper limits on the potential fish harvest, for example, but it also has interesting implications for the global carbon cycle. Since export will result in a loss of carbon from the system, while regeneration is accompanied by
respiration which will return carbon to the atmosphere, new production is a critical factor in
the global CO₂ budget. New production can be viewed as a "biological pump", sequestering
CO₂ in the deep ocean for long time scales; somewhat cynically, Platt et al. (1992b) have
likened it to sweeping the "pollution of the atmosphere...under the rug of the thermocline".
Eppley and Peterson (1979) extended the concept of new production further by noting that
systems with higher total primary production also have a greater fraction of new production;
this is expressed as the f-ratio, the ratio of new production to total production. Based on this
analysis, productive upwelling zones, where nitrate-rich water is brought to the surface may
well be the most important areas for new production (Dugdale and Wilkerson 1992), but there
is also evidence that nitrate is upwelled episodically in vast areas of the open ocean, and so
they may also contribute substantial new production (see Lewis et al. 1986, Glover et al.
is much controversy which arises because of problems in methodology (see Platt et al. 1989),
and the assumption that oceanic regions are in steady state may not be strictly valid (Platt et al.
1989). For example, Jaques (1991) pointed out that the Southern Ocean has additional
complications such as the removal of nitrate from the euphotic zone when deep water is
formed.
precipitation
deposition
(NO$_3^-$, NH$_4^+$)

$\text{NO}_3^-$ $\rightarrow$ PON
(phytoplankton)

$\text{NO}_3^-$ $\rightarrow$ remineralization

$\text{NO}_3^-$ $\rightarrow$ N$_2$ fixation

$\text{N}_2$ $\rightarrow$ $\text{NO}_3^-$

upwelling

plankton flux

fish, etc.

sedimentation

Figure 1. Diagram of the main processes involved in new and regenerated production in the upper water column.

See the text for explanations. (PON = particulate organic nitrogen, DON = dissolved organic nitrogen).
Traditional methods of estimating biological rates and their problems

As previously mentioned, many of the concepts of marine ecosystems and their functioning arise from, or have been biased by, the methods used to estimate biological rates in natural environments. There are problems common to methods of assessing growth rates and rates of new production, and problems distinct to each set of methods.

14C Incubations

The most common method of assessing production rates is by timed incubation. Clearly, given the enormous spatial and temporal variability in the oceans, any attempt to take serial samples from the ocean and monitor increases in in situ biomass (e.g. particulate carbon, nitrogen, or chlorophyll a), or decreases in in situ nutrients (e.g. nitrate or ammonium) will result in poor resolution. Thus, incubations of contained water samples are most often used. For growth rates, photosynthesis or carbon fixation is monitored. Harris (1984) cautioned that growth is not simply equal to photosynthesis, nor is photosynthesis simply equal to carbon fixation or oxygen production. Traditionally, oxygen production has been measured, but this method gives poor sensitivity and relies on a photosynthetic quotient to convert from oxygen production to carbon fixation, which has associated problems of its own (see Laws 1991). However, there is some evidence that, in certain areas of the ocean, the oxygen method may be better than alternatives (e.g. Harris 1984). Currently, the standard method involves use of 14C, a radioactive isotope of carbon. Radio-labeled bicarbonate is introduced into the incubation vessels and time-dependent incorporation into particulate material is measured (see Steemann-Nielsen 1952, Parsons et al. 1984a). Many of the problems with this method arise from the necessity of collecting and incubating cells in containers; these problems have been extensively reviewed (see Venrick et al. 1977, Li and Goldman 1981, Leftley et al. 1983, Harris 1984, Gieskes and Kraay 1982, Li 1986, Harris et al. 1989, Collos et al. 1993). For example, the process of collecting cells and placing them in incubation bottles may damage delicate species (e.g. Krupatkina 1990), and that once inside the bottles, collisions of cells with
the walls of the bottle, or the changes in turbulence scales will cause cell damage (e.g. Allen 1977). Upon containment, the parcel of water is separated from processes such as introduction of nutrients, or removal of waste products by advection, and thus the biological behaviour of the sample may change markedly. Grazers of phytoplankton may be selectively removed, and thus production can be enhanced (see Collos et al. 1993), or the containment of a grazer may improve grazing ability leading to artificially lower rates. Alternatively, removal of grazers may decrease production by preventing nutrient regeneration. Trace metals found in incubation bottles and sampling equipment (even wire lines) may present additional problems, both by inhibiting sensitive species, and by enhancing production where metals are limiting (see Fitzwater et al. 1982, Price et al. 1986). All of these problems may account for the sometimes dramatic shifts in species composition seen in contained versus natural samples (e.g. Venrick et al. 1977). The volume of water contained in an incubation bottle can also make a large difference, especially in oceanic waters where larger bottles give higher production estimates (see Gieskes et al. 1979), but the volume effect may not be significant in coastal regions (Leftley et al. 1983). The length of incubation is also an issue. Collos et al. (1993) found that containment altered regeneration rates within bottles and also carbon isotope fractionation. They advocated keeping incubations less than 3 h. Shorter times minimize certain containment problems, but make extrapolations to longer (e.g. 24 h) time scales difficult. The light climate during the incubation is also a problem. Traditionally, in situ methods have bottles returned to the same depths from which they were sampled, on some sort of fixed array. The problem here is that the bottle or container may change the natural light spectrum (e.g. borosilicate glass will screen out UV radiation) (see Smith et al. 1980), and on oceanographic cruises it is frequently impossible to stop for extended incubation periods. An alternative is the simulated in situ method where samples are placed in shipboard incubators at appropriate light levels. Very often, however, the light is controlled by neutral density screening, while in situ, a change in light spectrum towards more blue light would occur. Laws et al. (1990) found that primary production rates estimated using neutral density screening were half of those estimated using in situ incubations. Comparisons of the in situ
and simulated *in situ* methods indicate that there are differences; Lohrenz *et al.* (1992) found that differences were greatest during short term incubations, and advocated longer incubations, but longer incubations would increase many of the containment effects (e.g. grazing) previously discussed. In both methods, cells are held at a single light level. This is not natural because cells would normally circulate in the upper mixed layer of the ocean and be exposed to a varying light field. There is evidence that this can make a large difference. Ferris and Christian (1991) reviewed seven studies using fluctuating versus steady light regimes; production in the fluctuating versus the steady light was decreased in 2 cases, enhanced in 3 cases and showed no difference in 2 cases. Mallin and Paerl (1992) reported that varying light reduced photoinhibition and resulted in markedly higher growth and production rates in estuarine phytoplankton.

After incubation, there is the problem of how to collect the cells and remove unincorporated 14C. Collection is usually done by filtration, but there is evidence of problems with filter retention of cells (e.g. Stockner *et al.* 1989). Equally, the rupture of delicate cells can lead to losses, depending on filter type and filtration pressure (Venrick *et al.* 1977). Acid fuming or acid rinsing of cells can be used to remove the unincorporated label, but there are unaccountable losses using these methods (see Hilmer and Bate 1989). Alternatively, the whole sample can be acidified and bubbled to remove unincorporated label. Riemann and Jensen (1991) reported up to 57% higher incorporation rates using this method. To correct for non-specific carbon uptake, a control in which light is blocked is often run, but there can be substantial carbon uptake in the dark (Leftley *et al.* 1983). Alternatively, the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) can be added (Legendre *et al.* 1983), or blanks filtered without incubation (i.e. a zero time blank) can be used. The issue of excretion of dissolved organic carbon (DOC) from phytoplankton could be critical. Zlotnik and Dubinsky (1989) found that light and temperature both caused variation in DOC release in laboratory cultures of phytoplankton. The range of excretion fell between 1 and 55% of primary production. Harris *et al.* (1989) showed that up to 50% of carbon fixed could be lost
in an 8-12 h dark period in the tropics. Bacterial respiration of excreted carbon, and grazing by microheterotrophs were thought to be responsible.

When added up, the magnitude of the error caused by these problems can be extreme. Li and Goldman (1981) reported that in a test using laboratory cultures, the $^{14}$C method routinely over-estimated growth for certain species by up to 40%, yet under-estimated other species by 40-100%. They pointed out that the species used were probably hardier than the majority of species in the field, so the situation with natural populations may be even worse.

$^{15}$N Incubations

In the case of new production determinations, incubations with the stable isotope $^{15}$N as a tracer are used, typically provided as either nitrate or ammonium (see Dugdale and Goering 1967, Dugdale and Wilkerson 1986). As an incubation technique, this method shares the same problems of containment effects, and filtration collection illustrated in the $^{14}$C method. Added bicarbonate will probably not affect incubations in the $^{14}$C method, but additions of labeled nutrients is a greater problem, particularly in nutrient-depleted waters (Dugdale and Wilkerson 1986). In addition to these problems, there are problems with the loss of $^{15}$N label. Bronk and Glibert (1991, 1993) looked at release of dissolved organic nitrogen (DON) during incubations and concluded that both uptake and release of DON occur. Releases measured were between 5 and 54% of uptake. Collos et al. (1992b) found that laboratory cultures released up to 63% of the nitrate they took up in the first hours of the light period as DON, but took up most of this DON again in the following dark period. This could lead to serious problems with short-term incubations.

Finally, it should be pointed out that all of these techniques are population measurements; measuring any of these parameters at the level of a species or individual taxon is impossible without modifications.
Alternative approaches to estimating growth rates

Recognition of the problems outlined above has led to a variety of ingenious ways to overcome them (see Ceccaldi 1981). Furnas (1990) has divided such methods into four categories: a) biochemical or cell cycle markers, b) biochemical rate measurements and relative growth indices, c) cage and bottle incubations, and d) mathematical models.

Cell Cycle Markers

A variety of methods are based on determining the frequency of occurrence of cells in a specific stage of the cell cycle in phytoplankton populations. In general, the higher the frequency, the higher the growth rate. Such "frequency of dividing cell" methods rely on being able to identify a particular stage of the cell cycle (usually by microscopic examination of stained cells) and on knowing the time spent in this stage, a variable which can be difficult to determine (MacDuff and Chisholm 1982). In theory, no incubation is required, and species-specific information can be obtained (Chang and Carpenter 1988). Chang, Carpenter and others have explored this idea in great detail, moving from theory to the lab, to the field (Chang and Carpenter 1988, 1990, 1991, Antia et al. 1990). It has also been applied to cyanobacteria and picoplankton (Li and Dickie 1991 and Vaulot 1992). Flow cytometry can permit the method to be applied to individual cells (Chisholm et al. 1986).

Biochemical Rate Measurements and Relative Growth Rate Indices

Biochemical and growth rate indices have also been a fruitful area of research (note that enzymatic indices will be considered separately). There have been a variety of methods based on chlorophyll a fluorescence. Eppley and Sloan (1966) attempted to use light absorption by chlorophyll to estimate production, but species variation, different photosynthesis versus irradiance relationships and temperature effects caused problems. Chamberlin and Marra (1992) have obtained good correlations between natural fluorescence and photosynthetic rates, but a lack of basic understanding of the processes involved has hampered progress. By
enhancing the fluorescence of chlorophyll by adding DCMU, Furuya and Li (1992) found a relationship between fluorescence and photosynthetic capacity, but for different species the relationship showed over 5-fold variation. By stimulating cells with light pulses, information about natural maximum photosynthetic rates has also been obtained (Falkowski et al. 1986, Falkowski et al. 1992). There have been detailed investigations of cell ATP (Sheldon and Sutcliffe 1978, Laws et al. 1985, Noges 1989) or GTP (Karl 1980) contents (or various ratios) as growth rate indices, but these vary with the specific limitation and are relatively insensitive. Content of RNA, or various ratios of RNA, DNA, and protein have been considered in laboratory and field populations (Dortch et al. 1983, Dortch et al. 1985) and sensitive and convenient methods to determine DNA and RNA are available (Berdalet and Dortch 1991). Unfortunately, these methods are frequently highly specific to individual taxa, and are biased by detrital material (see Dortch et al. 1985).

*Modified Incubation Techniques*

Modifications to the traditional $^{14}$C method, and variations on incubation techniques have been proposed to overcome problems. Jespersen et al. (1992) concluded from a review of carbon-specific incorporation rates that they often gave growth rates which were too high. Use of $^{3}$H incorporation into DNA using labeled adenine was proposed, but rates estimated by this method were usually much too low. Use of $^{3}$H-labelled thymidine has also been proposed, but incorporation of this compound in natural samples appears to be exclusively due to bacteria (Moriarity 1986). Using traditional $^{14}$C methods, but looking at pigment-specific activities can allow carbon-specific and species-specific production rates to be inferred (see Welschmeyer and Lorenzen 1984, Gieskes and Kraay 1989) Alternatively, DNA-specific dyes have been used to monitor increases in DNA instead of increases in carbon in incubations (see Falkowski and Owens 1982). Irwin (1991) proposed that coulometric methods (measuring consumption of CO$_2$) could be used alongside $^{14}$C measurements to provide an independent check, or on their own when isotope use was inappropriate or difficult. Estimating protein synthesis rates using radioactive sulphur has also achieved some success (e.g. Bates 1981,
Cuhel and Lean 1987). In theory, protein is better conserved along food chains and so may be a better index of net phytoplankton production (see Lean et al. 1989). There are, however, methodological problems involving blank activities (Bates 1981). Alternatively, by using post-incubation separations, Madariaga et al. (1991) found population characteristics of photosynthesis and growth rate could be determined using the ratio of $^{14}$C incorporated into protein over that incorporated in low molecular weight metabolites. Another approach to growth rate in incubations involves removal of grazers. This can be accomplished by filtration, or selective heterotrophic inhibitors, but a less invasive method is simply to dilute the sample sequentially and measure grazing effects (see Li 1986). The y-intercept of a plot of growth rate versus degree of dilution should give the growth rate in the absence of grazing, although there are a number of problems, including nutrient depletion in the absence of regeneration (see Li 1986). Unfortunately, all of these methods still share the variety of problems associated with incubations previously discussed. In terms of modifying incubation methods themselves, Langdon et al. (1992) and Dandonneau and Bouteiller (1992) have both proposed remote, in situ samplers which would permit collection and inoculation of isotope into vessels without the problems associated with sample manipulation. Furnas (1991) had success using specially constructed diffusion chambers in place of static bottle incubations.

**Mathematical Models**

Beyond these experimental methods, modeling offers promise as well. Models predicting growth from irradiance alone, or combinations of irradiance, nutrients and temperature are very popular and diverse and include both mechanistic and empirical formulations (see Laws et al. 1985, Keller 1989, Laws and Chalup 1990, Sakshaug et al. 1991). Cullen (1990) reviewed several models that attempt to predict growth rates from irradiance, concluding that most of the many available mechanistic models are based on nearly identical theoretical bases. Species-specific differences and the problem of short-term unbalanced growth present the greatest difficulties. In a different approach, Lande et al.
(1989) estimated growth rates in situ from depth profiles of cell concentrations and turbulent diffusion measurements. Such methods will be aided by new techniques that increase the lower limit of nitrate detection (e.g. Garside 1982, Raimbault et al. 1990, McCarthy et al. 1992). When theoretical and empirical models are combined, it may be possible to predict production from surface ocean temperature and ocean colour (see Campbell and O'Riely 1988, Prasad et al. 1992) However, these methods may only be sensitive to processes in the very surface layer, and must still rely on calibration and validation by surface measurements (see Platt and Sathyendranath 1988).

**Alternative approaches to estimating new production**

For new production techniques, several alternatives exist to $^{15}$N incubations. Since export production is of interest, measuring the rate of organic sedimentation below the euphotic zone using moored or free-floating sediment traps is a direct approach (Eppley 1989, Platt et al. 1992b), although there are problems concerning trap recovery and efficiency (see Gardner 1980, Honjo et al. 1992). Bulk properties such as the increase in oxygen in the euphotic zone due to photosynthesis, or the decrease in oxygen below the euphotic zone due to decomposition of sedimented material (apparent oxygen utilization) have been proposed (see Platt et al. 1992b). These can be given a time scale by measuring the $^{3}$H:$^{3}$He ratio as an index of time since departure from the surface mixed layer, but may fail in the presence of small scale mixing and heterotrophic activity. They are also difficult to correlate meaningfully with biological processes on short time scales. By modeling circulation and mixing, the flux of nitrate from deeper water can be predicted, giving some estimate of new production (Platt et al. 1989, 1992b). Isotopic disequilibrium is also a promising method. In the isotope pair $^{238}$U and $^{234}$Th, apparently only Th (thorium) adsorbs to organic particles. When these particles sink from the euphotic zone, the change in the $^{238}$U:$^{234}$Th ratio is therefore a predictor of export of organic carbon (Platt et al. 1992). In all these cases, however, relationships between carbon and nitrogen must be assumed to allow interconversion. Laws (1991) pointed out that this is not always suitable; C:N ratios frequently exceed those predicted
by the Redfield ratio (i.e. 6.6 C:1 N). Thus, when based on nitrate data, the export of carbon could be 15-30% higher than would be predicted using the Redfield ratio. The satellite image methods discussed above may be extended to estimate new production by assuming a relationship between nitrate and surface temperature (see Campbell and Aarup 1992). In many of these methods, the question of whether the parameters are being measured on a time scale relevant to the organisms performing the processes is difficult to resolve.

**Enzyme activity: a theoretical basis for predicting biological rates**

In addition to methods considered previously, enzymes may provide a unique approach to estimating biological rates. Since enzymes are catalysts of the biological reactions of interest, and since they are adapted in character and concentration to meet prevailing demands of organisms (Hochachka and Somero 1984), the theoretical basis for studying enzymes is strong. There are however practical limitations.

Enzyme activity can be measured in two basic ways: the quantity of enzyme protein can be estimated by purification, or immunoassay, or the activity of the enzyme can be measured. Assay of enzyme concentration using immunoassays is currently complicated and subject to many of the same problems as the assay of enzyme activity, but offers extremely high sensitivity and is becoming more routine (see Balch et al. 1988, Rosalki 1989, Parker 1990; see also Chapter 2). Practically, when enzyme activity is assayed, in order to be reproducible, it is the maximal activity ($V_{\text{max}}$) that is measured. This is done with all substrates and cofactors at saturating levels, so that under these conditions the maximal activity should be proportional to the enzyme concentration (see Rosomando 1990). However, a number of factors may cause this not to be true. To begin with, enzyme extraction may damage the protein, or assay conditions may not support optimal activity (Newsholme and Crabtree 1986). Furthermore, the *in vitro* activity of the enzyme may bear little relationship to *in vivo* activity because in addition to varying enzyme activity by changing enzyme concentration, activity can be altered by changing the chemical nature of the enzymes (i.e. by manufacturing proteins with different characteristics that perform the same function, i.e.
isozymes), or by altering the environment of the protein in situ by changing variables such as cell surface to volume ratio, or cell membrane composition (see Raven 1981). On a finer scale, the enzyme itself may be covalently modified in processes such as adenylation or phosphorylation, or non-covalent mechanisms such as allosteric modification, or regulation of activity by substrate supply may prevail (Raven 1981). Each of these mechanisms may act on different time scales and may or may not be detected in $V_{\text{max}}$ assays, depending on the precise homogenization procedures and assay conditions. Despite the variety of methods of regulation and cell metabolic control, there is still evidence that maximal enzyme activity can be used to accurately estimate maximal in vivo rates of metabolism (see Newsholme and Crabtree 1986).

**Application of enzyme activity measurements to planktonic organisms**

The potential of enzyme determinations has not been lost on oceanographers. In all but a few cases, it is enzyme activity and not enzyme concentration that has been measured (but note Balch et al. 1988, Orellana et al. 1988, Wood 1988).

**ETS Measurements**

In phytoplankton, electron transport system (ETS) activity has been proposed as an index of respiration, or general metabolism (Packard et al. 1971, Kenner and Ahmed 1975a, 1975b, Packard 1985), and ETS may also be related to growth rate (Martinez 1992). It has been used in marine (Romano et al. 1987, Packard et al. 1988) and freshwater systems (Rai 1988), although the precise relationship between ETS and respiration is often in question (see Kenner and Ahmed 1975b, Martinez 1992).

**Enzymes Involved in Carbon Metabolism**

As an estimate of carbon fixation, the enzyme ribulose 1,5-bisphosphate carboxylase (RUBISCO) has proved useful. RUBISCO and photosynthesis have been correlated in laboratory cultures (Hellebust and Terbough 1967, Descolas-Gros 1982, Hobson et al. 1985, Smith and Platt 1985, Rivkin 1990), and in the field (Priscu and Goldman 1983, Li et al.
Glover and Morris (1979) found that RUBISCO activity was highly variable in field situations; it could account for less than half the variation in photosynthetic rate. However, Orellana and Perry (1992) showed good correlations between maximal photosynthetic rates and RUBISCO concentration in cultures, using an immunoassay. Assays of some other carboxylases involved in carbon fixation, phosphoenolpyruvate carboxylase (PEPCase) and phosphoenolpyruvate carboxykinase (PEPCK), have provided insight into rates of C4 carbon fixation, and carbon isotope fractionation (Morris et al. 1978, Descolas-Gros 1982, Descolas-Gros and Fontugne 1985, Descolas-Gros and Fontugne 1988).

**Enzymes Involved in Nitrogen Metabolism**

Enzymes of nitrogen metabolism have been explored as indices of nitrogen incorporation rates. Nitrate reductase (NR) has been used to predict nitrate incorporation rates (e.g. Eppley et al. 1969, Blasco et al. 1984), but the relationships have been highly variable and generally difficult to relate to other field measurements. NR immunoassays have also been proposed (Balch et al. 1988). Nitrite reductase (NiR) has also been explored, with limited success (see McCarthy and Eppley 1972). Enzymes involved in ammonium incorporation have also received attention. Glutamate dehydrogenase (GDH) was initially identified as a key enzyme of interest (see Ahmed et al. 1977), but activity seldom correlated well with incorporation rates (McCarthy and Eppley 1972, Dortch et al. 1979). Subsequently, it was shown that glutamine synthetase (GS) is more likely to be the pathway for ammonium incorporation (Miflin and Lea 1976). The emphasis shifted to this enzyme both in culture (Falkowski and Rivkin 1976, Thomas et al. 1984, Everest et al. 1986, Slawyk and Rodier 1986, 1988) and in the field (Clayton and Ahmed 1987). In some cases, GS activity has correlated well with ammonium uptake, but this is not true during starvation, or perturbations in nutrient levels (Slawyk and Rodier 1986).
Other Enzymes

Other enzymes have been used as indicators of specific states, rather than indices of rates. Alkaline phosphatase (APase) has served as an indicator of phosphate deficiency in marine and fresh waters (Perry 1972, Jansson 1976, Sakshaug et al. 1984, Davies and Smith 1988). Cell surface amino acid oxidases have also been studied, as indicators of the ability of cells to use combined amino nitrogen (Palenik and Morel 1990). As well, Price and Morel (1990) describe a number of other exoenzymes, including metal reductases, and proteases.

Enzymes in Other Planktonic Organisms

The oceanographic use of enzyme activities as biochemical indices of metabolic rates has not been limited to phytoplankton. Bacterial exoenzymes, involved in degradation of organic compounds, have been used as indices of bacterial activity (Chrost et al. 1989, Smith et al. 1992, Martinez and Azam 1993). Aspartate transcarbamylase (ATCase), a key enzyme in nucleotide synthesis, has been measured as an index of secondary production in zooplankton (Bergeron 1983, 1986, 1990) although a relationship between ATCase and secondary production has not been shown under controlled conditions. ETS and GDH activities have also been applied to zooplankton to estimate respiration and excretion rates, respectively (Bidigare and King 1981, Bidigare et al. 1982, Mayzaud 1987), but there are indications that these enzyme indices may be biased by size effects on metabolic rates (see Berges et al. 1993). As well, digestive enzymes such as amylase and trypsin have been studied in zooplankton in an effort to estimate feeding rates (Samain et al. 1983, Mayzaud et al. 1984, Hasset and Landry 1990a, 1990b), but there are problems with the complex spatial variability of enzyme activity in the field (see Hirche 1989) and the slow response time of the enzymes to new conditions (see Roche-Mayzaud et al. 1991). Berges (1989) and Berges et al. (1990) suggested that nucleoside diphosphokinase (NDPK), might be useful as a predictor of zooplankton growth rate. As with other enzymes, the use of NDPK activity does not necessarily yield straightforward results. For example, NDPK response varied with animal growth stage,
perhaps due to differences between stages of growth by cell proliferation versus stages of cell size increase (Berges 1989).

Despite a strong rationale for the use of enzyme activity, and many cases where enzyme assays have been applied, the success of these enzyme methods has been equivocal. Relationships of enzyme activities with other indices of biological rates have been highly variable. A major problem has been that the enzyme index is frequently applied to a field situation before there has been adequate laboratory investigation. Leftley et al. (1983) criticized marine ecologists for their over-enthusiasm in taking fledgling methods to the field. The problem is that in the field there is usually no way to independently measure the biological rate of interest, except by using the very methods whose inadequacy prompted development of the enzyme methods in the first place. When traditional methods and enzyme-based methods disagree, it is unclear which is correct, or in fact if either is correct. In general, detailed laboratory studies have not been conducted first.

**Organization and goals of this thesis**

In this thesis, work began with laboratory studies using unialgal cultures under steady state conditions. Complexity of experiments was gradually increased until enough confidence in the techniques and in the relationships between enzymes and their associated rate processes had been gained so that preliminary field work could be attempted.

For these studies, the diatom *Thalassiosira pseudonana* was selected as the principal experimental organism. This diatom occurs as single cells and has regular dimensions, facilitating counting of cells and cell volume determinations. As well, it is fast growing (up to three divisions per day) and can be easily maintained in the laboratory. Finally, because this species has been the subject of numerous previous studies, it is well characterized and a large body of specific information is available. The particular clone considered in these studies, 3H, was originally isolated by Guillard in 1958, from a coastal embayment in Long Island, New York (see Guillard and Ryther 1962). *Thalassiosira pseudonana* has worldwide distribution; there are isolates from locations ranging from along the Atlantic seaboard of the U.S., to
European estuaries, tropical Atlantic reefs and Australian coastal waters (see Nelson and Brand 1979). However, it may not be the most ecologically relevant species to use, since it is rarely a dominant member of the plankton (but see Guillard and Ryther 1962, and Gallegos 1992 for records of blooms), and is usually confined to enriched waters. There is also evidence that the 3H isolate has lost heterozygosity at several loci since isolation (Murphy 1978). However, there is no reason to believe that the fundamental physiology of *T. pseudonana* is different from that of any other diatom. Its availability, ease of culture and the great deal of previous research conducted with this organism, make it a prime candidate for a reference species, much as the white rat serves this purpose for medical research.

In Chapter 1, the relationship between growth rate and nucleoside diphosphate kinase (NDPK) activity is examined in *T. pseudonana*. The goals are: a) to characterize the enzyme in this species in terms of optimal assay conditions, and kinetic and thermodynamic constants, and b) to determine whether NDPK activity is related to light limited growth rate in a predictable manner that might allow the enzyme to be used to estimate growth rate.

In the following chapters, the enzyme nitrate reductase (NR) is examined as a means to estimate nitrate incorporation rates.

In Chapter 2, the goals are: a) to develop and optimize an assay for NR activity in *T. pseudonana*, b) to characterize the enzyme in terms of kinetic constants, substrate specificity and cofactor requirements in *T. pseudonana*, as well as another diatom *Skeletonema costatum*, and a dinoflagellate, *Amphidinium carterae*, c) to validate the NR assays in these three species by determining whether NR activity is sufficient to account for observed nitrate incorporation rates in cultures growing on excess nitrate under light limitation, and d) to determine whether the assay developed in *T. pseudonana* is applicable to a range of other phytoplankton species, using the criterion that NR activity must equal or exceed measured rates of nitrate incorporation.

Chapter 3 compares NR activity in *T. pseudonana* in steady state light-limited and nitrate-limited cultures. The goals of this chapter are to determine: a) if NR activity is related
to nitrate incorporation rates, and b) how cell composition changes under different limiting conditions in order to select a scaling variable for enzyme activity.

In Chapter 4 more complex, but ecologically relevant cases are considered, where *T. pseudonana* is grown on light:dark cycles, or under different light spectra, or where cells are starved of nitrate, or where cultures are provided with ammonium as a nitrogen source. The goals of the chapter are to determine in each case whether the different conditions affect the relationships between NR activity and nitrate incorporation rates seen under steady state conditions.

Finally, in Chapter 5, the NR assay is taken to the field in a preliminary study under carefully controlled conditions. The goals of this chapter are: a) to determine whether the NR assay developed in Chapter 2 can be applied in the field, b) to determine the characteristics of NR activity in natural populations in terms of kinetic constants, substrate specificity and cofactor requirements, and c) to compare NR activities to other indices of nitrate incorporation rates including nitrate disappearance from the medium, particulate nitrogen increase, or $^{15}$N uptake. These comparisons are made over diel cycles in irradiance, and in the presence or absence of ammonium.
CHAPTER 1: RELATIONSHIP BETWEEN NUCLEOSIDE DIPHOSPHATE KINASE ACTIVITY AND LIGHT-LIMITED GROWTH RATE IN THE MARINE DIATOM THALASSIOSIRA PSEUDONANA

INTRODUCTION

Selecting an enzyme to serve as an index of growth rate is not a simple matter. There is evidence that many different enzyme activities correlate with growth rate in a variety of organisms. For example, Pedersen et al. (1978) reported that in the bacterium Escherichia coli, 102 of 140 proteins (representing 2/3 of the protein mass of the cell) catalogued on chromatography plates showed nearly linear increases with increasing growth rate. In yeast cells, Sebastian et al. (1973) demonstrated a correlation between RNA polymerase I activity and growth rate, while Yao et al. (1985) found that ornithine decarboxylase activity in the ciliate Tetrahymena thermophila was also correlated with growth. However, because phytoplankton growth is often unbalanced (see Eppley 1981), increases in cell number may not be equal to specific rates of elemental increase (e.g. carbon incorporation), or by the rate of synthesis of an individual component (e.g. an amino acid). Thus, an enzyme associated with synthesis of a particular component may not be suitable as a growth rate index under all circumstances. A more general index of growth rate is desirable.

Hochachka and Somero (1984) divided metabolism in animal cells into three blocks: a) a catabolic block where energy was provided as ATP or NAD(P)H (and presumably corresponding to photosynthetic reactions in plant cells), b) an anabolic block where ATP and NAD(P)H drive basic biosynthetic reactions and chemical and mechanical work, and c) a block involving growth and integration. Interestingly, in general, growth and integration do not use ATP directly, but instead use other nucleoside triphosphate (NTP) compounds, e.g. GTP for protein synthesis, CTP for synthesis of certain lipid compounds, and UTP for synthesis of complex carbohydrates (Lehninger 1982, Hochachka and Somero 1984). The specialization of these NTP forms probably aids in proper allocation of ATP among different metabolic needs (see Atkinson 1977). Furthermore, NTP compounds are also required for the
DNA and RNA synthesis which must accompany growth (Parks and Agarwal 1973). With the exception of ATP and a small portion of GTP, all nucleoside triphosphates are synthesized by nucleoside diphosphate kinases (E.C. 2.7.4.6., NDPK) (Ingraham and Ginther 1978). NDPK catalyses the reversible reaction:

\[
\text{ATP} + \text{NDP} \leftrightarrow \text{ADP} + \text{NTP}
\]

where NDP and NTP are the high energy di- and triphosphate forms of the nucleosides cytidine, guanosine, uridine, or thymidine. It might be hypothesized that the increased requirements for NTP compounds at higher growth rates would necessitate increases in NDPK activity.

**Characteristics of NDPK**

NDPK is found in all cells, and has been measured in a wide variety of organisms (Parks and Agarwal 1973). Characteristics of the enzyme are summarized in Table 1.1. The enzyme is usually found as a hexamer of about 100 kDa, but Jong and Ma (1991) have reported a tetrameric form in yeast. There also appear to be many isozyme forms of the enzyme with distinct characteristics; however, Gilles et al. (1991) have shown that some NDPK isoforms may be an artifact of enzyme purification procedures. In their study, human erythrocytes were found to contain only one form of NDPK, a hexamer composed of two distinct polypeptide chains. When purified under denaturing conditions (using isoelectric focusing), these subunits dissociated and could randomly re-associate to produce two or more apparently different enzymes (Gilles et al. 1991). NDPK is relatively non-specific for different nucleoside di-and triphosphates. \( K_m \) values and reaction rates with different nucleosides are generally within the same order of magnitude (Ingraham and Ginther 1978).

The NDPK reaction described above has an equilibrium constant near 1.0; values range from 0.6 in *Bacillus subtilis* (Sedmak and Ramaley 1971) to 1.28 in yeast (Parks and Agarwal 1973), depending on assay conditions.
Table 1.1. Characteristics of nucleoside diphosphate kinase (NDPK) from various sources (ISOZYME = isoelectric point of the isozyme where available, otherwise the authors’ description; MW = molecular weight, * indicates the weight of a monomer; AE = apparent activation enthalpy below/above the transition temperature; – = not provided by the authors).

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>ISOZYME</th>
<th>MW (kDa)</th>
<th>SUBUNITS</th>
<th>$K_m$ ATP (mM)</th>
<th>$K_m$ TDP (mM)</th>
<th>AE (kJ mol$^{-1}$)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>8.4</td>
<td>100</td>
<td>–</td>
<td>0.15</td>
<td>–</td>
<td>1.15/2.46</td>
<td>Sedmak and Ramaley 1971</td>
</tr>
<tr>
<td>brewers yeast</td>
<td>8.0</td>
<td>102</td>
<td>–</td>
<td>0.31</td>
<td>–</td>
<td>–</td>
<td>Parks and Agarwal 1973</td>
</tr>
<tr>
<td>* Saccharomyces cervisiae</td>
<td>70</td>
<td>4</td>
<td></td>
<td>1.66</td>
<td>0.16</td>
<td>–</td>
<td>Jong and Ma 1991</td>
</tr>
<tr>
<td>rat liver</td>
<td>m 6.0</td>
<td>–</td>
<td>–</td>
<td>1.0</td>
<td>0.055</td>
<td>–</td>
<td>Kimura and Shimada 1988</td>
</tr>
<tr>
<td>c 6.0</td>
<td>18*</td>
<td>6</td>
<td></td>
<td>1.33</td>
<td>0.19</td>
<td>–</td>
<td>*</td>
</tr>
<tr>
<td>* beef brain</td>
<td>8.6</td>
<td>120</td>
<td>–</td>
<td>0.23</td>
<td>0.26</td>
<td>–</td>
<td>Robinson et al. 1981</td>
</tr>
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<td>human erythrocytes</td>
<td>5.4</td>
<td>80</td>
<td>–</td>
<td>0.2</td>
<td>0.11</td>
<td>–</td>
<td>Parks and Agarwal 1973</td>
</tr>
<tr>
<td>*</td>
<td>5.8</td>
<td>93</td>
<td>–</td>
<td>1.0</td>
<td>0.55</td>
<td>–</td>
<td>Parks and Agarwal 1973</td>
</tr>
<tr>
<td>*</td>
<td>6.3</td>
<td>84</td>
<td>–</td>
<td>3.0</td>
<td>0.22</td>
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<td>*</td>
</tr>
<tr>
<td>*</td>
<td>6.8</td>
<td>80</td>
<td>–</td>
<td>0.25</td>
<td>0.20</td>
<td>–</td>
<td>Parks and Agarwal 1973</td>
</tr>
<tr>
<td>*</td>
<td>7.3</td>
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<td>–</td>
<td>1.08</td>
<td>0.30</td>
<td>1.00/2.03</td>
<td>*</td>
</tr>
<tr>
<td>*</td>
<td>8.3</td>
<td>103</td>
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<td>0.17</td>
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<td>–</td>
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<td>*</td>
<td>–</td>
<td>17*</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Gilles et al. 1991</td>
</tr>
<tr>
<td>Ehrlich Ascites tumor cells</td>
<td>–</td>
<td>76</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Koyama et al. 1984</td>
</tr>
<tr>
<td>Spinacea oleracea</td>
<td>I 16*</td>
<td>6</td>
<td></td>
<td>2.0</td>
<td>–</td>
<td>–</td>
<td>Nomura et al. 1991</td>
</tr>
<tr>
<td>II 18*</td>
<td>6</td>
<td></td>
<td>0.89</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>*</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>I – 100</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Klein and Follmann 1988</td>
</tr>
<tr>
<td>II – 100</td>
<td>–</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>*</td>
</tr>
</tbody>
</table>
NDPK and Growth Rate

The fact that NDPK is a near-equilibrium enzyme (i.e. the reaction is freely reversible) indicates that it is unlikely that the enzyme is substrate saturated in vivo and thus rate-limiting (see Newsholme and Crabtree 1986). This suggests that the maximal activity of NDPK, measured in vitro with saturating substrate cannot be quantitatively related to an in vivo rate. Nevertheless, a correlation between maximal NDPK activity and growth rate might still be possible. Brown (1991) has argued that there are adaptive pressures on cells to minimize their protein content (since protein is usually near the solubility limit within the cell). Thus, if a given enzyme was not rate-limiting and it was in greater concentration than necessary, there would be an advantage to reducing its concentration. As a result, even non-rate limiting enzymes should respond as the fluxes through metabolic pathways change.

Although NDPK has been measured in a wide range of organisms from bacteria to higher plants to mammalian cells (Parks and Agarwal 1973), there is only one report of a measurement in a unicellular autotroph (Klein and Follmann 1988), and no cases of measurements in marine phytoplankton. There has been speculation about the importance of NDPK in cell growth processes during development (Dickinson and Davies 1971), including a correlation with growth rate in mammalian tumor cells (Koyama et al. 1984), and evidence of relationships between NDPK and growth rate in crustaceans (Berges 1989, Berges et al. 1990). In multicellular organisms, however, the relationship between NDPK and growth is complicated by changes in body size and composition during development. Such relationships may be clearer in a unicellular organism.

The objectives of this chapter are to examine: a) the general characteristics of NDPK in Thalassiosira pseudonana, b) the relationship between maximal NDPK activity (which should be proportional to enzyme concentration) and growth rate under light (energy) limitation, and c) the relationship between various cell components and growth rate in order to determine to which biomass parameter NDPK activity is best scaled.
MATERIALS AND METHODS

Culture conditions

The marine diatom *Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal (3H clone) was obtained from the Northeast Pacific Culture Collection, Department of Oceanography, University of British Columbia. Cultures were grown in semi-continuous batch culture in enriched artificial seawater (ESAW) based on the recipe by Harrison *et al.* (1980), with sodium glycerophosphate replaced with an equimolar concentration of sodium phosphate, ferrous ammonium sulphate with an equimolar concentration of ferric chloride and additions of selenite, nickel and molybdate to achieve 1 nM final concentration. Temperature was maintained at 17.5 ± 0.5°C using a circulating water bath. Cultures were grown in 1 L glass flasks, stirred at 60 rpm with Teflon-coated stir bars and bubbled with air filtered through a 0.22 μm membrane filter. Continuous illumination was provided by Vita-lite™ fluorescent tubes and attenuated by distance or neutral density screening to give a range of irradiances from 6 to 120 μmol quanta m⁻² s⁻¹ measured in air inside empty culture vessels using a LiCor model 185 meter. During the course of experiments, cultures were never dense enough to reduce average irradiance by more than 10%. Growth rates were followed by *in vivo* fluorescence, measured twice daily using a Turner Designs™ Model 10 fluorometer and cell counts using a Coulter Counter™ model TAI equipped with a population accessory. All sampling was conducted in early to mid logarithmic growth phase.

Cell composition

In all experiments, cell carbon and nitrogen quotas were determined by filtering samples onto pre-combusted 13 mm Gelman type AE glass fiber filters and analyzing them using a Carlo Erba CNS analyzer. Samples for protein determination were collected on pre-combusted Whatman GF/F filters. Homogenates were prepared as described by Dortch *et al.* (1984). They were ground with 3% trichloroacetic acid (TCA) and solublized in 1 N NaOH.
Protein was determined by the method of Bradford (1976) using the micro-assay procedure of the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, 500-0001) with bovine serum albumin (BSA, Sigma Chemical Co. A 7638) as a standard. Cell volumes were calculated from Coulter Counter measurements and calibrated using 5 μm latex microspheres, following Thompson et al. (1991).

**Cell homogenization and enzyme assay**

Samples were collected on 25 mm Whatman GF/F glass fibre filters using filtration pressures less than 100 mm Hg. Filters were immediately placed in 1 mL of ice cold extraction buffer consisting of 50 mM imidazole, pH 7.4, 2 mM dithiothreitol, 2 mM EDTA, 1% (w/v) BSA and 0.1% (v/v) Triton X-100. Cells and filters were ground in a 5 mL glass-Teflon tissue homogenizer for 2 min. Homogenates were centrifuged in a Sorval RCB-2B centrifuge at 4°C for 5 min at 750 g and used immediately in assays. Preliminary experiments showed that no NDPK activity remained in the pellet. This might have been anticipated since the enzyme from higher plants has been shown to be predominantly in the cytosol (Dancer et al. 1990).

Assay conditions were adapted from Berges et al. (1990). Assays were conducted in 1 mL volumes in disposable plastic cuvettes. All assay components were obtained from Sigma Chemical Co. and were the purest grade available. ADP produced in the NDPK reaction was coupled to NADH oxidation through pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Agarwal et al. 1978). Substrate concentrations were optimized by increasing the concentration of each reaction component until no further increase in NDPK activity was observed. This was routinely verified over the course of the experiments in cultures growing at low and high irradiances. Further increases in substrate concentrations were avoided, since they also increased rates of side reaction and thus decreased precision. Final concentrations in the assay were 50 mM imidazole buffer (pH 7.4), 0.2 mM NADH, 20 mM MgCl₂, 70 mM KCl, 1.1 mM phosphoenol pyruvate, 2.0 mM ATP, 0.7 mM TDP, 10 U lactate dehydrogenase (Sigma L 2500) and 1 U pyruvate kinase (Sigma P-1506). Reactions were
started by adding TDP. Controls were run without homogenate and without TDP, and rates were corrected accordingly (Agarwal *et al.* 1978). Reactions were followed by monitoring the decrease in absorbance at 340 nm due to NADH oxidation using a LKB Ultrospec II UV spectrophotometer with a six position water-cooled turret interfaced to an IBM personal computer (see Appendix B). Typically, it was necessary to monitor reactions for 5 to 10 min to establish the initial, linear rate of reaction. Temperature was maintained at 17.5 ± 0.1 °C (the growth temperature of the cultures) using a Lauda RM6 water circulating bath. NDPK activity was expressed in units (U), where 1 U represents the quantity of enzyme catalyzing the conversion of 1 μmol of substrate to product per minute, using a millimolar extinction coefficient of 6.22.

**Enzyme characterization**

Assays were conducted over a range of ATP and TDP concentrations to determine $K_m$ values for the algal enzyme. Data for NDPK activity versus substrate concentration were fitted to a Michaelis-Menten model using a non-linear fitting routine (NONLIN, Wilkinson 1990; see also Appendix C). Assays were also conducted over a range of temperatures from 10 to 25°C. An Arrhenius transformation was used to calculate an apparent activation enthalpy ($\Delta H^+$) of the enzyme (Hochachka and Somero 1984).

**Steady state experiments**

On six separate occasions, four to six semi-continuous batch cultures were grown at different irradiances ranging from 6 to 120 μmol quanta m$^{-2}$ s$^{-1}$. Cultures were acclimated for a minimum of 10 generations except in cultures growing at $\mu < 0.4$, where 6 to 8 generations were allowed. Cultures were sampled for cell volume, cell nitrogen, carbon and protein quotas (i.e. cell contents), and NDPK activity. These parameters were plotted against growth rate and examined using linear correlation analyses (Wilkinson 1990).
Transition Experiments

On two occasions, six *T. pseudonana* cultures were acclimated in the same manner as in steady state experiments, three to 15 μmol quanta m⁻² s⁻¹ and three to 135 μmol quanta m⁻² s⁻¹. Samples identical to those in the steady state experiment were taken; then the cultures were transposed. In the first experiment, cultures were sampled at 24 h intervals for 72 h after transposition. In the second experiment, sampling continued for 210 h after the transition. Changes over time in cell carbon, nitrogen and protein quotas, cell volume, growth rate, and NDPK activity were examined.

RESULTS

Enzyme characterization

*Kₘ* values for the substrates, calculated from six separate homogenates, were 0.24 ± 0.01 mM for TDP and 0.86 ± 0.06 mM for ATP (Fig. 1.1).

The slope of the regression lines of Arrhenius plots of log NDPK activity versus the inverse of temperature gave an apparent activation enthalpy of 0.841 ± 0.026 kJ mol⁻¹ (Fig. 1.2).

Steady state experiments

Steady state growth rates versus irradiance data were collected over a period of 18 months and demonstrate the constancy of the growth rate-irradiance relationship over the experimental period (Fig. 1.3). Fitting a Michaelis-Menten type curve to the data gave a *μ*ₘₐₓ of 1.64 d⁻¹ and a half-saturation constant (*Kₐ*) of 23 μmol quanta m⁻² s⁻¹. From Fig. 1.3, *Iₖ* (the onset of light saturation as defined in Parsons *et al.* 1984b) was estimated to be approximately 40 μmol quanta m⁻² s⁻¹.

Carbon, nitrogen and protein cell quotas (pg cell⁻¹) were not significantly correlated with growth rate (*P > 0.3, P > 0.06, and P > 0.5*, respectively) (Fig. 1.4). However, there
Figure 1.1. Nucleoside diphosphate kinase (NDPK) activity versus substrate concentration for A) thymidine 5'-diphosphate (TDP) and B) adenosine 5'-triphosphate (ATP) in homogenates of *Thalassiosira pseudonana*. Curves are fit to rectangular hyperbolae. $K_m$ values are 0.24 mM for TDP and 0.86 mM for ATP.
Figure 1.2. Arrhenius plot of NDPK from *Thalassiosira pseudonana*.

The solid line represents a least squares regression fit to the data.

Apparent activation enthalpy is 0.841 kJ mol\(^{-1}\).
Figure 1.3. Growth rate versus irradiance curve for *Thalassiosira pseudonana*. Curve is fit to a rectangular hyperbola. $\mu_{\text{max}} = 1.64 \text{ d}^{-1}$ and $K_I = 23 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Each point represents a single culture. Error bars represent the standard error of the mean of 3 to 6 growth rate measurements, or if not seen are smaller than the size of the symbol.
Figure 1.4. Cell composition versus light-limited specific growth rate in *Thalassiosira pseudonana*. A) Cell carbon quota, B) cell nitrogen quota, C) cell volume, and D) cell protein quota. Each point represents the mean of duplicate determinations from a single culture.
was a significant positive linear relationship between cell volume and growth rate (P < 0.01). If carbon, nitrogen and protein were expressed per unit cell volume (i.e. pg µm⁻³), there were significant negative relationships with growth rates for carbon (P < 0.05) and nitrogen (P < 0.05), but not protein (P > 0.09). In addition, there were no significant correlations between NDPK activity (on a per cell basis) and carbon cell quota, nitrogen cell quota, protein cell quota or cell volume (P > 0.2 in all cases; data not shown).

The relationships between NDPK activity on a per cell basis and either specific growth rate or growth rate in terms of carbon (the product of specific growth rate and carbon cell quota, which is analogous to a ¹⁴C measurement) were highly variable (Fig. 1.5). However, NDPK activity was significantly and positively correlated with growth rate. When a linear model was used, NDPK activity per cell was significantly correlated with specific growth rate (P < 0.05) and carbon growth rate (P < 0.04). NDPK activity at low growth rates (µ < 0.4) appeared to increase. Using a quadratic model, the correlation improved (P < 0.01 for both cases). Expressing NDPK activity per unit cell volume, carbon, or protein did not change the pattern of the relationship, although the variability increased significantly (Fig. 1.6 A, B, D). When NDPK activity was expressed per unit nitrogen, however, the quadratic term in the NDPK-growth rate relationship was no longer significant (P > 0.07), indicating that the relationship was more linear.

**Transition Experiments**

Transition experiments provided another way to assess whether growth rate and NDPK activity were related. By measuring NDPK activity in individual cultures before and after a transfer from low to high light or vice versa, changes in enzyme activity could be followed and an approximate time for changes to occur determined. Transition experiments were repeated twice. In the first case, the time course was followed for only 72 h, in the second case for 210 h. Results were nearly identical in both experiments; for clarity only the results of the 210 h time course are presented. Under steady state conditions, the growth rate of high light cultures was 1.45 d⁻¹, while low light cultures grew at 0.56 d⁻¹ (Fig. 1.7). Cultures were switched at
Figure 1.5. NDPK activity versus A) light-limited specific growth rate, and B) growth rate in terms of carbon in *Thalassiosira pseudonana*. Each data point represents a single culture. Error bars show the standard error of the mean of two enzyme assays or a minimum of three growth rate determinations.
Figure 1.6. NDPK activity versus specific growth rate in *Thalassiosira pseudonana*.

Activity is expressed per unit carbon (A), nitrogen (B), cell volume (C) or protein (D). Error bars show the standard error of the mean of two enzyme assays or a minimum of three growth rate determinations.
Figure 1.7. Cell composition versus time in terms of A) cell carbon quota, B) cell nitrogen quota, C) cell volume, and D) cell protein quota in *Thalassiosira pseudonana.*

(○) Cultures grown under high light (135 μmol quanta m⁻² s⁻¹) and moved to low light (15 μmol quanta m⁻² s⁻¹) at t = 32 h (marked by the arrow). (●) Cultures grown under low light and switched to high light at t = 32. Error bars represent standard errors of the mean of three replicate cultures. Statistically significant differences (P < 0.05) are indicated by asterisks (*).
t = 32 h, and growth rates had changed by 48 h. Composition between treatments was
compared using a repeated measures ANOVA followed by LSD comparisons at the 95% level
(Steel and Torrie 1980, Wilkinson 1990). Results were similar to the steady state experiments
in that there were no differences between high and low light cells for carbon quota or nitrogen
quota. Protein quota differed only in one case. Cell volume was significantly higher in high-
light grown cells before the transition, and by 150 h cell volumes in transition cultures were
effectively the same as those found under the corresponding low or high light steady state.
Steady state NDPK activities were significantly higher in high light cultures when expressed
per cell, or per unit carbon, or nitrogen, but not significantly different when expressed per unit
protein (Fig. 1.8). Following the transition, although the NDPK activity dropped significantly
for high light to low light transition, the treatments were not significantly different at the end
of the experiment.

DISCUSSION

Enzyme characterization

Although NDPK is found in a broad range of organisms, its characteristics are very
similar (Parks and Agarwal 1973). Differences between characteristics of the crude enzyme
homogenate from Thalassiosira pseudonana and those published in the literature might have
been anticipated, since the majority of work has been done on the purified enzyme. Further,
the algal NDPK may be a mixture of isoforms. For example, Nomura et al. (1991) described
two NDPK isozymes from spinach (Spinacea oleracea). Despite these considerations, the
kinetic and thermodynamic constants appear almost identical to those published (see Table
1.1). A $K_m$ for TDP of 0.24 mM was obtained for T. pseudonana, which is close to the
values found in yeast (Jong and Ma 1991) and in human erythrocytes (Agarwal et al. 1978).
For ATP, a $K_m$ of 0.86 mM was calculated in the present study, which is very near that found
by Nomura et al. (1991) in spinach leaves and within the range found in human erythrocyte
isoforms (Agarwal et al. 1978). Arrhenius plots of the enzymes
Figure 1.8. NDPK activity scaled to A) cell number, B) cell carbon quota, C) cell nitrogen quota, D) cell volume, and E) cell protein quota, versus time for transition experiments with *Thalassiosira pseudonana*. Symbols are the same as in Figure 1.7.
often display biphasic behavior, although this is dependent on the particular isoform (Agarwal et al. 1978). It is possible that this was obscured in the present study by a mixture of isoforms or because of the relatively low number of temperatures assayed. The pI 7.3 isoform of NDPK from human erythrocytes shows two phases with a break at 31°C (Agarwal and Parks 1971), while for NDPK from Bacillus subtilis a transition occurs at 25°C (Sedmak and Ramaley 1971). Both of these transitions occur at or above the highest temperature tested in the present study, but the activation enthalpy for T. pseudonana (0.841 KJ mol⁻¹) is close to the values determined in other species, below their transition points (see Table 1.1). In all cases, the optimal substrate concentrations and assay conditions are remarkably similar; conditions determined for crustacean tissue (Berges et al. 1990) proved optimal for NDPK from Thalassiosira pseudonana.

**Cell composition**

No consistent relationships were found between light-limited growth rate and carbon, nitrogen or protein quota. Cell volume, however, was positively correlated with growth rate. Although there were significant relationships between carbon, and nitrogen per cell volume, these are probably caused by the significant change in volume alone. This illustrates a potential pitfall in using such ratios (see also Packard and Boardman 1988). Raven (1981) points out that variation in cell volume may be an important metabolic adaptation; in order to maintain the proper cellular concentration of metabolites and catalysts it may be necessary to change cell volume. Thompson et al. (1991) provide a detailed review of carbon and volume relationships with growth rate and show that there is a general, positive relationship for a variety of species. For Thalassiosira pseudonana, in particular, the volume-growth rate relationships in the present study agree well with theirs, but in contrast, Thompson et al. (1991) found a strong positive relationship between carbon quota and growth rate which was not observed in the present study. The reason for these differences is unclear, although the experiments in the present study were conducted over a much shorter period of time than those of Thompson et al. (1991), during which large differences in cell volume and carbon quotas
were seen. Such variability may result, in part, from size changes related to the sexual cycle of diatom species, although we observed no evidence of sexual reproduction in any of our cultures. Various authors have also demonstrated size and carbon quotas which decrease with growth rate (Thompson et al. 1991).

There is evidence in the present study of increases in cell volume, and cell quotas of carbon, nitrogen and protein at very low growth rates (μ < 0.25). This represents novel information since there is very little data in the literature for such low growth rates. Thompson et al. (1991), for example, had only one culture in this range of growth rates, and although Sakshaug and Andresen (1986) reported increases in cell carbon and nitrogen quotas at low irradiance in cultures of Skeletonema costatum, this effect was only prominent when cells were grown on light-dark cycles with short day lengths. Increased NDPK activity at low growth rate was also found in the present study. This is not due solely to increases in cell size, since the pattern persists even if data are scaled to cell volume or carbon, or protein cell quotas (Figure 1.6 A, B, D), but it may be related to cell nitrogen quota.

Composition data for the transition experiments agree well with the steady state data; only cell volume changed consistently throughout the transitions. This volume change is in agreement with data presented by Thompson et al. (1991), although these authors also found significant changes in carbon quota. In a similar light transition study, Post et al. (1985) noted that in turbidostat-grown cultures of Thalassiosira weisflogii, changes in carbohydrate occurred during light transitions, but no significant changes in protein were found. Similarly, Claustre and Gostan (1987) found changes in volume but not in protein during transitions with Isochrysis and Hymenomonas species.

A discussion of the causes and meaning of these cell composition differences is beyond the scope of this chapter, but they will be discussed further in Chapter 3. However, such changes have important implications for selecting a biomass variable on which to scale enzyme activity. Since there was no indication that NDPK activity was correlated with any index of cell composition measured, the usefulness of normalizing to facilitate comparisons within this species is questionable. Because NDPK activity per cell varied over a factor of 6 or 7, while
carbon or cell volume only varied by a factor of two, scaling enzyme activity to either carbon or volume does not substantially change the relationship between NDPK and growth rate, and may in fact add variation to the measurement. In addition, if the relationship between enzyme activity and carbon, cell volume or nitrogen is complex (e.g. curvilinear), the scaled enzyme activity becomes much more difficult to interpret (Packard and Boardman, 1988). NDPK activity per unit nitrogen appeared more linear and consistent with a monotonic increase in NDPK activity with growth rate. However, the relationship was highly variable; less than 25% of the variation in NDPK activity could be attributed to growth rate. Protein is commonly selected as a scaling variable in enzyme studies, but diatom species have many potentially interfering compounds such as amino acids, which complicate such a measurement (Dortch et al. 1984; Appendix A). Cell volume may also be unsuitable as a scaling factor because of methodological biases. Thompson et al. (1991) speculated that short-term diatom volume increases (such as those found in this study) are achieved by addition of intercalary bands, added between the valves of the diatom frustule, which would elongate the cell without an increase in width. This implies a change in cell geometry which will result in an error if the volume is measured by a particle counter such as a Coulter Counter™ (Kubitscheck 1987; Montagnes et al. submitted). For the present, when a single species is considered, expressing activity per cell and providing data on cell composition seems to be the most reasonable course. In cases where interspecific comparisons must be made the issue is clearly more complex and cell nitrogen may offer some promise.

NDPK and growth rate

Although statistically significant, the relationship between NDPK activity and growth rate is relatively poor and therefore of limited use in a predictive sense. Part of the reason for this variability may be that NDPK has other functions in the cell in addition to NTP interconversions. NDPK has been shown to be involved in cell signal transduction and regulatory processes. NDPK may directly interact with membrane G proteins and may be involved in the regulation of adenylate cyclase (Jong and Ma 1991). As well, NDPK has been
implicated in activating guanine nucleotide binding proteins (Jong and Ma 1991; Nomura et al. 1991). The importance of these processes in a unicellular organism is unclear. Because these processes are generally associated with the cell membrane, they may involve membrane bound forms of NDPK. Since the cytosolic forms of the enzyme appear to be more abundant, the role of NDPK in NTP interconversions may predominate.

Alternatively, it is likely that because NDPK is a near-equilibrium enzyme, it is not operating at $V_{\text{max}}$ in vivo. This can be supported by a simple calculation of the maximum NTP requirement of a growing cell. Consider a *T. pseudonana* cell of 10 pg C, that is doubling once a day ($\mu = 0.69 \text{ d}^{-1}$). Based on data from the literature, such a cell would be expected to have approximately 6 pg protein, 7.5 pg carbohydrate, 3.5 pg lipid, and 0.3 pg DNA and RNA (Darley, 1977, Dortch et al. 1984, Harrison et al. 1990b, Laws 1991, and the present study). In terms of protein requirements, it is assumed that 2 GTP per amino acid incorporated into protein are required (Morris 1974, Lehninger 1982; note that the requirement is actually slightly higher, but that a small amount of GTP is also produced by the succinyl CoA synthase reaction, and via phosphoenol pyruvate carboxykinase). For carbohydrate requirements it is assumed that all cell carbohydrates exist as chrysolaminarin, or other glucose-based polymers that require one UTP per monomer (Craigie 1974, Darley 1977; note that this is an overestimate since only ~ 60% of carbohydrate in diatoms exists as chrysolaminarin). It is also assumed that all cell lipid is found in membranes and is in the form of phospholipid, glycolipid, or other lipid forms that require one CTP per molecule for their synthesis (Lehninger 1982, Andrews and Ohlrogge 1990; note that these lipid forms may account for less than 50% of the total). Finally, it is assumed that the DNA and RNA contain 3 times as much of other nucleosides as ATP (according to Darley 1977, G+C residues account for 37-58% of the total). Given these assumptions, the total NTP requirement of the cell could not exceed approximately $2 \times 10^{-10} \mu\text{mol NTP min}^{-1} \text{ cell}^{-1}$. In the present study, *T. pseudonana* cells growing at this rate had NDPK activity in the range of $20-50 \times 10^{-10} \mu\text{mol NTP min}^{-1} \text{cell}^{-1}$, or at least an order of magnitude higher than the maximum requirement.
If NDPK is not operating at $V_{\text{max}}$, then enzyme activity in vivo may be regulated by factors including substrate concentration, or control mechanisms such as phosphorylation or adenylate energy charge. If NDPK was substrate-limited, i.e. reaction rates were a function of substrate and not enzyme concentration, it might be expected that ATP and NTP concentrations within the cell would fluctuate as a function of growth rate. For ATP this does not always appear to be true; a review by Karl (1980) showed that ATP content per cell is relatively constant over a wide range of growth conditions for prokaryotes, autotrophs and heterotrophs. While some studies have demonstrated a correlation between growth rate and ATP pools, this depends on whether ATP is scaled to cell number or carbon quota, and there is still controversy (Chapman and Atkinson 1977; Karl 1980, Sakshaug and Andresen 1986). The relationship may also depend on what is limiting growth. Karl (1980) cites data showing that in the diatom *Thalassiosira weissflogii* ATP per cell correlates with growth rate under nitrate or phosphate limitation but not when cells are limited by light or ammonium. Laws et al. (1983) showed that for the diatom *Thalassiosira weissflogii*, the ratio of ATP to carbon was constant over a wide range of light- and nutrient-limited growth rates. They speculated that ATP turnover, as opposed to concentration, might be a critical factor. In bacterial systems, the concentration of ATP and other nucleotides are at best a weak function of growth rate (Marr 1991). Karl (1980) suggests that adenine nucleotides are at or near saturating levels for most respiratory and metabolic enzymes. For other nucleotides there is also disagreement. Chapman and Atkinson (1977) found that other nucleotides followed patterns of ATP and did not vary with growth rate. Interestingly, they attributed this to rapid equilibration of other nucleotide pools and ATP through NDPK. Data presented by Marr (1991) support this view. Alternatively, Karl (1980) demonstrated that certain NTP pools, particularly GTP, vary with biosynthesis and growth rate. He suggested that the ratio of GTP/ATP might be useful as an index of growth rate. Pall (1985) also assigned GTP a key regulatory role in anabolic processes within the cell. At another level of enzyme regulation, phosphorylation control of NDPK has been suggested (Pall 1985) but has not been demonstrated.
There is strong evidence that the adenylate energy charge (AEC, defined as the ratio of the concentrations of ATP and one-half the concentration of ADP to the total concentration of ATP plus ADP plus AMP) plays a role in controlling NDPK activity. In general, the energy charge varies between 0.7-0.9 in healthy cells (Atkinson 1977, Plaxton 1990). Thompson and Atkinson (1971) have shown that for bovine liver NDPK, activity of the enzyme is maximal when the energy charge is near 1.0 and rapidly drops off as the ratio falls. Laws et al. (1983) demonstrated a significant positive correlation between energy charge and growth rate in *Thalassiosira weisflogii* cultures under a variety of limitations. This may explain the pattern in activity with growth rate observed in the present study. At moderate growth rates (between about 0.5 to 1.0 d\(^{-1}\)) there is little change in the *in vitro* activity of the enzyme. Over this range either substrate concentration or energy charge may be regulating activity. However, Dolezal and Kapralek (1976) showed that in a bacterium grown in a chemostat between 7 to 60% of maximal growth rate, there was little change in adenylate levels and no change in energy charge. A similar response was seen in the diatom *Skeletonema costatum* where cell content of ATP increased only when growth rates were 50% of \(\mu_{\text{max}}\) or greater (Sakshaug 1977). These two studies may not be directly comparable with Laws et al. (1983) or the present study, since they used chemostats and therefore the cells were nutrient rather than light limited. At higher growth rates, energy charge may be near its maximum, so that further increases in growth rate may necessitate increases in NDPK concentration. The reason for an apparent increase in nitrogen quota and NDPK activity per cell at very low growth rates remains unclear.

Another possible source of variability in NDPK activity is the stage of cell division. Berges (1989) found strong relationships between NDPK activity and growth rate in the brine shrimp *Artemia franciscana*, but such relationships were specific to different developmental stages. However, Klein and Follmann (1988) showed that for the green alga *Scenedesmus obliquus*, NDPK activity was constant throughout the cell division cycle.

It is apparent that neither NDPK activity nor nucleotide concentrations are entirely satisfactory as indices of *in situ* growth rate. If, however, the measurements were combined,
it is possible that their predictive value would improve, particularly if adenylate energy charge, ATP turnover rates, or substrate concentration regulate NDPK activity over a range of growth rates. Furthermore, measurement of nucleotides and NDPK activity could provide insight into the specific growth rate limitation that cells experience in situ. The present study has examined only light-limited growth rates. Since data presented by Karl (1980) suggest that light, phosphorus, nitrate or ammonium limitation result in different ATP-growth rate relationships, examining NDPK activity with respect to these cases would also be interesting.

In summary, NDPK in the diatom Thalassiosira pseudonana appears to be relatively similar to other NDPK enzymes previously investigated. Maximal NDPK activity is a poor index of cell growth rate, although the two variables are significantly correlated. Finally, because cell composition varies with growth rate, and because of difficulties in measuring cell volume or cell protein in phytoplankton species, scaling enzyme activity to different biomass variables is problematic. In culture, NDPK activity per cell volume appears to be a useful expression, but expressing activity per unit nitrogen might also be suitable.
CHAPTER 2: OPTIMIZATION AND VALIDATION OF AN ASSAY FOR NITRATE REDUCTASE ACTIVITY IN MARINE PHYTOPLANKTON

INTRODUCTION

As detailed in the introduction, several researchers have noted that NR does not appear to be satisfactory as an index of nitrogen uptake or incorporation rates (see e.g. Eppley et al. 1969, Packard et al. 1971, Collos and Slawyk 1976, Collos and Slawyk 1977, Dortch et al. 1979, Blasco et al. 1984; but also note the good agreement found by Morris and Syrett 1965, and Hersey and Swift 1976). There are essentially three possible explanations for these discrepancies: a) the extractions and assays of NR are inadequate, b) there is no relationship between NR and nitrate incorporation rate, or c) the presence of regulatory mechanisms mean that the measured maximal activity of NR is not a good indicator of the actual rate of nitrate reduction in vivo. In this chapter and the following two chapters, each of these possibilities will be examined.

In this chapter, an extraction and assay procedure for nitrate reductase (nitrate:nitrite NADH oxioreductase, E.C. 1.6.6.1, NR) is optimized and validated using marine phytoplankton. At this point in the thesis, the specific roles of and place of nitrate reductase in marine phytoplankton will be only generally outlined; Chapter 3 will address these issues in greater detail. Similarly, aspects of the regulation of NR will be considered only as they pertain to assay methods; Chapter 4 will deal with these regulatory mechanisms and their implications in greater detail.

The place of NR in algal nitrate metabolism

The general nitrogen metabolism of microalgae has been considered in the Introduction. Comprehensive reviews of these processes are provided in Morris (1974), McCarthy (1980), Collos and Slawyk (1980), Syrett (1981), Wheeler (1983), Falkowski (1983), and Syrett (1989). In this chapter, only nitrate metabolism will be considered. In addition to the reviews cited above, specific reviews of nitrate metabolism are available for higher plants (Hewitt et...
The terminology surrounding the uptake, reduction and subsequent incorporation of nitrate into cellular constituents is confusing, because different authors have chosen different terms. In this thesis, the following terms will be used to describe the different processes within the cell (after Wheeler 1983). *Uptake* will be used to describe the removal of nitrate from the medium, whether judged by disappearance from the medium, or appearance within the cells. Note that for higher plants, the presence of intercellular spaces, particularly in root tissue make this more difficult to define (see Redinbaugh and Campbell 1991). *Assimilation* will be used to describe the conversion of nitrate to nitrite to ammonium to small organic nitrogen components, such as amino acids and small, soluble peptides. *Incorporation* will be reserved for the process in which small organic components are synthesized into macromolecules, such as proteins and DNA. Functionally, it is difficult to distinguish assimilation from incorporation. For the purposes of this study, nitrogen will be considered to have been incorporated when it is retained in filtered samples and detectable by carbon-nitrogen analyzers. Note that the rupture of cells during this process would result in an underestimate of incorporation, while including inorganic nitrogen contained in the vacuoles of filtered cells might result in an overestimate of this process.

Nitrate metabolism in eukaryotes begins with the uptake of nitrate into the cell. There is relatively little information about this process. Based on electrochemical and thermodynamic considerations, nitrate transport must be an active process (Pilbeam and Kirby 1990). There is thought to be a specific nitrate transport protein (also referred to as a permease). Such a protein has been isolated in cyanobacteria (Omata 1991, Lara et al. 1993), but is poorly characterized in eukaryotes (see Redinbaugh and Campbell 1991, Miyagi et al. 1992). In higher plants, there is evidence for a $2H^+:1NO_3^-$ symport (Deane-Drummond 1990, Collos et al. 1992c), but a $NO_3^-:OH^-$ antiport has also been proposed (Deane-Drummond 1990, Lara et al. 1993). For marine phytoplankton, Falkowski (1975) showed that there was an ATP requirement for nitrate transport in a marine diatom. There appears to
be a strong dependence of nitrate uptake on sodium in marine species (Syrett 1989). For organisms living in an alkaline environment (seawater pH is near 8.0), it has been suggested that maintaining gradients of Na\(^+\) instead of H\(^+\) may require less energy (Lara et al. 1993). Siddiqi et al. (1990) described a two-phase system in barley roots, where there was a high-affinity inducible system operating at low nitrate concentration, and a constitutive transport system at high concentration, and there is also evidence of such a system in marine diatoms (Collos et al. 1992c). A direct role for NR in the uptake of nitrate in higher plants was suggested, based on membrane associations of NR and the close link between uptake and reduction of nitrate (Butz and Jackson 1977). However, Warner and Huffaker (1989) have demonstrated that the induction of transport and the uptake kinetics provided no evidence for a role of NR in nitrate uptake. Nonetheless, in certain fungi, NR activity and nitrate uptake are highly coordinated (Goldsmith et al. 1973), and evidence from Tischner et al. (1989) showing that antibodies to NR protein inhibit nitrate uptake in the green alga *Chlorella sorokiniana* suggest that if NR is not responsible for uptake, the two processes are closely linked.

The reduction of nitrate to ammonium proceeds in two steps; a two electron donation catalyzed by NR (note that earlier ideas about alternate pathways for nitrate reduction, e.g. Dortch et al. 1979, Clayton 1986, have been largely discredited), followed by a six electron donation by nitrite reductase (E.C. 1.7.7.1, NiR). Several reviews suggest that NR is the rate-limiting process in nitrate incorporation (e.g. Beevers and Hageman 1980, Campbell 1988, Wray and Fido 1990), but other authors disagree. Noting that in certain species internal nitrate pools do not accumulate, Tischner (1990) argues that nitrate uptake is in fact the limiting step (but note that Fuggi (1989) presents a mechanism whereby leakage of nitrate would allow NR to be limiting without a build-up of nitrate within cells). However, even among those who maintain that uptake is rate-limiting, there is at least recognition that NR is a key point of control of the process (see De la Rosa et al. 1989). Other authors have noted a build-up or efflux of nitrite from cells under conditions of senescence, low CO\(_2\) (Azura and Aparicio 1983), or light-dark transitions (Stulen and Lanting 1976, but see also Sanchez and Heldt 1990) and suggest that NiR may be rate-limiting, especially in the dark when the
physiological source of reductant for NiR (ferredoxin) cannot be produced. Generally, however, NiR activity exceeds NR activity by up to a factor of 8, an indication that NiR is not limiting (Eppley et al. 1969, Aslam and Huffaker 1989; but note that Kessler and Czygan (1968) found similar levels of the two enzymes in green algae). There is no doubt, however, that NiR is also highly regulated in the cell. Evidence from analyses of mRNA and NiR protein suggest that induction of NR and NiR are nearly simultaneous (Galvan et al. 1992). In fact, nitrate appears to induce NiR as effectively as it does NR (Galvan et al. 1992). Because nitrite is toxic within the cell, it makes sense that the two enzymes should be closely coupled, and that NiR activity should exceed the activity of NR.

Following these reduction reactions, the ammonium produced may be incorporated into amino acids in one of two processes: into glutamate via glutamate dehydrogenase (GDH, E.C. 1.4.1.4), or into glutamine by the enzyme glutamine synthetase (GS, E.C. 6.3.1.2) and then to glutamate by the enzyme glutamate synthase (GOGAT, E.C. 1.4.7.1). In general, the GS/GOGAT is thought to be the favoured pathway based on evidence from labeling studies of first products, equilibrium considerations, inhibitor studies, and the high degree of regulation found for GS (Miflin and Lea 1976, Syrett 1981, Wheeler 1983, Syrett 1989). GDH is generally assigned a role in amino acid catabolism for internal reorganization of cell nitrogen (see Syrett 1989, Robinson et al. 1991), but under certain conditions it may still be important in assimilation (e.g. Ahmad and Hellebust 1985a, Callies et al. 1992), or in cellular control by adjusting the cells' glutamine/glutamate ratio (see Flynn 1991). The case for the GS/GOGAT pathway limiting nitrogen incorporation has also been made. Since GOGAT activity almost always exceeds that of GS, GS is thought to be rate-limiting (Syrett 1989).

In addition to its roles in nitrate assimilation, there is also evidence that NR may perform other functions in the cell. Jones and Morel (1988) found a cell membrane-associated NR in the diatom Thalassiosira weisflogii, and hypothesized a role for NR in controlling plasmalemma redox. The presence of a NR of different molecular weight (representing about 0.8% of total cell NR) in membrane fractions of Chlorella sorokiniana was also noted by Tischner et al. (1989) and Tischner (1990). Azura and Aparico (1983) showed that high rates
of nitrite excretion occurred under high light and low CO₂ conditions in *Chlamydomonas reinhardtii*. They suggested that nitrate might be acting as an electron acceptor under these conditions to adjust levels of reducing power in the cells. Castigetti and Smarrelli (1984) and Smarrelli and Castigetti (1988) have suggested that NR may be involved in reducing siderophores which are responsible for acquiring metals for cell nutrition. This process may be quantitatively more important in microalgae in metal-deficient aquatic environments (see Price *et al.* 1991) than for higher plants in soil environments.

**Structure and characteristics of NR**

Distinct types of NR exist in prokaryotes, where nitrate is used in place of oxygen as a terminal electron acceptor (dissimilatory forms), or in photosynthetic bacteria and cyanobacteria, where nitrate is used as a nitrogen source (assimilatory forms) (Guerrero *et al.* 1981). The dissimilatory enzymes are classified as to whether or not chlorate inhibits the nitrate reducing activity (type A) or not (type B), and they are smaller enzymes containing much more iron than assimilatory forms (Hewitt 1975). The assimilatory enzymes of cyanobacteria and photosynthetic (and perhaps chemosynthetic) bacteria differ from NR in eukaryotes in that they use reduced ferredoxin as an electron donor and cannot use pyridine nucleotides (e.g. NADH or NADPH).

In contrast, NR in eukaryotes is a large, soluble, multi-centered redox enzyme that exists in three distinct forms (not including isozymes), based on the source of reducing power: NADH-NR (E.C. 1.6.6.1), the most common form, found in higher plants and algae, NAD(P)H-NR (E.C. 1.6.6.2), which is found in higher plants and green algae, and NADPH-NR (E.C. 1.6.6.3), which occurs only in fungi (Campbell and Kinghorn 1990). Within these general categories there is evidence of isozymes (Callaci and Smarrelli 1991); Schuster *et al.* (1989) for example showed that there were 4 distinct forms of NR in mustard (*Sinapis alba*) cotyledons. Table 2.1 gives a comparison of the molecular weights and substrate specificities of the purified enzyme from different sources. Comprehensive reviews of the structure of NR are provided by Guerrero *et al.* (1981), Solomonson and Barber (1989, 1990), and Wray and
Table 2.1 Characteristics of nitrate reductase from various sources. (MOL WT = molecular weight, REDUCTANT = physiological electron donor, MONOMERS = number of monomers in the native protein, -- = information not determined by the authors)

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>MOL WT (kDa)</th>
<th>REDUCTANT</th>
<th>MONOMERS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cyanobacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anacystis nidulans</em></td>
<td>83</td>
<td>ferredoxin</td>
<td>1</td>
<td>Andriesse et al. 1989</td>
</tr>
<tr>
<td><strong>Higher Plants</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Spinacea oleracea</em></td>
<td>230</td>
<td>NAD(P)H</td>
<td>2</td>
<td>Hewitt 1975</td>
</tr>
<tr>
<td><em>Nicotiana plumbaginifolia</em></td>
<td>214</td>
<td>NAD(P)H</td>
<td>2</td>
<td>Moureaux et al. 1989</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>197</td>
<td>NADPH</td>
<td>2</td>
<td>Hewitt 1975</td>
</tr>
<tr>
<td><em>Funaria hygrometrica</em></td>
<td>232</td>
<td>NADPH</td>
<td>2</td>
<td>Padidam and Johri 1991</td>
</tr>
<tr>
<td><strong>Microalgae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>370</td>
<td>NAD(P)H</td>
<td>4</td>
<td>Solomonson and Barber 1987</td>
</tr>
<tr>
<td><em>Ankistrodesmus braunii</em></td>
<td>500</td>
<td>NAD(P)H</td>
<td>8</td>
<td>Solomonson and Barber 1990</td>
</tr>
<tr>
<td><em>Thalassiosira pseudonana</em></td>
<td>330</td>
<td>NADH</td>
<td>--</td>
<td>Amy and Garrett 1974</td>
</tr>
</tbody>
</table>
Fido (1990). The functional size of the enzyme ranges from 200 to 500 kDa, although there is some variation according to the method used to determine the size (Solomonson and Barber 1990). The enzyme is composed of single polypeptide chains of about 100 kDa each, which may associate as dimers, tetramers or octamers, depending on the species (Solomonson and Barber 1990). Hyde et al. (1991) demonstrated that the functional domains of the enzyme are very similar across different species, although the sequence homology is not as highly conserved as in the case of ribulose 1,6-bisphosphate carboxylase oxygenase (RUBISCO) for example (see Newman and Cattolico 1990). Antibodies raised against NR from squash cross-reacted with the NR of most higher plants, but not with that from *Chlorella pyrenoides*, or from the fungus *Neurospora crassa* (Cherele et al. 1986). The molecular weight of the polypeptide predicted from the DNA sequence is very close to that of the NR protein, suggesting that there is little post-translational modification, aside from the insertion of cofactors (Sherman and Funkhouser 1989). Each polypeptide subunit has three linearly arranged domains: a flavin adenine dinucleotide (FAD) region nearest the C-terminal end of the protein, a central region with heme-iron contained in a b557-type cytochrome, and a N-terminal component containing molybdopterin (Wray and Fido 1990, Solomonson and Barber 1990). It is thought that the electron transfer between the cytochrome and molybdopterin is the rate limiting step in catalysis (Kay et al. 1991). As will be discussed further under the section on NR Assay Methods, the NR protein exhibits several so-called "partial activities" in addition to the full reaction that reduces nitrate to nitrite and oxidizes NADH to NAD.

In contrast to the reaction catalyzed by NDPK, which is near equilibrium, the NR reaction (nitrate to nitrite) is generally considered to be irreversible, with the equilibrium constant ($K_{eq}$) on the order of $10^{25}$ (Hewitt 1976).

**Regulation of NR**

The regulation of nitrate reductase is complex and beyond the scope of this chapter, in which only a general discussion is given. Excellent reviews are provided by Fernandez and Cardenas (1989), Crawford and Davis (1989), Solomonson and Barber (1990), and Crawford
et al. (1992). As Solomonson and Barber (1990) point out, there is probably no single mode of regulation of NR, but several modes acting simultaneously, or in sequence.

The enzyme appears to be largely regulated by synthesis and degradation of the protein (Sherman and Funkhouser 1989). Sequential induction of transcription (i.e. appearance of NR mRNA's) and translation (i.e. appearance of NR immuno-reactive protein) followed by increases in NR activity have been demonstrated for higher plants (Stewart and Rhodes 1977, Lillo 1991, Li and Oaks 1993), green algae (Sherman and Funkhouser 1989, Diez and Lopez-Ruiz 1989), and marine diatoms (Smith et al. 1992). The factors involved in the regulation of NR synthesis are still under debate. NR synthesis has long been held to be induced by nitrate (Stewart and Rhodes 1977, Faure et al. 1991), but there is evidence that nitrate may not be required. Kessler and Osterheld (1970) found that in the green alga Ankistrodesmus braunii NR activity increased when ammonium-grown cells were transferred to N-free medium. This was also found by Amy and Garret (1974) in Thalassiosira pseudonana and Diez and Lopez-Ruiz (1989) in a green alga, leading to the idea that ammonium may repress NR synthesis, but nitrate does not induce it. This effect may be isoform-specific; Calacci and Smarrelli (1991) have shown that of three isoforms, only the pH 7.5 variant of NR in soybean is induced by nitrate. It is also worth noting that Oaks et al. (1990) showed that trace nitrate contamination of soil was responsible for a "no nitrate" induction of NR in higher plants. Light (Faure et al. 1991, Gao et al. 1992), alternate carbon sources (e.g. citrate in cucumber cotyledons, Stewart and Rhodes 1977), and some alternate nitrogen sources (e.g. uric acid in certain species of microalgae, Syrett and Hipkin 1973) can also induce NR. NR synthesis is repressed by the presence of ammonium (Syrett 1989) or other nitrogen sources such as amino acids (Liu and Hellebust 1974), but Harrison (1976) reported a NR from a marine dinoflagellate that was not repressed completely by ammonium. Rasjasekar and Oelmuller (1987) also found that there was a non-repressible NR in corn. In terms of NR regulation by degradation, a wide range of NR-specific proteases are known from higher plants and fungi (Wallace 1977).

Alternatively, there is a range of situations in which NR activity changes in the absence of protein synthesis or degradation. In some cases it may be difficult to distinguish these
processes. Tischner and Hutterman (1978) initially thought that light activation of *Chlorella* NR was dependent on protein synthesis, based on the inhibition of this activation by the protein synthesis inhibitor cyclohexamide. They later discovered that the action of cyclohexamide was non-specific; it inhibited the activation mechanism as well. In green algae, cyanide or superoxides produced at times in photosynthesis appear to convert NR to an inactive form (Pistorius et al. 1976). Blue light, flavins and mild oxidation with ferricyanide can be used to reactivate the enzyme (Franco et al. 1987, Corzo and Neill 1992b). This mechanism does not appear to operate in marine diatoms (Serra et al. 1978a). Light activates the enzyme, probably through a phytochrome (Ninneman 1987, De la Rosa et al. 1989), and there is a diel periodicity in NR (Packard et al. 1971, Smith et al. 1992). Specific allosteric modification by adenylylates was thought to occur (Eaglesham and Hewitt 1975), but later evidence shows that this may be mediated through other mechanisms such as phosphorylation. Phosphorylation has been shown to occur in the spinach enzyme during light-dark transitions (Huber et al. 1992a). Tischner (1984) could distinguish two enzyme forms in *Chlorella*, a low activity form present at the end of the dark cycle, and a high activity form which appeared about one hour into the light period. This was hypothesized to be an intramolecular change (Tischner 1984), and it may represent a phosphorylation event. Alternatively, adenylylates may play other roles in enzyme activity modification (Kaiser and Spill 1991, Kaiser et al. 1992). There may also be a direct inhibition of NR by ammonium, probably through a product of ammonium incorporation (Syrett 1981, Flynn 1991). Larsson et al. (1985) showed that in the green alga *Scenedesmus*, inactivation of NR was too rapid to be due simply to degradation of NR and must involve some inactivation mechanism.

In addition to protease action, there are reports of proteins in higher plants that bind to NR and irreversibly inactivate the enzyme, but do not appear to be proteases (Solomonson and Barber 1990, Yoshimura et al. 1992).

Other controlling factors may include competition between NR and GDH for reductant (Stewart and Rhodes 1977), the glu/gln ratio in the cells (Flynn 1991), and carbon limitation
(Pace et al. 1990) In one fungus, Mo limitation of NR activity has also been demonstrated (Padidam et al. 1991).

**NR assay methods**

Any consideration of NR activity and its regulation is complicated by the bewildering range of assays, extraction buffers and assay conditions that have been employed in different studies (see Table 2.2). Assays can be broadly divided into two categories, those that use intact cells (*in situ* assays), and those that use cell homogenates (*in vitro* assays).

**In Situ Assays**

The *in situ* assay is often termed *in vivo* in the literature. In principle, cells are permeabilized, provided with nitrate and incubated under conditions where nitrite cannot be further reduced to ammonium. The nitrite produced is then measured colorimetrically (Hageman and Reed 1980). In this thesis, the term *in situ* will be used in preference to *in vivo* since cells permeabilized in this manner are not usually viable (see discussion in Corzo and Neill 1992b). The term *in vivo* is probably better reserved for truly non-invasive monitoring procedures such as nuclear magnetic resonance (see Roberts 1984).

There are many variations in the *in situ* procedure including the permeabilizing agent used (freezing, propanol, toluene, Triton X-100, cetyl-trimethylammonium bromide) and its concentration, whether a buffer is used, the concentration of nitrate provided, and whether a reductant or carbon source is provided (see Table 2.2). There are several problems with these assays. For a true measurement of NR activity, it must be assumed that it is the enzyme activity which limits the reaction rate, not the ability of nitrate to reach the enzyme (transport), or the ability of nitrite to move out of the cell. It is difficult to verify this assumption (see Hog et al. 1983). Reducing power must not be limiting, but NADH is not readily transported across membranes; Lillo (1983) found it necessary to add glucose to barley leaves and allow glycolysis to provide reductant. In addition, the assay must be conducted anaerobically and in the dark to prevent nitrite from being converted to ammonium via NiR (Lillo 1983). Using
Table 2.2. Selected assay mixtures for *in vitro* or *in situ* nitrate reductase assays. (DTT = dithiothreitol, CYS = cysteine, FAD = flavin adenine dinucleotide, PVP = polyvinyl pyrrolidone, * = NADPH used in place of NADH; ? = information not provided by authors)

| ORGANISM | °C | BUFFER | pH | DTT (w/v) | Mg²⁺ (mM) | EDTA (mM) | CYS (mM) | FAD (µM) | PVP (w/v) | NO₃⁻ (mM) | NADH (mM) | OTHER ADDITIONS | REFERENCE |
|----------|----|--------|----|----------|----------|-----------|---------|---------|----------|----------|------------|------------|----------------|-----------|
|          |    |        |    |          |          |           |         |         |          |          |            |            |                |           |
| **IN VITRO ASSAYS** |    |        |    |          |          |           |         |         |          |          |            |            |                |           |
| Sphaerosilbe repens  | 20 | 100 mM PO₄³⁻ | 7.5 | --       | --       | 1.0       | --       | 10      | 5%       | 10       | 0.4*       | 1 µM Mo    | 1% casein     | Essgouri and Botton 1990 |
| Spinacea oleracea    | 24 | 50 mM PIPES   | 7.6 | --       | 10       | 10        | --       | --      | --       | 1.0      | 0.5        | ATP        | liquid N₂    | Kaiser et al. 1992 |
| Spinacea oleracea    | 25 | 50 mM PO₄³⁻ | 7.5 | 1 mM     | --       | 0.1       | 1.0      | 10      | --       | 5.0      | 0.1        | ATP        | leupeptin     | Sanchez and Heldt 1990 |
| Lemna gibba          | 29 | 50 mM PO₄³⁻ | 7.8 | --       | --       | 1.0       | 1.0      | 50      | --       | 25       | 0.4        | 10 µM leupeptin | 3% casein | Ingemarsson 1987 |
| Zea mays             | 30 | 100 mM PO₄³⁻ | 7.4 | --       | --       | 1.0       | 1.0      | --      | --       | 11.7     | 0.47       | 0.1% Triton | 15% glycerol | Pace et al. 1990 |
| Hordeum vulgare      | 27 | 25 mM PO₄³⁻ | 7.5 | --       | --       | 1.0       | --       | --      | --       | 10       | 0.2        | --         | --           | Lillo 1983 |
| Hordeum vulgare      | 30 | 100 mM PO₄³⁻ | 7.5 | --       | --       | --       | 5.0      | --      | --       | 10       | 0.4        | --         | --           | Tischner et al. 1986 |
| Chlamydomonas reinhardtii | 25 | 50 mM TRIS   | 7.5 | 0.1 mM   | --       | 1.0       | --       | 10      | --       | 10       | 0.2        | --         | --           | Franco et al. 1987 |
| Chlorella vulgaris    | 20 | 67 mM PO₄³⁻ | 7.6 | --       | --       | --       | --       | --      | --       | 6.6      | 0.6        | --         | --           | Pistorius et al. 1976 |
| Skeletonema costatum | 25 | 200 mM PO₄³⁻ | 7.9 | 1.0%     | 0.844    | --       | --       | --      | 0.3      | 3.61     | 0.167      | liquid N₂   | Clayton 1985 |
| f.w. phytoplankton   | 25 | 150 mM PO₄³⁻ | 7.6 | --       | --       | --       | --       | --      | --       | 10       | 0.65       | 3% toluene  | Hochman et al. 1986 |

* IN VITRO ASSAYS |
Table 2.2 (Continued)

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>°C</th>
<th>BUFFER</th>
<th>pH</th>
<th>DTT (w/v)</th>
<th>Mg$^{2+}$ (mM)</th>
<th>EDTA (mM)</th>
<th>CYS (mM)</th>
<th>FAD (µM)</th>
<th>PVP (w/v)</th>
<th>NO$_3^-$ (mM)</th>
<th>NADH (mM)</th>
<th>OTHER ADDITIONS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>marine phytoplankton</td>
<td>20</td>
<td>200 mM PO$_4^{3-}$</td>
<td>7.9</td>
<td>1 mM</td>
<td>--</td>
<td>--</td>
<td>5.85</td>
<td>--</td>
<td>29</td>
<td>0.29</td>
<td>--</td>
<td>Everest et al. 1986</td>
<td></td>
</tr>
<tr>
<td>marine phytoplankton</td>
<td>20</td>
<td>200 mM PO$_4^{3-}$</td>
<td>7.9</td>
<td>1.0%</td>
<td>30-100</td>
<td>--</td>
<td>--</td>
<td>0.6</td>
<td>10</td>
<td>0.2</td>
<td>--</td>
<td>Eppley et al. 1969</td>
<td></td>
</tr>
<tr>
<td>marine phytoplankton</td>
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<td>200 mM PO$_4^{3-}$</td>
<td>7.9</td>
<td>1 mM</td>
<td>--</td>
<td>--</td>
<td>4.7</td>
<td>--</td>
<td>29</td>
<td>0.029</td>
<td>--</td>
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<td></td>
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<tr>
<td>Acetabularia mediterranea</td>
<td>20</td>
<td>100 mM PO$_4^{3-}$</td>
<td>7.5</td>
<td>1 mM</td>
<td>--</td>
<td>0.5</td>
<td>20</td>
<td>0.42</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>liquid N$_2$ 10 µM leupeptin</td>
<td>Balandin and Aparicio 1992</td>
</tr>
<tr>
<td>Fucus gairdnerii</td>
<td>20</td>
<td>200 mM PO$_4^{3-}$</td>
<td>8.2</td>
<td>1 mM</td>
<td>10</td>
<td>--</td>
<td>--</td>
<td>0.2</td>
<td>11</td>
<td>0.2</td>
<td>--</td>
<td>Thomas and Harrison 1988</td>
<td></td>
</tr>
<tr>
<td>Enteromorpha intestinalis</td>
<td>20</td>
<td>200 mM PO$_4^{3-}$</td>
<td>8.0</td>
<td>1 mM</td>
<td>10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>11</td>
<td>0.2</td>
<td>--</td>
<td>Thomas and Harrison 1988</td>
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<td>IN SITU ASSAYS</td>
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<tr>
<td>Chlamydomonas reinhardtii</td>
<td>25</td>
<td>25 mM HEPES</td>
<td>7.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>250</td>
<td>--</td>
<td>--</td>
<td>1.6</td>
<td>--</td>
<td>2 mM toluene</td>
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</tr>
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<td>29</td>
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<td>7.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>200</td>
<td>--</td>
<td></td>
<td>5% propanol</td>
<td>Corzo et al. 1991</td>
</tr>
<tr>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>20</td>
<td>--</td>
<td></td>
<td>4% propanol</td>
<td>Smith et al. 1992</td>
</tr>
<tr>
<td>Ulva rigida</td>
<td>30</td>
<td>100 mM PO$_4^{3-}$</td>
<td>8.0</td>
<td>--</td>
<td>0.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>30</td>
<td>10 µM glucose</td>
<td>--</td>
<td>1.1% propanol</td>
<td>Corzo and Neill 1992</td>
</tr>
</tbody>
</table>
the \textit{in situ} assay, Thomas and Harrison (1988) also found that NR activity was very dependent on the length of incubation with the permeabilizing agent propanol. As well, Brinkhuis \textit{et al.} (1989) point out that uptake of nitrite by cells must also be accounted for in this assay. Sawhney \textit{et al.} (1978) cautioned that \textit{in situ} NR assays are not true reflections of what goes on physiologically; light, ATP concentration and mitochondrial respiration all affected NR in wheat leaves, and all these parameters were altered under assay conditions. Based on these considerations, it was decided that an \textit{in vitro} assay offered better quantification of NR activity for the present study.

\textbf{In Vitro Assays}

\textit{In vitro} NR assays also present difficulties. The \textit{in situ} assay is often adopted when activity cannot be found using an \textit{in vitro} technique (e.g. Thomas and Harrison 1988, Corzo and Neill 1992b), although Lillo (1983) compared \textit{in situ} and \textit{in vitro} assays in barley leaves and found that \textit{in vitro} activity was up to 5 times higher. The reasons for failure to detect activity may have to do with problems associated with the stability of the enzyme when it is extracted. Morris and Syrett (1965) and Hersey and Swift (1976) believed that only a portion of NR was recovered on extraction from \textit{Chlorella}, and two marine dinoflagellates, respectively. Morris and Syrett suggested that NR from nitrogen-deficient cells was even more unstable. Eppley \textit{et al.} (1969) calculated that only 25\% of NR activity necessary to support observed rates of nitrate incorporation was recovered from marine phytoplankton. On the other hand, it is possible that these assay conditions themselves may have excluded a necessary cofactor, or been conducted under non-optimal conditions.

There are many aspects of homogenization techniques that bear consideration. Cells can be collected by filtration, although this may cause cell rupture and loss of enzyme (Hochman \textit{et al.} 1986). Centrifugation is another method of cell collection, although it is time-consuming, and difficult to use with larger volumes of dilute culture; at high speed it may be no more gentle than low pressure filtration. Cell disruption has been accomplished by freeze-thawing in liquid nitrogen (Balandin and Aparicio 1992), grinding in a mortar and
pestle (Eppley 1978), homogenizing with a glass-glass or glass-Teflon tissue homogenizer (Hochman 1982), sonicating (Pistorius et al. 1976), or using a French press pressure cell (Pistorius et al. 1976). For larger enzymes such as NR, however, there is evidence that sonication may cause damage to the protein (Pistorius et al. 1976). Hochman (1982) found that in a freshwater dinoflagellate, sonication and French press methods both gave much lower activities than grinding in a glass-glass culture tube.

For in vitro assays the buffer into which the NR enzyme is extracted is critically important. There is extensive evidence that sulphydryl groups in the active site of the protein must be protected by thiol compounds such as cysteine, mercaptoethanol or dithiothreitol (Cleland 1964; Newsholme and Crabtree 1986). Eppley et al. (1969) noted that DTT was more effective than cysteine with marine diatoms, and there is also evidence in barley leaves that the use of cysteine may stimulate thiol proteases within the cell (Tischner et al. 1986).

Phenolic compounds which are common in the tissues of algae (Thomas and Harrison 1988) may also inactivate proteins; use of polyvinyl pyrroldione to bind phenolic compounds has been recommended (Loomis and Battile 1966, Gegenheimer 1990). Heavy metals as reagent contaminants or on glassware may also be problematic, and the use of EDTA can overcome such problems (Newsholme and Crabtree 1986). Finally, proteolytic inactivation may occur with NR, since this is known to occur in fungi, higher plants and green algae (Wallace 1977), although it has not been specifically addressed in marine microalgae. A wide range of protease inhibitors are available (Gegenheimer 1990), some of which have been used in NR extraction including leupeptin (Wray and Kirk 1981, Ingemarsson 1987), chymostatin (Long and Oaks 1990), and phenylmethyl sulfonyl fluoride (PMSF, Essagouri and Botton 1990). The addition of protein to extraction buffers has also provided protection from proteases. Both bovine serum albumin (BSA) and casein have been used at concentrations ranging from 0.1-3% (w/v) (Sherrard and Dalling 1978, Ingemarsson 1987, Pace et al. 1990).

Assay conditions for NR are extremely variable (see Table 2.2). In terms of buffers, phosphate has been found to enhance NR activity 10-30% compared with buffers such as TRIS (Serra et al. 1978a). In fact, Eppley et al. (1969) and Everest et al. (1984) cautioned against
the use of TRIS buffer, which gave lower NR activities. There is also a wide range of buffers currently available that may have advantages over traditional buffers such as phosphate or TRIS (see Good et al. 1966). The temperature of the assay is another consideration. In theory, lower activity can be easily amplified by increasing the assay temperature. Thus, NR assays are often conducted at 25-30°C, which can be more than 10°C higher than the in situ temperatures the organisms experienced. In theory, activity can be back-corrected to the in situ rate using an activation energy derived from an Arrhenius plot (Hochachka and Somero 1984; see Packard et al. 1971a), but it is necessary to determine the activation energy.

Packard et al. (1971a) found that for Skeletonema costatum grown between 16-19°C, activity measured at 25°C was only 25% of the activity measured at 15°C. To make matters worse, in some cases authors have not even reported the assay temperature. Clearly, these problems can be avoided by conducting assays at the in situ temperatures. Additions of MgSO_4 have been reported to increase NR activity, although this response is variable (Eppley et al. 1969).

Findings by Huber et al. (1992a) that Mg^{2+} inhibits the activity of the phosphorylated NR complicate this issue. Kaiser et al. (1992) reported that the effect of Mg^{2+} was overcome by additions of high EDTA (5 mM). In preliminary experiments, the additions occasionally increased activity up to 10%, however they also increased assay variability. With the routine addition of 5 mM EDTA to assays these effects disappeared; thus in the experiments reported in this thesis, MgSO_4 was not added. The issue of the concentrations of NADH (or NADPH) and nitrate to be added must be considered. Determination of maximal activity (V_{max}) clearly demands that substrates be saturating, yet frequently authors do not confirm saturation. A review of the literature shows a wide range of substrate concentrations (Table 2.2). Finally, additions of flavin adenine dinucleotide (FAD) should be considered as they have long been known to enhance NR activity under certain circumstances, especially after partial purification of the enzyme (Evans and Nason 1953).

Activation of NR in assays must also be considered. Additions of ferricyanide have been shown to increase NR activity, particularly in green algae (Pistorius et al. 1976). There
is also an activation mechanism involving pre-incubation with cysteine, above and beyond its role as a thiol protectant (Smarreelli and Campbell 1980).

Optimization of an assay must be conducted carefully. Clearly, it is possible to increase activity by using a higher temperature, or by shifting pH outside of the physiological range. However, if an index of what is occurring in vivo is required, the physiological constraints of the system must be accepted. In this study, assays were conducted at the in situ temperature and at a pH of 7.9, which may be to the higher end of the cellular norm (see Guern et al. 1991), but Amy and Garrett (1974) found that there was a broad pH optimum of 7-8 for NR from the diatom *Thalassiosira pseudonana*.

The activity to be monitored is also an issue. The overall reaction (NADH as electron donor, NO₃⁻ as acceptor, NADH-NR) is monitored by either measuring nitrite produced, or NADH oxidized (Wray and Fido 1990). In addition to this activity, NR displays several partial activities. For example, in place of NADH, reduced flavin mononucleotide, reduced methyl or benzyl viologens, or reduced bromphenol blue can donate electrons, resulting in the reduction of nitrate (FMN-NR, MV-NR, BV-NR, or BPB-NR, respectively) (Wray and Fido 1990). Nitrite produced is monitored in these assays. Alternatively, electron acceptors other than nitrate can be used, for example, cytochrome c (NADH-cytochrome c reductase, also called diaphorase), or ferricyanide (NADH-ferricyanide reductase). NADH oxidized or cytochrome c reduced are monitored spectrophotometrically in these assays (Wray and Fido 1990). Since some of these activities do not rely on an intact enzyme, they may to some extent overcome the loss of activity due to protease action (in fact, these activities have been used along with proteases to elucidate the structure of the enzyme, Wray and Fido 1990). Ingemarsson (1987) compared these partial activities in *Lemna*. He found that FMN-NR, MV-NR and NADH-cytochrome c reductase activities were all higher than NADH-NR by 20-50%, although the general trends in activity were similar. BPB-NR activity may be 10-15 times higher than NADH-NR (Wray and Fido 1990). In a freshwater dinoflagellate, MV-NR was found to be 3-6 times higher than the NADH-NR activity and BV-NR was 1.5-3 times greater (Hochman 1982). However, there may be problems trying to compare activities.
Yamagishi et al. (1988) for example, noted that NR inactivator protein in spinach completely suppressed NADH-NR activity, but there was no loss of MV-NR activity. Tischner (1984) also noted that MV-NADH and diaphorase activities in Chlorella did not respond to regulatory mechanisms in the same way as NADH-NR activity. Thus, it appears most sensible to monitor the full NADH-NR activity.

Another class of in vitro assays is the immunoassay (see Whitford et al. 1987). In theory, immunoassays do not necessarily require a functional protein since the enzyme reaction need not be run, and immunoassays may be extremely specific, depending on the specificity of the antibody employed. Although immunoassays offer extremely high sensitivity, with detection of enzyme within single cells possible, they still require a means to convert enzyme concentration to activity (Balch et al. 1988). However, from a practical point of view, if the enzyme is cleaved by proteolysis, or if the site the antibody recognizes is modified by some regulatory feature, the antibody may not recognize it. Alternatively, if the binding site is non-essential for NR activity, then degraded or inactive enzyme may be detected. There are cases where the two methods agree well (Maki et al. 1986, Watt et al. 1992); however there are other examples where the mismatch between activity and immunoprotein level is difficult to reconcile (Sherman and Funkhouser 1989, Smith et al. 1992). Immunoassays and enzyme activity assay are therefore distinct and complementary approaches which together provide more detailed information than either alone.

Since highly purified NR from marine species of phytoplankton was not available, and thus antisera could not be easily raised, the research in this thesis has focused on the usefulness of in vitro activity assays by measuring the full NADH-NR activity. The goal of this chapter is to provide a framework for the rest of the thesis by determining an optimal assay for NR in marine phytoplankton. To simplify considerations at this point, only light-limited semi-continuous batch cultures with nitrate as the sole nitrogen source have been used. Scaling of activity was not essential since in a pure culture exhibiting balanced growth, the NR activity and the actual nitrate incorporation rates of the algae could be measured and calculated on a per cell basis. The criteria selected for an optimal assay were that: a) activity be as high as
possible within physiological conditions (i.e. saturating substrates), b) activity be stable at least over the assay period, and c) activity of NR equal or exceed the calculated rate of nitrate incorporation.

MATERIALS AND METHODS

General culture conditions

All species used were obtained from the Northeast Pacific Culture Collection (NEPCC), Department of Oceanography, University of British Columbia. Cultures were grown in semi-continuous batch culture in artificial seawater medium (ESAW, based on Harrison et al. 1980, modified as in Chapter 1) at 17.5 ± 0.5°C under continuous light, with nitrate (550 μM) as the sole nitrogen source, as described in Chapter 1.

Enzyme optimization experiments

For initial experiments to optimize the NR assay, semi-continuous 1 L batch cultures of the diatom Thalassiosira pseudonana were used. These cultures were harvested in mid-log phase at cell densities of less than 5.0 x 10^5 cells ml^-1.

Validation of spectrophotometric assay

To begin with, the NR assay of Eppley (1978) was used. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the purest grade available. The extraction medium (Buffer A) consisted of 200 mM phosphate buffer, pH 7.9, 1 mM dithiothreitol, and 0.3% (w/v) polyvinyl pyrrolidone (PVP). Cells were harvested on 25 mm GF/F filters and homogenized in 1.0 ml of extraction buffer using a glass-Teflon homogenizer, as described in Chapter 1. Homogenates were clarified by centrifugation at 750 g at 4°C in a refrigerated Sorval RCB centrifuge for 5 min. The supernatants were removed using disposable Pasteur pipettes and used immediately for assays. Assays were conducted in 1 cm disposable plastic cuvettes in a total volume of 1.0 ml. The assay mixture contained
final concentrations of 200 mM phosphate buffer, pH 7.9, 0.2 mM NADH and 100-400 µl homogenate. Reactions were initiated by adding 10 mM KNO₃.

For spectrophotometric assays, the oxidation of NADH was followed at 340 nm (Hageman and Reed 1980), using an LKB Ultrospec II spectrophotometer with a temperature-controlled turret, interfaced to an IBM personal computer (see Appendix B). Enzyme activities were always determined at the in situ temperature. The initial rate of absorbance change was followed for 5 min before addition of KNO₃; the rate after KNO₃ addition was corrected for this background to give the nitrate-specific rate, which was converted to a rate of NADH oxidation using a millimolar extinction coefficient of 6.22 (Hageman and Reed 1980). In the same samples, nitrite production was also determined, using a modification of the method of Eppley (1978). The reaction was stopped at a specific time (generally 10-15 min) using 2.0 ml of 550 mM zinc acetate. This concentration is substantially higher than that used by Eppley (1978), but Eppley also added 6.0 ml of ethanol. The method used in this study completely stopped the reaction, but minimized the dilution of the homogenate. The homogenate was centrifuged at maximum speed in a clinical centrifuge. Samples (0.5 ml) of the clear supernatant were removed for assays. Excess NADH which could interfere with the subsequent assay of nitrite was oxidized by adding 20 µl of 125 µM phenazine methosulphate (PMS, Scholl et al. 1974). Nitrite produced was determined colorimetrically using 0.5 ml each of sulfanilamide and N-(1-napthyl)-ethylenediamine 2 HCl solutions and reading the resulting colour at 543 nm (Eppley 1978).

Although there is little doubt that the relationship between nitrate reduction and NADH oxidation is stoichiometric (Evans and Nason 1953, Hageman and Reed 1980), this may not be true for a crude homogenate versus a purified sample of NR. In order to verify this, activities calculated using NADH oxidation and nitrite production were compared in eight different cultures grown to a range of cell densities. Throughout subsequent experiments, the equivalence of these methods was routinely verified, particularly when investigating a new species or a new culture condition.
Cell collection, Homogenization, and Assay Optimization

Variations on homogenization and extraction procedures were made. In one experiment, 6 samples were collected from a single culture by filtration as previously described. An additional 6 samples were collected by centrifuging cell suspensions at 18 000 g for 15 min at 4 °C. The pellets were resuspended in 1.0 ml extraction buffer. To account for volume differences and to improve homogenization, dry 25 mm GF/F filters were added to centrifuged cell suspensions. Three samples from each collection method were ground as previously described. The other three samples were sonicated for 60 s using a Branson model 20 sonic disruptor with a microprobe, on the 40% power setting. Homogenates were centrifuged and assayed as previously described. Activities were compared using t-tests (Steel and Torrie 1980). A second experiment ascertained whether extraction of NR from cell detritus was complete. Six samples were collected by filtration from a single culture. Three samples were placed in normal NR extraction buffer (buffer A), and three were placed in Buffer A plus 0.1% Triton X-100. All samples were ground and centrifuged as previously described. The resulting supernatants were removed and the pellets were rehomogenized in an additional 1.0 ml of extraction buffer. NR was determined in each fraction and activities compared using an analysis of variance (ANOVA) followed by Tukey’s least significant difference (LSD) test. For subsequent experiments, homogenates were collected by filtration and homogenized by grinding with 0.1% Triton X-100 added.

The effect of different buffer compounds on NR activity was compared. Four sets of three samples each were collected from a single culture and homogenized and assayed in either 200 mM phosphate, 50 mM 3-(N-Morpolino)propanesulfonic acid (MOPS), 50 mM Tris(hydroxymethyl)aminomethane (TRIS), or 50 mM imidazole buffers, all adjusted to pH 7.9. In this experiment, and in subsequent optimization experiments, NR activities were compared using an analysis of variance (ANOVA) followed by Tukey's least significant difference (LSD) test. Subsequent experiments were all conducted using 200 mM phosphate buffer, pH 7.9.
The effects of additions to the extraction buffer were tested. Triplicate samples from a single culture were prepared in: a) buffer (200 mM phosphate plus 0.1% Triton X-100), b) buffer plus 5 mM EDTA, c) buffer plus 0.03 % (w/v) DTT, d) buffer plus 0.3 % (w/v) PVP, or e) buffer plus EDTA, DTT, and PVP. For subsequent experiments the extraction buffer consisted of: 200 mM phosphate buffer, Triton X-100, EDTA, DTT, and PVP (buffer B).

The effects of various activating compounds on activity were also evaluated. Triplicate samples from a single culture were prepared and assayed: a) without additions, b) after a 5 min incubation with 5 mM cysteine, c) with 0.1 mM flavin adenine dinucleotide (FAD), or d) after a 5 min incubation with 0.2 mM potassium ferricyanide.

The stabilities of enzyme extracts were assessed using three treatments. Sets of triplicate samples from a single culture were homogenized using: a) buffer B alone, b) buffer B plus 3% BSA, or c) buffer B plus a protease inhibitor mix recommended by Gegenheimer (1990) consisting of 1 mM PMSF, 1 mM benzamide, 1 mM benzamidine-HCl, 5 mM ε-amino-n-caproic acid, 10 mM EGTA, 1 μg ml⁻¹ antipain, 1 μg ml⁻¹ leupeptin, and 0.1 mg ml⁻¹ pepstatin. Extracts were assayed immediately, or after 20, 70 or 135 min. Activities were compared within each time interval using t-tests (Steel and Torrie 1980). Subsequent extractions were performed adding 3% BSA to the buffer (buffer C).

The substrate specificity of the enzyme from *T. pseudonana* was investigated. Assays were conducted on triplicate homogenates with either 0.2 mM NADH, 0.2 mM NADPH or a mixture of 0.1 mM NADH plus 0.1 mM NADPH.

The effect of liquid nitrogen freezing on NR activity was assessed. Four sets of triplicate samples were collected on 25 mm GF/F filters. One set was homogenized and assayed immediately. The other samples were placed in 1.5 ml Eppendorf microcentrifuge tubes and placed in liquid nitrogen. One set was immediately withdrawn from the liquid nitrogen, homogenized and assayed. The other sets of samples were withdrawn and assayed after 48 or 96 h.
Enzyme Kinetics

For the *T. pseudonana* enzyme, assays were performed with constant NADH (0.2 mM) and KNO₃ concentrations ranging from 0 to 10 mM, and with constant KNO₃ (10 mM) and NADH concentration ranging from 0 to 0.6 mM. Kₘ values for nitrate and NADH were estimated by fitting the data to a Michaelis-Menten model using a non-linear fitting routine (NONLIN, Wilkinson 1990; see Appendix C). Mean Kₘ values for each substrate were estimated from determinations on at least 3 separate homogenates.

Enzyme Desalting

To assess the effects of endogenous compounds in the homogenate (e.g. endogenous nitrate), triplicate homogenates of *T. pseudonana* were desalted using Sephadex G-25 prepared in columns in 30 ml disposable syringes. The resin was equilibrated with buffer, then homogenates were centrifuged through the syringes at 4° C in a refrigerated centrifuge at 750 g. NR assays were performed on homogenates before and after passing them through the column, with and without additions of FAD.

Enzyme characterization in different species

Semi-continuous batch cultures of the diatom *Skeletonema costatum* (Greville) Cleve (NEPCC 18) and the dinoflagellate *Amphidinium carterae* Hulburt (NEPCC 629) were grown as described for *T. pseudonana*. Cells were harvested, and NR extracted and assayed as previously described, except that in the case of *A. carterae* GF/F filters were replaced with 934 AH filters to prevent clogging by this larger species. As well, because *S. costatum* forms chains of cells, it proved necessary to sonicate samples of this species in a bath sonicator in order to break up chains for cell counts (Falkowski and Stone 1975). Cell volumes for *S. costatum* were calculated from the linear dimensions determined under the microscope, assuming a cylindrical shape.
For each species, assays were conducted with additions of FAD, activation with FeCN, and NADPH in place of NADH, as described for *T. pseudonana*.

K_m values for nitrate and NADH were determined for each species as described for *T. pseudonana*.

**Comparisons of NR activity with growth rate**

For *T. pseudonana*, *S. costatum*, and *A. carterae* light-limited growth rate experiments were conducted as described in Chapter 1, except that irradiances ranged up to 220 μmol quanta m^-2^ s^-1^.

Growth rates were monitored using fluorescence and growth irradiance curves prepared as before (Chapter 1). Cultures were harvested in log phase growth. For each culture, duplicate NR measurements were made and cell nitrogen quotas measured as described previously (Chapter 1). The nitrate reduction rate necessary to support observed growth (nitrate incorporation rate) was calculated from the product of cell growth rate (μ) and nitrogen quota (Q_N), assuming that nitrate was the only nitrogen source and that nitrogen quota and internal nitrate pools were constant (after an acclimation period) over the course of the experiment. This was converted to units of NR activity and the two variables were compared for each culture using linear regression analyses (Steel and Torrie 1980).

A preliminary survey of NR activity was conducted for 12 species: the diatoms *Thalassiosira weisflogii* (Gru.) Fryxell et Hasle (NEPCC 636), *Ditylum brightwellii* (t. West) Grunow in Van Huerck (NEPCC 8), and *Phaeodactylum tricornutum* Bohlin (NEPCC 31); the chlorophytes *Dunaliella tertiolecta* Butcher (NEPCC 1), and *Chlamydomonas* sp. (NEPCC 73); the prasinophyte *Tetraselmis* sp. (NEPCC 46); the cyanobacterium *Synechococcus* sp. (NEPCC 539); the dinoflagellates *Prorocentrum minimum* (Pavillard) Schiller (NEPCC 623), and *Gymnodinium sanguineum* Hirasaka (NEPCC 354); and the prymnesiophytes *Pavlova lutheri* (Droop) Hibberd (NEPCC 2), *Emiliania huxleyi* (Lohm.) Hay et Mohler (NEPCC 659), and *Isochrysis galbana* Parke (NEPCC 633). Cultures were grown on continuous light in ESAW as previously described in this chapter, but in 50 ml glass tubes without stirring or aeration. Growth rate was monitored by *in vivo* fluorescence as previously described.
Quadruplicate cultures were acclimated for a minimum of 8 generations, then sampled for NR activity and carbon and nitrogen content as previously described in this chapter. Nitrate incorporation rate was calculated as for the other species and compared with NR activity using linear regression analysis. In addition, the ability of each species to use NADPH in place of NADH was tested by conducting NR assays with 0.2 mM NADPH in place of NADH.

RESULTS

Enzyme optimization experiments

Validation of spectrophotometric assay

Figure 2.1 A shows a typical plot of absorbance versus time in the spectrophotometric NR assay. Typically, the rate of decrease in absorbance before addition of nitrate was 20% of the rate after nitrate addition. The absorbance decrease was linear for up to 40 min under the conditions described, and increased linearly with amount of homogenate added.

The spectrophotometric assay agreed very well with the nitrite production assay (Fig. 2.1 B). The slope of the NADH oxidation vs. nitrate reduction regression was 0.98 (not significantly different from 1.0, \( P < 0.001 \)) and the coefficient of determination \( (r^2) \) was 0.99. This was verified repeatedly during the course of experiments.

Cell collection, Homogenization, and Assay Optimization

NR activities in homogenates collected by centrifugation and grinding were not significantly different \( (P > 0.5) \), but centrifuged samples were more variable (Fig. 2.2 A). Regardless of the collection method, sonication gave consistently lower activities than grinding (Fig. 2.2 A; \( P < 0.01 \) for filtration, \( P < 0.001 \) for centrifugation). On centrifugation without Triton X-100, significant NR activity (~10% of supernatant) remained in the pellet. Addition of Triton removed this activity, although it did not significantly increase activity in the supernatant \( (P > 0.08) \).
Figure 2.1. Validation of spectrophotometric assay for nitrate reductase (NR). A) Time course of reaction before and after addition of 10 mM KNO$_3$ (indicated by arrow).

B) Comparison of activity calculated from NADH oxidation rate (corrected for NADH-oxidation in the absence of nitrate), and that calculated based on production of nitrite. Regression equation is: $Y = -0.81 + 0.98X$ ($r^2 = 0.99$).
Figure 2.2. Comparison of NR homogenization and extraction procedures in homogenates of *Thalassiosira pseudonana*. A) Relative NR activities in samples collected by filtration onto glass fibre filters, or centrifugation at 7500 g. In each case, replicate samples (n = 3) were homogenized by grinding or by probe sonication. B) Relative NR activity in the supernatant and pellet fractions of homogenates of cells collected by filtration and homogenized by grinding. Homogenizations were performed with or without 0.1% Triton X-100. Centrifugations were done at 750 g for 5 min.
NR activity in phosphate buffer was significantly higher than in TRIS or imidazole (P < 0.02 and P < 0.01, respectively), but no higher than in MOPS buffer (P > 0.06) (Fig. 2.3).

Single additions of EDTA, DTT, or PVP significantly increased NR activity (Fig. 2.4 A, P < 0.05 in all cases). NR activity was numerically highest with the addition of all three reagents, but not statistically different from any of the single additions (P > 0.08 in all cases). Additions of FAD had no effect on NR activity (Fig. 2.4 B, P > 0.2), but cysteine or ferricyanide preincubations resulted in significantly lower activities (Fig. 2.4 B, P < 0.01 in both cases).

Addition of BSA to the extraction buffer gave over 50% higher NR activity than homogenization without BSA, or homogenization with protease inhibitors (Fig. 2.6, P < 0.04 in both cases). Whereas activity dropped significantly by 60 min in homogenates without BSA (P < 0.05 in both cases), no significant decrease in activity in the BSA extract was seen at the same time (P < 0.05). By 135 min, however NR activity in the BSA extract had significantly declined (P < 0.05).

Activity of the T. pseudonana enzyme with NADPH as reductant was less than 10% of the activity with NADH, and not significantly different from zero (Fig. 2.6, P > 0.07). Activity with 0.1 mM NADH and 0.1 mM NADPH was significantly lower than that with NADH alone (P < 0.04) and similar to what would be expected with 0.1 mM NADH alone (see Fig. 2.8 A).

Filtered samples which were frozen in liquid nitrogen had identical NR activity to those that were not (Fig. 2.7, P > 0.5). No decrease in activity was seen in samples stored for 48 or 96 h (P > 0.5 in both cases), although variability of the assay appeared to increase.
Figure 2.3. Relative NR activity in homogenates of *Thalassiosira pseudonana* prepared in 200 mM phosphate buffer, 50 mM MOPS buffer, 50 mM TRIS buffer, or 50 mM imidazole buffer. In all cases, pH was 7.9. n = 3 for each buffer treatment.
Figure 2.4. Effects of different additions on nitrate reductase (NR) activity in homogenates of *Thalassiosira pseudonana*. A) Activity in homogenates with only 200 mM phosphate buffer and 0.1% (v/v) Triton X-100 (1) versus: 5 mM EDTA (2), 0.3 g l\(^{-1}\) DTT (3), 3.0 g l\(^{-1}\) PVP (4), or DTT, EDTA and PVP (5).

B) NR activity in homogenates prepared using only buffer 5, or with additions of 0.1 mM FAD or 0.2 mM ferricyanide (\(n = 3\) in all cases).
Figure 2.5. Stability of NR activity over time in homogenates of *Thalassiosira pseudonana* homogenized without additions (●), with addition of 3% BSA (■) or with additions of protease inhibitors as described in the text (▼).

Points represent means plus standard errors of 3 separate homogenates.
Figure 2.6. Relative NR activity in homogenates of *Thalassiosira pseudonana* provided with different reductants: 0.2 mM NADH, 0.2 mM NADPH, or 0.1 mM NADH plus 0.1 mM NADPH. Error bars represent standard errors of the mean of 3 separate homogenates.
Figure 2.7. NR activity in homogenates of *Thalassiosira pseudonana* before (t = 0 h) and after (t = 48, 96 h) freezing and storage in liquid nitrogen. Points represent the mean and standard error of 3 separate samples.
**Enzyme Kinetics**

For *T. pseudonana*, the $K_m$ for nitrate was found to be 0.047 (±0.006) mM (Fig. 2.8 A). For NADH, an inhibition of activity was seen at levels greater than 0.2 mM. Calculating $K_m$ only for concentrations lower than this gave a $K_m$ of 0.017 (±0.003) mM.

**Enzyme Desalting**

Results of desalting experiments are shown in Table 2.3. Whether assessed by NADH oxidation or nitrite production, Sephadex-treated samples had lower NR activity ($P < 0.01$). Addition of FAD made no difference to homogenates that had not been Sephadex-treated ($P > 0.4$), but activity of Sephadex-treated samples did not differ from that of normally treated samples with added FAD ($P > 0.4$).

**Enzyme characterization in different species**

For *Skeletonema costatum*, ferricyanide treatment resulted in an almost complete loss of NR activity (Fig. 2.9). FAD, however, increased NR activity. As Figure 2.9 shows, the enhancement of NR activity was highly variable: in one trial NR increased by 50%, but in a second trial the increase was over 250%. No activity was found when NADPH was substituted for NADH. For *S. costatum*, the $K_m$ for KNO$_3$ was calculated as 0.146 (±0.022) mM, and the $K_m$ for NADH was 0.048 (±0.005) mM (Fig. 2.10). Unlike the case for *T. pseudonana*, high NADH levels did not inhibit NR activity.

In *Amphidinium carterae*, FAD addition caused a decrease in activity (Fig. 2.11), although this response differed between homogenates, resulting in high variability. In contrast to the case in other species, ferricyanide had no effect on NR activity (Fig. 2.11, $P > 0.3$). No activity was found when NADPH was substituted for NADH (Fig. 2.11). For *A. carterae*, the $K_m$ for KNO$_3$ was calculated as 0.075 (±0.012) mM (Fig. 2.12 A). High levels of nitrate (>1 mM) appeared to inhibit NR activity, but this response varied between extracts. The $K_m$ for NADH was 0.150 (±0.045) mM (Fig. 2.12 B). High NADH levels did not inhibit NR activity, as in *S. costatum*, but not *T. pseudonana*. 
Figure 2.8. NR activity versus substrate concentration for: A) KNO₃ and B) NADH in homogenates of *Thalassiosira pseudonana*. Curves are fit to rectangular hyperbolae. $K_m$ values are 0.0165 mM for NADH and 0.0471 mM for KNO₃.
Table 2.3. Effects of addition of 0.1 mM FAD on nitrate reductase activity in homogenates of *Thalassiosira* pseudonana. NR activity was determined by NADH oxidation rate or nitrite production rate and either analyzed directly (Normal) or desalted using a Sephadex G-25 column (Sephadex). Values represent means and standard errors of 3 replicate assays.

<table>
<thead>
<tr>
<th>Homogenate treatment</th>
<th>Assay addition</th>
<th>NADH oxidation</th>
<th>$\text{N}0_2^-$ production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>none</td>
<td>2.90 ± 0.13</td>
<td>2.86 ± 0.08</td>
</tr>
<tr>
<td>Sephadex</td>
<td>none</td>
<td>1.13 ± 0.13</td>
<td>1.29 ± 0.17</td>
</tr>
<tr>
<td>Normal</td>
<td>FAD</td>
<td>2.73 ± 0.09</td>
<td>3.40 ± 0.13</td>
</tr>
<tr>
<td>Sephadex</td>
<td>FAD</td>
<td>2.84 ± 0.13</td>
<td>3.31 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 2.9. Comparison of effects of addition of FAD and FeCN on NR activity in homogenates of *Skeletonema costatum*. Activity is expressed relative to the activity in the homogenate without additions. Trials 1 and 2 represent two separate experiments using two different cultures. Error bars represent standard errors of the mean of 3 homogenates.
Figure 2.10. Nitrate reductase (NR) activity versus substrate concentration for: A) KNO₃ and B) NADH in homogenates of *Skeletonema costatum*. Curves are fit to rectangular hyperbolae. $K_m$ values are 0.146 mM for KNO₃ and 0.0476 mM for NADH.
Figure 2.11. Comparison of the effects of additions of activators on NR activity in homogenates from *Amphidinium carterae*. Additions to the standard buffer are: 0.1 mM FAD, or 0.2 mM ferricyanide (FeCN). Error bars represent the standard error of the mean of 3 separate homogenates.
Figure 2.12. Nitrate reductase (NR) activity versus substrate concentration for: A) KNO₃ and B) NADH, for homogenates of *Amphidinium carterae*. Curves are fit to rectangular hyperbolae. $K_m$ values are 0.075 mM for KNO₃ and 0.150 mM for NADH.
Comparisons of NR activity with growth rate

Growth irradiance curves for *T. pseudonana*, *S. costatum*, and *A. carterae* are presented in Figure 2.13. For *T. pseudonana*, $\mu_{\text{max}}$ was 1.84 d$^{-1}$ and $K_I$ was 33 $\mu$mol quanta m$^{-2}$ s$^{-1}$. For *S. costatum*, two separate experiments gave very different results. In the first experiment, higher growth rates were achieved giving $\mu_{\text{max}} = 1.08$ d$^{-1}$ and $K_I$ of 79 $\mu$mol quanta m$^{-2}$ s$^{-1}$, while in the second experiment, $\mu_{\text{max}}$ was 0.33 d$^{-1}$ and $K_I$ was 25 $\mu$mol quanta m$^{-2}$ s$^{-1}$. For *A. carterae*, $\mu_{\text{max}}$ was 1.07 d$^{-1}$ and $K_I$ was 68 $\mu$mol quanta m$^{-2}$ s$^{-1}$.

Figure 2.14 shows the comparison between NR activity and the calculated rate of nitrate incorporation for each species. For *T. pseudonana* there was a highly significant relationship ($r^2 = 0.99$, $P < 0.001$) in which the slope of 0.95 ($\pm 0.40$) was not significantly different from 1.0 ($P > 0.3$). For *S. costatum* the relationship was not significant ($P > 0.5$), however, if a single culture was dropped from the analysis (see asterisk, Fig. 2.14 B), the relationship became significant ($r^2 = 0.95$, $P < 0.02$) and the slope of 0.87 ($\pm 0.06$) was not different from 1.0 ($P > 0.05$). For *A. carterae* there was a significant relationship ($r^2 = 0.71$, $P < 0.04$), but the slope of the relationship, 0.19 ($\pm 0.06$) was significantly lower than 1.0 ($P < 0.01$) indicating that NR activity accounted for less than 20% of the calculated nitrate reduction rate.

In Figure 2.15, the NR activity is compared to the calculated nitrate reduction rate for 12 species of marine phytoplankton. The regression is significant ($P < 0.05$), with a slope of 0.786 which is significantly lower than 1.0 ($P > 0.06$). This implies that for these species there is a tendency for NR to underestimate the calculated rate of nitrate incorporation. However, for individual species, there is wide variation. Diatoms tend to fall close to the 1:1 relationship, but species such as *Dunaliella tertiolecta* and *Emiliania huxleyi* show much higher NR activity than can be accounted for by calculated rates. Alternatively, only very low NR activity was detected in dinoflagellates tested, and no activity was found in the cyanobacterium *Synechococcus* sp.

As shown in Table 2.4, species from the Chlorophyceae and Prasinophyceae (i.e. green algae) were able to use NADPH in place of NADH. Some activity was found in the
Figure 2.13. Growth rate versus irradiance curves for: A) *Thalassiosira pseudonana* (•), B) *Skeletonema costatum* (■), and C) *Amphidinium carterae* (▼). Curves are fit to rectangular hyperbolae (parameters are given in the text).

Each point represents the mean and standard error of three growth rate determinations from a separate culture. Note two experiments are included in B).
Figure 2.14. Nitrate reductase (NR) activity versus $\text{NO}_3^-$ incorporation rate calculated from growth rate and nitrogen quota for: A) *Thalassiostra pseudonana* (●), B) *Skeletonema costatum* (■), and C) *Amphidinium carterae* (▼). Points represent means and standard errors of 2 enzyme measurements from individual cultures. Dashed lines represent least squares regressions. Solid lines represent the 1:1 relationships. Regression parameters are given in the text.
Figure 2.15. Nitrate reductase (NR) activity versus nitrate incorporation rate (calculated from cell growth rate and cell nitrogen quota) for 12 species of marine phytoplankton. Solid line represents the least squares regression. Dashed line represents the 1:1 relationship. Points represent mean NR activity with standard error for 2 NR assays from duplicate cultures. ○ chlorophytes, ● diatoms, □ prasinophytes, ■ prymnesiophytes, ▽ cyanobacteria, ▼ dinoflagellates. Species are indicated by abbreviations as explained in Table 2.4. Equation of the regression line is: $Y = -8.34 + 0.786 X \ (r^2 = 0.71)$. 
Table 2.4. NR activity in various species of phytoplankton using NADPH as a reductant. Activities are expressed as a mean percentage (± standard errors, n = 2 cultures) of activity found using NADH.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ABBREVIATION</th>
<th>% ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillariophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phaeodactylum tricornutum</em></td>
<td>Pt</td>
<td>4.37 ± 1.59</td>
</tr>
<tr>
<td><em>Thalassiosira weisflogii</em></td>
<td>Tw</td>
<td>2.31 ± 3.99</td>
</tr>
<tr>
<td><em>Ditylum brightwellii</em></td>
<td>Db</td>
<td>29.9 ± 16.9</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dunaliella tertiolecta</em></td>
<td>Dt</td>
<td>93.9 ± 3.4</td>
</tr>
<tr>
<td><em>Chlamydomonas sp.</em></td>
<td>Csp</td>
<td>79.0 ± 23.3</td>
</tr>
<tr>
<td>Cyanophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Synechococcus sp.</em></td>
<td>Ssp</td>
<td>--</td>
</tr>
<tr>
<td>Prymnesiophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pavlova lutheri</em></td>
<td>Pl</td>
<td>35.2 ± 9.6</td>
</tr>
<tr>
<td><em>Isochrysis galbana</em></td>
<td>Ig</td>
<td>0.58 ± 8.01</td>
</tr>
<tr>
<td><em>Emiliania huxleyi</em></td>
<td>Eh</td>
<td>1.03 ± 0.47</td>
</tr>
<tr>
<td>Prasinophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tetraselmis sp.</em></td>
<td>Tsp</td>
<td>39.0 ± 14.6</td>
</tr>
<tr>
<td>Dinophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gymnodinium sanguineum</em></td>
<td>Gs</td>
<td>1.69 ± 1.29</td>
</tr>
<tr>
<td><em>Prorocentrum minimum</em></td>
<td>Pm</td>
<td>0.30 ± 0.31</td>
</tr>
</tbody>
</table>
prymnesiophyte *Pavlova lutheri*; however, in all other cases activities were not significantly different from zero (P > 0.1 in all cases).

**DISCUSSION**

**Enzyme assay optimization**

The spectrophotometric assay for NR is simple, rapid and appears to provide estimates identical to those obtained using the nitrite production assay. This was also found by Evans and Nason (1953) and verified by Amy and Garrett (1974) who used the assays interchangeably. It offers the additional advantages that time-dependence and linearity of the assay can be confirmed for a single assay.

For *T. pseudonana*, collection of cells by filtration appeared to be as effective and potentially more reproducible than centrifugation. Filtration is more feasible when dealing with large-volume samples. Homogenization by sonication gives lower NR activity than grinding. This is similar to findings by Pistorius *et al.* (1976) and Hochman (1982). Hochman (1982) found that NR activity in a freshwater dinoflagellate was improved by grinding in a glass-glass versus a glass-Teflon homogenizer, but Hochman did not use filtered samples. The inclusion of a filter with the cells probably improved the grinding technique; certainly Eppley *et al.* (1969) and Serra *et al.* (1978a) remarked on its favorable effects, noting full release of activity within 30 s. Dortch *et al.* (1984) confirmed by microscopy that this techniques resulted in complete fragmentation of cells. The issue of whether the resulting homogenate should be centrifuged must be considered. The risk is that NR might not be fully released from cells that are sedimented, or might be adsorbed to particulate material such as filter fragments. However, use of Triton X-100 appears to release NR completely from the pellet. As well, having excess protein in the homogenate (as BSA) may mean that non-specific binding will remove BSA and minimize the loss of the less concentrated NR protein. The removal of membrane fractions may also minimize side reactions that might oxidize NADH.
Phosphate buffer clearly gives highest NR activity. MOPS also seems acceptable, but as previously noted (Serra et al. 1978a) TRIS is unsuitable and imidazole worse yet. Good et al. (1966) have pointed out that buffer choice is largely individual to enzymes; there is probably no single "best" buffer for all circumstances. The vast majority of researchers have selected phosphate buffer for NR assays (see Table 2.2). In terms of the extraction buffer, additions of EDTA, DTT and PVP seem justified, based on their enhancement of activity. Everest et al. (1984) found that 2-4 mM EDTA gave highest activity in several marine phytoplankton species. In a cyanobacterium, Herrero et al. (1984) proposed that sulfhydryl compounds stabilized enzyme activity by keeping NR amino acid residues reduced. Oxidized NR was apparently more susceptible to proteolytic attack; nitrate itself could stabilize activity. Effects of PVP and DTT have been found to enhance NR activity in a variety of organisms, although there are conflicting and species-specific results. For example, Eppley et al. (1969) found that cysteine was sufficient for flagellate NR protection, but that diatom species required DTT. On the other hand, in macroalgae Thomas and Harrison (1988) noted that PVP was required to obtain activity in Fucus species, but actually inhibited activity in Enteromorpha species. Where possible, and certainly in laboratory studies, assays should be optimized in individual species. Although combined additions of EDTA, PVP and DTT increased activity no more than single additions, activity was no lower, and therefore all additions were routinely made in subsequent assays.

BSA increased NR activity in T. pseudonana assays, and improved the stability of the enzyme. From the literature it is possible that this is due to protection from proteases that degrade NR either specifically or non-specifically (Wallace 1977). The fact that protease inhibitors had no effect in the present study does not necessarily refute this idea because the effectiveness of the tested protease inhibitors varies with the species, and proteases resistant to the particular inhibitors used in this study may have been present. For example, leupeptin is effective against proteases found in Lemna (Ingemarsson 1987) and in barley (Hamano et al. 1984), while PMSF is not. But PMSF was effective in preventing loss of NR activity in a
fungus (Essgaouri and Botton 1990). In corn roots, chymostatin was required (Long and Oaks 1990). The effectiveness of BSA has also been noted by Ingemarsson (1987) and Tischner et al. (1986), but in these cases casein was also effective; this was not true in the present study (data not shown). Casein also stabilized NR in wheat leaves (Sherrard and Dalling 1978), and in tomato plants (Ramon et al. 1989). BSA may act by providing a protein in higher concentration that the proteases can degrade in preference to NR, but there may be other effects. Schrader et al. (1974) proposed that casein and BSA stabilized NR in corn by binding inhibitory compounds. Some enzymes also show increased activity when the concentration of protein in the assay is increased. Without added protein, the total protein concentration in the assay is likely much lower than that found in vivo, and this dilution may adversely affect certain enzymes (Newsholme and Crabtree 1986, Aragon and Sols 1991). Alternatively, BSA may decrease non-specific adsorption of NR protein, or it may also be effective in binding phenolics that are not trapped by PVP (Gegenheimer 1990). Even in the presence of BSA, NR was still not completely stable; after 120 min, activity had dropped by almost 20%. This is an improvement over the decay seen by Hersey and Swift (1976), where dinoflagellate NR declined by half in 2-3 h. Similar time-dependent decreases in activity after homogenization have been seen by Essgaouri and Botton (1990) where 50% of fungal NR was lost in 1 h at 20 °C. Everest et al. (1984) reported that NR from marine phytoplankton was stable for up to 24 h at 0°C, and Harrison (1976) found that NR from *Gonyaulax polyedra* had a half-life of 24-30 h, but in these cases the assay incubations were as long as 1 h. Substantial enzyme degradation may have occurred during this time; subsequent degradation may have happened more slowly, giving results which were interpreted as indicating enzyme stability. In *T. pseudonana*, NR was stable for up to 60 min and assays could typically be performed within the 15 min of the extraction; thus activity assays were probably better than in previous studies, but there is still room for improvement.

The improved assay was reflected in terms of NR preservation in liquid nitrogen. Ahmed et al. (1976) found that ETS activity and GDH activity in whole cells of marine phytoplankton could be preserved without loss for up to one year in liquid nitrogen. In
contrast, Clayton (1986) reported losses of activity in Skeletonema costatum NR of up to 40% immediately after freezing. Such losses were not found in this study and this suggests that in field situations samples could be maintained frozen and await future analyses.

**Enzyme characterization in different phytoplankton species**

The use of NADH versus NADPH and the kinetic constants for NADH and nitrate provide some basis for comparing enzymes from different species. In this study the only species able to use NADPH were the green algae (chlorophytes and prasinophytes), although there was some evidence that at least one prymnesiophyte might also be able to use NADPH. The cyanobacterium could apparently use neither. This is in accord with a review by Syrett (1981) showing that green algae alone used NADPH. However, Hochman (1982) noted that NADPH-NR activity represented only 16.5% of NADH activity in the freshwater dinoflagellate Peridinium cinctum, and Serra *et al.* (1978) found some low activity (about 16% of NADH) in the diatom Skeletonema costatum. One possible explanation for this discrepancy might involve a membrane bound NADH:NADPH transhydrogenase (see Jackson 1991). If such an enzyme were present in cell homogenates, added NADPH could be converted to NADH and used by NADH-specific NR. Since this is most likely to happen in crude homogenates that are not centrifuged, the results of these studies must be considered carefully and weighed against results obtained with the purified enzyme. The meaning of differences in pyridine nucleotide specificity is uncertain. Evolutionarily, nitrate utilization was probably first dissimilatory, before oxygen was present in high concentration on Earth (see Mancinelli and McKay 1988). Since cyanobacteria do not use pyridine nucleotides, it is likely that the division of NR enzymes into NADH and NADPH forms arose later on. Although well beyond the scope of this study, it is tempting to speculate that different NR forms may have had different functions. Classically, NADPH is thought to be used primarily in biosynthetic pathways, while NADH is used in degradative energy-producing pathways (Hochachka and Somero 1984). This may have represented a division between, for example, assimilatory and dissimilatory nitrate reduction, although some present day bacteria also use NADH as an
electron donor for dissimilatory nitrate reduction (Stouthamer et al. 1980). Evidence of other functions of NR is accumulating (see Jones and Morell 1988, Castigetti and Smarrelli 1986). The fact that fungi have a NADPH-specific NR (Hewitt 1975) is also interesting.

In terms of kinetic behavior of NR, there appear to be distinct species differences. There was evidence that greater than 0.2 mM NADH inhibited NR from T. pseudonana, but S. costatum and A. carterae were unaffected even at 0.4 mM. NADH inhibition of NR has been noted by Serra et al. (1978) in S. costatum, but only at concentrations above 0.6 mM. Hochman (1982) found no NADH inhibition of a freshwater dinoflagellate. Alternatively, A. carterae NR appeared to be inhibited by high nitrate. Despite this finding in two separate kinetic experiments, in the growth rate study, NR activities determined at 1 mM and at 10 mM KNO₃ were no different. The reason for this difference is unclear. Kₘ values for NADH and nitrate obtained in the present study are well within the range of those previously found (Table 2.5). According to Packard (1979) Kₘ values for nitrate are typically between 0.05 and 0.15 mM in marine microalgae. All values in the present study fall in this range. Since uptake of nitrate typically has a much lower Kₛ (on the order of 0.0001 to 0.01 mM, Syrett 1981) this has been taken as evidence that there must be a high-affinity uptake system for nitrate.

In terms of FAD additions, no difference in NR activity was found for T. pseudonana, but S. costatum activity was increased. This increase was highly variable; from 275% of the control in one experiment to less than 10% in growth rate experiments. This variability has been noted previously in marine phytoplankton species. Epbley et al. (1969) found no effects of FAD, but Dortch et al. (1979) found increased activity. Everest et al. (1984) reported different effects of FAD with different marine phytoplankton species. Insight into these differences is provided by experiments in which T. pseudonana NR was desalted using a Sephadex column. There was a loss of activity on desalting which could be completely restored by adding FAD. This effect has been previously reported by Evans and Botton (1953) in higher plants and in Chlorella by Vennesland and Solomonson (1972). It appears to be an effect of dissociation of the FAD cofactor from the enzyme protein. This may be a function of the species (or even the strain, see Vennesland and Solomonson 1972), but also the
<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>Km NO₃⁻ (mM)</th>
<th>Km NADH (mM)</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher Plants</td>
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<td></td>
</tr>
<tr>
<td>Spinacea oleracea</td>
<td>0.013</td>
<td>0.007</td>
<td>Wray and Fido 1990</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphaerostilbe repens</td>
<td>1.4</td>
<td>0.032</td>
<td>Essgouri and Botton 1990</td>
</tr>
<tr>
<td>Microalgae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyta vulgaris</td>
<td>0.064</td>
<td>-</td>
<td>Guerrero et al. 1981</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>0.062</td>
<td>0.015</td>
<td>Amy and Garrett 1974</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>0.063-0.083</td>
<td>0.008</td>
<td>Packard 1979</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>0.040</td>
<td>0.088</td>
<td>Clayton 1985</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>-</td>
<td>0.017</td>
<td>Serra et al. 1978</td>
</tr>
<tr>
<td>Diatoma brightwelli</td>
<td>0.24</td>
<td>0.020</td>
<td>Eppley et al. 1969</td>
</tr>
<tr>
<td>Peridinium cinctum</td>
<td>0.10</td>
<td>0.28</td>
<td>Hochman 1982</td>
</tr>
</tbody>
</table>
homogenization procedure used. It might be interesting to examine the effect of FAD addition on NR activity after homogenization by French press or sonication. In *Amphidinium carterae* FAD addition produced highly variable results; NR activity was numerically lower with FAD, but this difference was not statistically different. It appears that routine FAD addition may therefore be a sensible precaution, even if not an absolute requirement for assays.

No species tested demonstrated increased NR activity with ferricyanide additions, and in fact, the diatoms showed a decrease in activity. NR activation by ferricyanide has been found in green algae (e.g. Pistorius *et al.* 1976), but could not be demonstrated in *S. costatum* (Serra *et al.* 1978a), nor in the freshwater dinoflagellate *Peridinium cinctum* (Hochman 1982). Thus, this form of NR activation may be confined to green algae.

**Comparisons of NR activity with growth rate**

With the improved assay, measured NR activity could be equated with calculated NR incorporation rates in *T. pseudonana*. This has not been consistently achieved before, and suggests that improvements in the NR assay are responsible. If this is true, it might be reflected in a comparison of NR activity found in the present study to other NR activities available in the literature. Unfortunately, this is not possible for a number of reasons. To begin with, assays have been conducted on cells grown at different temperatures, under different light levels or light:dark cycles, or with alternate nitrogen sources in the medium. Even when culture conditions are clearly specified, growth rates of cells are rarely provided. To make matters worse, activities are often scaled to cell number without any indication of cell size or nitrogen content, to variables such as dry cell weight or packed cell volume that are nearly impossible to compare with data from the present study. Furthermore, where activities are scaled to cell protein, the variability in protein assays due to the assay used, the extraction method used, or the protein standard employed, may make comparisons impossible (see Appendix A). For example, if protein extraction was incomplete (i.e. the sample was homogenized in distilled water or buffer alone) NR activities might be inflated, while if TCA precipitation were not used, protein would likely be overestimated, with the result that NR
activity would appear to be lower. In a few cases where a comparison is possible, results are variable (Table 2.6). For *T. pseudonana*, NR activities of up to 150 x 10^{-12} U cell^{-1} were found in the present study, which are equal to 0.25 U mg protein^{-1}. These clearly exceed those of Amy and Garrett (1974) and Smarrelli and Campbell (1980). NR activities in *S. costatum* were generally less than 120 x 10^{-12} U cell^{-1}, or 0.025 U mg protein^{-1}. These values exceed Serra et al. (1978a), Clayton (1986) and Smith et al. (1992) (see Table 2.6). In all these studies, culture growth rates exceeded those in the present study. In fact, based on literature values, the *S. costatum* cultures in the present study were not growing optimally; rates of up to 2.0 d^{-1} should be possible (Sakshaug and Andresen 1986). Despite this, activities in the present study are higher, although cell size differences cannot be discounted because adequate information on relative cell sizes is not provided by these authors. In contrast, Kristensen (1987) reported NR values of up to 150 x 10^{-12} U cell^{-1} in the same species which are more similar to the results of the present study. Again, details for adequate comparison are missing. As for *T. pseudonana*, NR activity equaled or exceeded the calculated nitrate incorporation rates in this species. With one exception, these rates were very closely related. NR activities in *A. carterae* in the present study were less than 250 x 10^{-12} U cell^{-1}. Hersey and Swift (1976) showed activities considerably higher: up to 6000 x 10^{-12} U cell^{-1}. It is difficult to resolve this difference. Hersey and Swift (1976), grew cultures at growth rates of up to 1.2 d^{-1}, or twice as high as those in the present study, but nitrogen quotas were near 25 pg cell^{-1} and thus smaller than the range of 50-60 obtained in the present study. It is unclear why these differences occurred, but it may be related to the fact that cells in the Hersey and Swift (1976) study were grown on a light:dark cycle. In *A. carterae*, in contrast to the other species, NR activity accounted for less than half the calculated nitrate incorporation rates in most cases.

These results were mirrored in the multi-species comparisons. It must be emphasized that this experiment was meant only as a broad test of the applicability of the NR assay developed for *T. pseudonana*; the assay was not optimized for any of the survey species. NR activity was quite close to calculated nitrate incorporation rates in the diatom species tested,
Table 2.6. Representative nitrate reductase activities from eukaryotic microalgae, determined by assay *in vitro* or *in situ*.

<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>ASSAY TYPE</th>
<th>ACTIVITY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella sp.</em></td>
<td><em>in situ</em></td>
<td>0.007 U mg protein^{-1}</td>
<td>Hochman <em>et al.</em> 1986</td>
</tr>
<tr>
<td><em>Chlorella stigmatophora</em></td>
<td><em>in vitro</em></td>
<td>0.0087 U mg protein^{-1}</td>
<td>Everest <em>et al.</em> 1986</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td><em>in vitro</em></td>
<td>0.030 U mg protein^{-1}</td>
<td>Morris and Syrett 1965</td>
</tr>
<tr>
<td><em>Dunaliella primolecta</em></td>
<td><em>in vitro</em></td>
<td>0.036 U mg protein^{-1}</td>
<td>Everest <em>et al.</em> 1986</td>
</tr>
<tr>
<td><em>Dunaliella tertiolecta</em></td>
<td><em>in vitro</em></td>
<td>0.0225 U mg protein^{-1}</td>
<td>Everest <em>et al.</em> 1986</td>
</tr>
<tr>
<td><em>Stichococcus bacillaris</em></td>
<td><em>in vitro</em></td>
<td>0.030 U mg protein^{-1}</td>
<td>Everest <em>et al.</em> 1986</td>
</tr>
<tr>
<td><em>Brachiononas submarina</em></td>
<td><em>in vitro</em></td>
<td>0.0058 U mg protein^{-1}</td>
<td>Everest <em>et al.</em> 1986</td>
</tr>
<tr>
<td><em>Nannochloropsis oculata</em></td>
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<td>0.0032 U mg protein^{-1}</td>
<td>Everest <em>et al.</em> 1986</td>
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<td><em>Platymonas subcordiformis</em></td>
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<td>0.0067 U mg protein^{-1}</td>
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</tr>
<tr>
<td><em>Platymonas tetraithale</em></td>
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<td>2.12 x 10^{-12} U cell^{-1}</td>
<td>Syrett and Hipkin 1973</td>
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<tr>
<td><em>Pavlova lutheri</em></td>
<td><em>in vitro</em></td>
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<td><em>Isochrysis galbana</em></td>
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<tr>
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<td>ACTIVITY</td>
<td>REFERENCE</td>
</tr>
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<td>----------------------</td>
<td>------------</td>
<td>---------------------</td>
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<td>Eppley et al. 1969</td>
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<tr>
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<td>Hersey and Swift 1976</td>
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<tr>
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<td>$1670 \times 10^{-12}$ U cell$^{-1}$</td>
<td>Harrison 1976</td>
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but NR activity overestimated the rates for most of the prymnesiophytes and one of the chlorophytes. In contrast, there was insufficient NR activity to account for observed rates of nitrate incorporation in *Tetraselmis* sp., and in the dinoflagellates, the activity was near zero. NR has previously been found to be difficult to extract from certain dinoflagellates. Hersey and Swift (1976) detected no activity in *Pyrocystis noctiluca* or *Dissodinium lunula*, while *Gymnodinium sanguineum* was also a problematic species (Cochlan, Dortch and Doucette, unpublished). However, this appears to be species specific since Harrison (1976) and Hochman (1982) found NR activity in two different dinoflagellate species. It is noteworthy that in the present study NR activity was detected in *Pavlova lutheri*, a species in which Everest *et al.* (1986) failed to find activity.

**Remaining problems with NR assays**

It is clear that for certain species, NR assays remain problematic. This may be a function of the fact that the NR assay was only fully optimized for one species. Clearly, the amount of work necessary to do this for every species would be enormous and is beyond the scope of this study. For cyanobacteria, as demonstrated in this study, NADH and NADPH cannot be used as electron donors for NR. According to the literature, these species require reduced ferredoxin (Guerrero *et al.* 1981). Provision of this compound, however, would also support NiR activity. This might decrease the NR activity measured using nitrite production, since the NiR enzyme could then use ferredoxin and reduce nitrite to ammonium (Wray and Fido 1990).

It appears that, at least for diatoms and particularly for *T. pseudonana*, the assay is acceptable. In the following two chapters, the relationship between NR activity and nitrate incorporation rates under different conditions will be explored in this species.
CHAPTER 3: RELATIONSHIPS BETWEEN NITRATE REDUCTASE ACTIVITY AND GROWTH RATE UNDER STEADY-STATE LIGHT OR NUTRIENT LIMITATION IN THALASSIOSIRA PSEUDONANA

INTRODUCTION

From Chapter 2, it is apparent that for *T. pseudonana* under steady state light limitation, NR activity matched observed rates of nitrate incorporation quite closely. In this chapter, the goal is to explore, in detail, the relationships between growth rate, cell composition and nitrate reductase activity under conditions of steady state light limitation, transitions in irradiance and steady state nitrate limitation, and to determine whether NR adequately predicts nitrate incorporation rates under these situations. For light-limited cases, cell composition data from *S. costatum* and *A. carterae* were also used. Chapter 4 considers the response of NR in more complex cases (i.e. diel cycles of irradiance, different light spectra, nutrient starvation and growth on ammonium), when enzyme regulatory mechanisms might become important.

NR and the control of nitrate metabolism

If NR is to be useful in predicting rates of nitrate incorporation, it must be quantitatively related to these rates. From a general point of view, in the majority of cellular reactions, total concentration of enzymes is generally positively correlated with steady state rates of metabolism (Acerenza and Kacser 1990). For a particular pathway, however, the only true prediction of *in vivo* rate is the rate of the limiting enzyme in the pathway (but see also Chapter 1 for a discussion of the usefulness of non-rate-limiting reactions). It is important to recognize that a given enzyme may only be limiting in a reaction sequence under a particular set of conditions because more than one enzyme may be involved in rate control as conditions change. A great deal of effort has been expended by biochemists trying to elucidate which steps of pathways control observed fluxes. Recent summaries of their conclusions are given by Crabtree and Newsholme (1985), Kacser and Porteous (1987), Hofmeyr and Cornish-Bowden...
 Specific treatments in plant systems can be found in Preiss and Kosuge (1976), Davies (1977) and Raven (1981). One formalization of these theories, metabolic control theory, suggests that the degree of control of a pathway by a specific step (i) can be represented by a flux control coefficient \( C_i \), such that:

\[
C_i = \frac{\delta Y / Y}{\delta V_i / V_i}
\]

where \( \delta Y / Y \) is the rate of change of the flux, and \( \delta V_i / V_i \) is the rate of change of the enzyme activity at that step. Thus if the enzyme is a rate-limiting step, \( C_i \) should be close to 1.0, i.e. a change in enzyme activity results in a proportional change in flux.

Although it is rarely certain that \textit{in vitro} rates of enzyme activity equal rates \textit{in vivo}, Newsholme and Crabtree (1986) give examples from the animal literature of how maximal activities of enzymes can be used to predict fluxes through metabolic pathways \textit{in vivo}. In theory, this can be most easily accomplished for enzymes that are rate-limiting. A number of characteristics may give an indication that a given enzyme is a rate-limiting step, although these characteristics do not in themselves constitute proof. Rate-limiting enzymes tend to have complex structure (e.g. multiple subunits), and have complex (i.e. allostERIC) properties (Preiss and Kosuge 1976, Davies 1977). They often catalyze non-equilibrium reactions with substantial, negative standard free energy changes (\( \Delta G^\circ \)) (Crabtree and Newsholme 1985).

As well, such enzymes are frequently subject to reversible covalent regulation (e.g. phosphorylation/dephosphorylation mechanisms) (Raven 1981). Sometimes, the complex nature of rate-limiting enzymes (e.g. allosteric or covalent regulation) means that simple \( V_{\text{max}} \) assays may not adequately represent what is happening \textit{in situ}. As Newsholme and Crabtree (1986) point out, it is difficult to predict \textit{a priori} whether a given enzyme will be useful in estimating metabolic fluxes. Thus, the validation of enzyme indices relies on empirical demonstrations that \textit{in vivo} fluxes and \textit{in vitro} enzyme activities correspond, i.e. that \( C = 1.0 \).

There is a great deal of evidence from higher plants and algae suggesting that NR is in fact rate limiting for nitrate incorporation. To begin with, NR fits many of the characteristics
listed above for a rate-limiting enzyme. NR has a complex structure and properties (see Guererro et al. 1981, Campbell 1988, Solomonson and Barber 1990, Crawford et al. 1992, and Chapter 2). The reaction NR catalyzes is non-equilibrium ($K_{eq}$ is on the order of $10^{25}$ to $10^{40}$), and $\Delta G^\circ$ is very large and negative (-140 to -230 kJ mol$^{-1}$) (Hewitt et al. 1976, Solomonson and Barber 1990). There is also evidence that NR is regulated by phosphorylation (Huber et al. 1992a). Furthermore, there are data showing that nitrate pools accumulate within tissues and cells, and that NR activities are usually much less than the activities of enzymes elsewhere in the pathway of nitrate assimilation. Limitation of nitrate incorporation by NR would suggest that nitrate should accumulate within cells, while the downstream product, nitrite, would seldom occur within cells. It is important to note that analyses of such internal pools may be deceptive. Ovadi (1991) points out that the mean distances between enzymes in cells is often on the order of the size of a typical tetrameric protein. This degree of crowding may mean that substrates are "channelled", or effectively passed from enzyme to enzyme with no apparent change in intermediate pools sizes. It is clear that this phenomenon is not always important (e.g. Stitt 1991), but it should be borne in mind. The build-up of nitrate pools and the absence of intracellular nitrite have been generally confirmed in higher plants (Campbell 1988). This is also true of marine phytoplankton, although it must be stressed that virtually nothing is known about the compartmentalization of nitrate pools in these species (e.g. cytoplasmic versus vacuolar pools). Nitrate pools of substantial size have been reported in nitrate-limited chemostat cultures of Skeletonema costatum (Dortch et al. 1979, Dortch 1982, and Thoresen et al. 1982), and in nitrate-sufficient cultures of Thalassiosira pseudonana (Dortch et al. 1984). In some cases, nitrate can represent up to 55% of nitrogen in cells of S. costatum, although this is more normally only a few percent (e.g. in nitrate-sufficient T. pseudonana nitrate pools were 2.8% of particulate nitrogen). Because the concentrations of these pools were larger that the measured $K_m$ for nitrate of NR, it was concluded that NR was probably operating at near $V_{max}$ in these cells (Dortch et al. 1979). Slawyk and Rodier (1986) also measured nitrate pools in Chaetoceros affiliis, and found that NR did not correlate with internal nitrate concentrations, suggesting that the enzyme was not
likely under substrate control. Scianandria (1991) also found nitrate pools in the dinoflagellate *Prorocentrum minimum*, but only when nitrate was supplied continuously. Collos and Slawyk (1980) failed to find evidence of internal nitrite pools in *S. costatum*. Further down the pathway of incorporation, ammonium pools do not generally accumulate (Collos and Slawyk 1980), although there may be problems with methodology for ammonium pools (Dortch 1982, Thoresen et al. 1982). In higher plants, low NR activities relative to other enzymes in the pathway of nitrate incorporation (e.g. NiR and GS) have also been noted (see Guererro et al. 1981, Crawford et al. 1992, and Chapter 2). For marine phytoplankton, this may also be true for many species, but there are very few data (see review by Collos and Slawyk 1980). The high degree of regulation of nitrate reductase, including evidence of translational, transcriptional, covalent and allosteric control, also suggests that it is a control point of nitrate metabolism (see Solomonson and Barber 1990, Crawford et al. 1992).

On the other hand, there is evidence that, in specific cases, NR may not be rate limiting. Guererro et al. (1981) point out that this is especially true in more structurally complex organisms such as higher plants, where translocation and storage become more important. The transport of nitrate into the cell may also be a control point. Guererro et al. (1981) suggested that ammonium inhibition of uptake of nitrate likely took place at the transport step in microalgae, and in some higher plants. Ingemarsson (1987) in *Lemna*, and Watt et al. (1992) in *Chlamydomonas reinhardtii* both present evidence that nitrate uptake is limiting, particularly at low nitrate supply. Sanchez and Heldt (1990) have also suggested that NR may be the rate-limiting step, but that supply of NADH (i.e. substrate limitation) and not NR concentration may be the limiting factor; maximal NR activity would not correlate well with incorporation rates if this was true. Limitation at the nitrite reductase (NiR) step may also occur. Seith et al. (1991) have pointed out that in higher plants NiR is regulated to a very high degree, suggesting that it has a rate-limiting function. Virtually nothing is known about the regulation of NiR in marine phytoplankton. Redinbaugh and Campbell (1991) note that because NiR and NR are co-induced in higher plants and apparently closely co-regulated,
distinguishing which is the rate-controlling step may be difficult. Coregulation is beneficial, since nitrite is toxic within cells and it must therefore be removed quickly. Another option, however, if NiR were rate limiting, would be to remove nitrite by extracellular excretion. Martinez (1991) investigated N-starved cultures of S. costatum and showed that nitrite excretion into the medium occasionally occurred on resupply and uptake of nitrate, but nitrite excretion also occurs during light to dark transition (see Collos and Slawyk 1980). Regulation at the ammonium assimilation step may also occur. Seguineau et al. (1989) proposed that GS played a key role in nitrate incorporation and nitrogen metabolism in general in Dunaliella primolecta, based largely on the very high degree of regulation of the enzyme. Alternatively, it may be that the entire pathway adapts to the prevailing nitrate incorporation rate. Stewart and Rhodes (1977) have shown that NR and GS activities closely parallel each other in higher plants, and a close coordination of NiR and GS has also been demonstrated (Weber et al. 1990). From theoretical considerations, Brown (1991) reasoned that having single rate-limiting steps in metabolic pathways was wasteful. Because there are constraints on total protein content of cells, enzymes which are in excess of routine requirements should be under selective pressure to decrease in concentration. As a result, it would be expected that control of a pathway would be distributed throughout the pathway, although this may be complicated by differential turnover rates of proteins, or other means of regulation (Brown 1991).

Light and nitrate limitation of growth rate

It is also important that an enzyme index of nitrate incorporation rate should respond predictably under different limitations on growth and metabolism. As described in the General Introduction, light- and nitrate-limited growth are probably two very common situations in the marine environment. These limitations may have different characteristics and require different culturing techniques to study them.

Light-limitation restricts the rate at which photosynthesis can provide the cell with energy in the forms of ATP and reductant (NAD(P)H), and fixed carbon. There are a variety of acclimations a cell can make to low irradiance so that it can overcome these limitations
(Richardson et al. 1983, Zevenboom 1986, Falkowski and LaRoche 1991a). These involve changes in cell composition (e.g. Post et al. 1985, Goldman 1986, Claustre and Gostan 1987) including the pigments and proteins of the photosynthetic apparatus (Prezelin 1981, Richardson et al. 1983). Light limitation experiments are usually performed in batch culture and, as pointed out by Rhee (1979), these closed systems represent an ever-changing environment in that nutrients decline, biomass increases, and waste products build up. However, by keeping cultures in logarithmic growth phase, the effects of these changing conditions can be minimized, so that a steady state is approached (Rhee 1979). A distinction between the effects of irradiance and the effects of changing growth rate on cell parameters must be made under these conditions. While, in absolute terms, it is irradiance effects that are being considered, changes in irradiance are reflected in changes in growth rates. For comparisons with nitrogen-limited cases, it is useful to consider growth rate as an independent variable. Therefore, in the present study, to simplify the discussion, relationships between cell composition or enzyme activity and growth rate, not irradiance, will be considered. A good relationship between light and growth rate has been described for the species considered (see Chapters 1 and 2). As long as regions of photoinhibition are avoided, regressing a variable against light as opposed to growth rate will only affect the shape of the curves and not the trends in responses (see e.g. Zevenboom 1986). Another approach to investigate cell responses to light is to perform transition experiments in which cells are switched from one light level to another. This requires repeated measurements to monitor changes in cell composition and rates of metabolism in cultures following the transition until a new steady state is reached.

The situation under nutrient limitation is more complex. Sciandria (1991) draws an important distinction between "limitation", the restricted supply of a nutrient, and "starvation" the removal of a nutrient. As will become apparent, these two situations may be very different. A nutrient-limited cell may be able to make a range of acclimations to low nutrients, including changes in composition and photosynthetic parameters (Goldman 1980, Herzig and Falkowski 1989, Lewitus and Caron 1990, Cullen et al. 1992) but these strategies may not be available to a starved cell. According to Sciandria (1991), a limited cell will show
cell quotas for the limiting nutrient less than the maximum quota, while a starved cell will show quotas near the minimum possible (see also Goldman 1980). However, this may not be generally true. Harrison et al. (1977) compared three diatom species under either starvation or limitation for ammonium or silicate. *Skeletonema costatum* and *Chaetoceros debilis* both showed almost two-fold higher silica quotas under silica starvation versus silica limitation, but *Thalassiosira gravida* showed slightly higher silica quotas under limitation versus starvation. On the other hand, *C. debilis* and *T. gravida* had much higher nitrogen quotas when starved of ammonium than when limited by it, while no differences in nitrogen quotas were seen in ammonium-starved versus ammonium-limited cultures of *S. costatum*. Harrison et al. (1977) speculated that because cell division in starved cells stopped one or two divisions after nutrient exhaustion, any cell composition changes had to occur in this period. In chemostat cultures, cells could divide for more than 10 generations before achieving a steady state; thus they had a greater scope for modifying their cell quotas. In any case, a lack of knowledge of the ranges of cell quotas may make application of such criteria impossible in most cases. Cullen et al. (1992) have compared these situations in terms of their implications for photosynthetic acclimation, and illustrate some of the differences. The case of starvation is distinct and will be reserved for consideration in Chapter 4. In order to investigate limitation, the chemostat is a convenient tool. Chemostats provide more realistic levels of nutrients and allow precise control of growth (by dilution rate) and biomass (by the concentration of the limiting nutrient) (Rhee 1979, but see also Burmaster 1979, and Di Toro 1980 for more mathematical treatments of chemostat properties), but they represent a steady state that is controlled by a single factor. Such a state is virtually never achieved in nature; apparent steady states are usually the result of a combination of nutrient supply rates, loss terms such as sinking, and trophic interactions (see Rhee 1979 and Eppley 1981). Because of the nature of a chemostat, transitions in nitrate limitation are relatively easily accomplished, but in practice the transient states following transitions are more difficult to follow. Each sampling will change culture volume and thus dilution rates of the cultures, and although this can be minimized by growing very large cultures (> 6 L), it may become impractical. Furthermore, the more times the culture must
be sampled after the transition, the greater the problem becomes. For this reason, such transitions have been avoided in the present study.

There is also an important interaction between light and nitrogen limitation. Photosynthetic acclimations are constrained by nitrogen availability (see Cullen et al. 1992). For each sub-saturating irradiance level, a range of nitrogen-limited growth rates are possible. Thus a culture may be nutrient-sufficient at a given irradiance, but if the irradiance were increased, the same nutrient supply rate might be insufficient. Alternatively, a nitrogen-limited culture may be photoinhibited by an irradiance that would normally be tolerated under nitrogen sufficiency. Furthermore, photosynthetic carbon and nitrogen metabolism are inextricably linked (see Turpin 1991).

Responses to light and nutrient limitation are species specific. To account for interspecific differences and the interaction of light and nitrogen, the concept of relative growth rate, defined as $\mu/\mu_{\text{max}}$ (where $\mu_{\text{max}}$ is the maximum growth rate at a given irradiance) has been developed (Goldman 1980, but see also Tett et al. 1985). In this study, because a single organism is used and nitrogen limitations are performed at a single irradiance, use of specific growth rate as opposed to relative growth rate is justified.

**Cell composition and scaling of enzyme activity**

In comparing light and nutrient limitation, the issue of cell composition becomes important. Different growth rates affect the composition of cells and there is evidence that these effects differ between light and nutrient limiting conditions (see Rhee 1979, Goldman 1980, Goldman and Mann 1980, Morris 1981, Sakshaug and Andresen 1986, Sakshaug et al. 1991, Laws and Chalup 1990, Thompson et al. 1991). As indicated in Chapter 1, the variable to which enzyme activity is scaled can affect the interpretation of the results. For example, Dortch et al. (1979) grew S. costatum at two nitrate-limited growth rates (0.8 and 1.6 d$^{-1}$) and compared NR activities. When NR activity was scaled to cell volume, activity was higher in the lower growth rate culture, but when NR activity was scaled to chlorophyll $a$, the high growth rate culture showed higher enzyme activity. Scaling NR activity to cell nitrogen or
protein quotas resulted in no difference in NR activity between the cultures. For culture work, this is not a problem, since NR can be compared directly with measured or calculated rates (see Chapter 2). In the field, however, this is not so, and clearly the issue of a scaling factor must be resolved.

**MATERIAL AND METHODS**

**General culture conditions**

Cultures were obtained from the North Eastern Pacific Culture Collection (NEPCC) and maintained on artificial medium (ESAW) at 17.5 °C under continuous light, as previously described (Chapters 1 and 2).

**Steady-state light-limited experiments**

For the cultures of *Thalassiostra pseudonana, Skeletonema costatum*, and *Amphidinium carterae* used in the growth rate experiments described in Chapter 2, specific growth rates, cell volumes, and carbon (C), nitrogen (N) and protein quotas were determined as previously described (Chapter 1). In addition, for *S. costatum* and *A. carterae*, chlorophyll *a* (chl *a*) quotas were measured by fluorometric methods after extraction in 90% acetone (Parsons et al. 1984a). C:N molar ratios and C:chl *a* weight ratios were also calculated. These constituents and ratios were plotted against specific growth rate, and analyzed by linear regression analyses using SYSTAT MGLH routines (Wilkinson 1990).

For *T. pseudonana*, NR activity data were taken from Chapter 2, plus six additional cultures grown and measured as before. NR activities were plotted against specific growth rates or nitrate incorporation rates calculated as the product of cell nitrogen quota and cell specific growth rates.
Light transition experiments

Transition experiments were conducted as described in Chapter 1. Six 1 L cultures of *T. pseudonana* were established, three at low light (15 μmol quanta m⁻² s⁻¹) and three at high light (90 μmol quanta m⁻² s⁻¹). These cultures were acclimated for a minimum of 8 generations, sampled at 0 h, and 24 h, then transposed (i.e. high to low irradiance, H→L, or low to high irradiance, L→H) and sampled again at approximately 24 h intervals for three more days. At each sampling, cell volumes and numbers, cell carbon, nitrogen and protein quotas, and NR activities were determined as before and C:N ratios calculated (Chapters 1, 2). Specific growth rates (μ) were calculated from changes in cell numbers between samplings. Cultures were maintained in logarithmic growth phase, diluting with fresh medium as necessary. Nitrate incorporation rates were also determined as before (Chapter 2, except that cell numbers instead of fluorescence were used to calculate growth rates). Within each time interval, differences in cell constituents, growth rates, NR activities and nitrate incorporation rates were tested using paired t-tests, as before (Chapter 1).

Steady-state nitrate-limited experiments

For three separate experiments with *T. pseudonana*, six nitrate limited chemostats were set up in 1 L flasks that were mixed with Teflon-coated stir bars and magnetic stirrers. Chemostats were provided with an inlet for medium, an overflow for excess culture and a sampling port. Chemostats were run by positive pressure; new medium was pumped into the culture using a multi-channel peristaltic pump (Manostat model 10A) and excess culture was forced out the outflow by pressure. Under these conditions, once steady state is achieved, growth rate is a function of the rate of new medium addition (dilution rate), and total biomass in the culture is set by the concentration of the limiting nutrient in the added medium (Rhee 1979). Thus, growth rate can be controlled by adjusting the rate of the peristaltic pump. Medium (ESAW) was prepared as before, except that nitrate concentration was lowered to 40 μM, and bicarbonate additions were doubled (to 4 mM) to prevent possible carbon limitation
in the cultures. In each experiment, medium for six cultures was provided from a common 20 L reservoir. Cultures were judged to have reached steady state when the cell fluorescence and the concentration of phosphate (a non-limiting nutrient) of the outflow remained constant over two days. At this point, cultures were sampled for cell volume and numbers, cell carbon, nitrogen, chl a and protein quotas and NR activities, as described previously. C:N ratios, C:chl a ratios, and the rate of nitrate incorporation were calculated. Cell constituents were plotted against specific growth rates (dilution rates/culture volume) and analyzed by linear regression analyses, as before. NR activities were plotted against specific growth rates, or calculated nitrate incorporation rates.

Scaling of NR activity

For T. pseudonana light- and nitrate-limited experiments, NR activity was scaled to cell volume, or cell carbon, nitrogen, chl a or protein quotas. These activities were plotted against specific growth rates and analyzed by linear regression analyses. In each case, regressions were performed for light-limited cultures alone, nitrate-limited cultures alone, and both sets of cultures together. Regression slopes and intercepts were compared following Steel and Torrie (1980).

RESULTS

Steady-state light-limited experiments

Responses of cell constituents to differences in growth rate varied with the species examined. Composition data versus growth rate in T. pseudonana is presented in Fig. 3.1. In one replicate experiment of three cultures which used a separate batch of seawater medium, the cultures showed unusual behaviour; cells became elongated and considerably greater in volume. This appeared to correspond to limitation by selenium, as previously described by Price et al. (1987). These cultures were excluded from regression analyses, but are shown as open symbols in Fig. 3.1. A summary of regression results is presented in Table 3.1.
Figure 3.1. Cell composition versus light-limited specific growth rate for *Thalassiosira pseudonana*. A) cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell protein quota, and E) cell C:N ratio. Each data point represents the mean of duplicate determinations from a single culture. Open symbols represent three cultures where selenium limitation may have occurred. Lines represent least squares regressions. Parameters are given in Table 3.1.
Table 3.1. First-order linear regression parameters for composition versus growth rate relationships in light-limited cultures of various marine phytoplankton. P-values represent the probability that the slope is equal to zero.

<table>
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<th>INTERCEPT</th>
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<td>--</td>
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<td>--</td>
<td>--</td>
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Significant and positive relationships were seen only for cell volume (Fig. 3.1 A, Table 3.1) and carbon (Fig. 3.1 B, Table 3.1), indicating that cells growing faster have higher volumes and carbon contents. Whereas in *T. pseudonana* replicate experiments gave similar results, in the other two species there was high inter-experimental variation. For this reason, experiments were usually considered separately. A relationship between carbon quota and growth rate was not seen in *S. costatum* (Fig. 3.2 B, Table 3.1) or in *A. carterae* (Fig. 3.3 B, Table 3.1), but there was a significant increase in cell volume in one experiment with *S. costatum*, and in both *A. carterae* experiments (Fig. 3.2 A, Fig. 3.3 A, Table 3.1). In one *S. costatum* experiment, there was a significant and positive relationship between cell nitrogen quota and growth rate (Fig. 3.2 C, Table 3.1). For both *S. costatum* and *A. carterae*, chl *a* significantly decreased as growth rate increased (Fig. 3.2 E, Fig. 3.3 E, Table 3.1). As well, for *A. carterae*, both C:N and C:chl *a* ratios increased with growth rates (Fig. 3.3 F, G, Table 3.1), although such a phenomenon was not seen in the other species.

Data in Figure 3.4 is repeated from Chapter 2 in a slightly different form. In *T. pseudonana*, NR activity was positively related to growth rate (NR = 7.46 + 41.6 (± 7.86) µ, r² = 0.67, P < 0.002) (Fig. 3.4 A). As for the composition data, the cultures that were potentially selenium-limited were left out of the analysis. The relationship between NR activity and the calculated rate of nitrate incorporation was not different from the 1:1 relationship (NR = 4.49 +0.98 (±0.03) nitrate incorporation rate, r² = 0.98, P < 0.001; Fig. 3.4 B). In this case, selenium-limited cultures were included, as no significant differences from other cultures were seen.

**Light transition experiments**

Composition data from the transition experiments did not follow all of the trends seen in the steady-state experiments. Cell volumes were no different between H→L and L→H cultures at any point during the experiment, although there was a non-significant trend toward L→H cells becoming larger, and H→L cells were significantly smaller after the transition (Fig. 3.5 A, P < 0.01). Some significant differences in carbon were seen. In agreement with
Figure 3.2. Cell composition versus light-limited specific growth rate for *Skeletonema costatum*. A) cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell protein quota, E) cell chlorophyll a quota, F) cell C:N ratio, and G) cell C:chlorophyll a ratio. Where there are 2 types of symbols, they represent two different experiments. Points represent the means of duplicate determinations from single cultures. Lines represent least squares regressions. Parameters are given in Table 3.1.
Figure 3.3. Cell composition versus light-limited specific growth rate for *Amphidinium carterae*. A) Cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell protein quota, E) cell chlorophyll a quota, F) cell C:N ratio, and G) cell C:chlorophyll a ratio. Where there are 2 types of symbols, they represent two different experiments. Points represent the means of duplicate determinations from single cultures. Lines represent least squares regressions. Parameters are given in Table 3.1.
Figure 3.4. Nitrate reductase activity versus light-limited growth rate in *Thalassiosira pseudonana*. A) NR versus specific growth rate, and B) NR versus calculated rate of nitrate incorporation. Each point represents the mean NR activity in a single culture. Error bars represent standard errors of the mean of two NR assays. Solid lines represent least squares regressions. Dashed line represents the 1:1 relationship. Open symbols represent cultures where selenium limitation may have occurred.
Figure 3.5. Changes in cell composition on transition from low light to high light (●) or high light to low light (○) in *Thalassiosira pseudonana*. 

A) Cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell C:N ratio. Transitions were made at the point indicated by the arrow. Each point represents the mean and standard error of three separate cultures. Asterisks indicate significant differences (P < 0.05).
steady-state experiments, H→L cells had higher carbon quotas than L→H cells before the transition, but changes in carbon quotas followed no pattern during the transition. In contrast to what was found in steady-state, L→H cells were significantly higher in nitrogen than H→L cells, before the transition. By the end of the experiment, the L→H cells had lower nitrogen quotas and H→L cells had significantly higher nitrogen quotas (Fig. 3.5 C). Largely because of differences in nitrogen, a similar pattern was seen in the C:N ratio (Fig. 3.5 D).

Figure 3.6 A shows the clear transition in growth rates when cultures were transposed. There was some indication that cells moved from low to high light actually increased their growth rate above that which the high light-grown cells had shown, but this difference disappeared by the end of the experiment. In terms of calculated rates of nitrate incorporation, a similar trend was seen (Fig. 3.6 B), and throughout the transition experiment, the NR activity matched the nitrate incorporation rate almost perfectly.

**Steady-state nitrate-limited experiments**

Chemostats stabilized within 5-6 days in all experiments. Examination of nutrient concentrations showed no nitrate in the outflow, except in the case of the two highest dilution rates in each experiment where nitrate was between 0.4 and 2 μM, and nitrite was approximately 0.2 μM.

Relationships between growth rate and cell composition were quite different during nitrate limitation than for steady-state light-limited experiments. Significant negative relationships were found for cell volume and carbon versus growth rate (Fig. 3.7 A, B, Table 3.2) in contrast to the positive relationships seen earlier. Once again, nitrogen and protein showed no significant relationships with growth rate. C:N ratios significantly declined as growth rate increased (Fig. 3.7 F, Table 3.2). Chl a content of cells decreased as growth rates increased (Fig. 3.7 D, Table 3.2), which, combined with carbon decreases, lead to significant decreases in the C:N ratio with increasing growth rate (Fig. 3.7 G, Table 3.2).

NR activities in chemostat cultures were positively related to growth rate (NR = 22.7 + 23.9 (± 6.95) μ, r² = 0.43, P < 0.004) (Fig. 3.8 A, and positively related to calculated
Figure 3.6. Effect of light transitions on: A) growth rate, and B) nitrate reductase activity (●, ○), or calculated nitrate incorporation rates (■, □) in *Thalassiosira pseudonana*. Transitions were made at the point indicated by the arrow. Each point represents the mean and standard error of measurements made in three separate cultures. Asterisks indicate significant differences (P < 0.05). Open symbols represent high to low light transitions. Closed symbols represent low to high light transitions.
Figure 3.7. Changes in composition with growth rate in nitrate-limited chemostat cultures of *Thalassiosira pseudonana*. A) cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell chlorophyll *a* quota, E) cell protein quota, F) cell C:N ratio, and G) cell C:chlorophyll *a* ratio. Each point represents the mean of two determinations from a single culture. Lines represent least squares regressions. Parameters are given in Table 3.2.
Table 3.2. First-order linear regression parameters for relationships between cell composition and growth rate in nitrate-limited chemostat cultures of *Thalassiosira pseudonana*. P-values represent the probability that the slope is equal to zero.

<table>
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<th>PARAMETER</th>
<th>SLOPE</th>
<th>INTERCEPT</th>
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<th>P-value</th>
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<td>cell volume</td>
<td>-10.8</td>
<td>40.2</td>
<td>0.46</td>
<td>&lt; 0.002</td>
</tr>
<tr>
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<td>0.41</td>
<td>&lt; 0.005</td>
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</tr>
<tr>
<td>chl a</td>
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</tr>
<tr>
<td>protein</td>
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</tr>
<tr>
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<tr>
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<td>299</td>
<td>0.54</td>
<td>&lt; 0.001</td>
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</tbody>
</table>
Figure 3.8. Relationship between nitrate reductase activity and A) specific growth rate, or B) calculated rate of nitrate incorporation, in nitrate-limited chemostats of Thalassiosira pseudonana. Each point represents the mean and standard error of duplicate NR assays from a single chemostat. Solid lines represent least squares regression fits to the data. Dashed line represents the 1:1 relationship. Parameters are given in the text.
rates of nitrate incorporation (NR = 24.2 + 0.485 (± 0.125) nitrate incorporation rate, \( r^2 = 0.48, P < 0.002 \)) (Fig. 3.8 B). However, in contrast to light-limited experiments, the slope of the relationship was less than 1:1; NR activity was significantly higher than the calculated nitrate incorporation rate at low growth rates.

Scaling of NR activity

NR activity scaled to cell volume (Fig. 3.9 A), carbon quota (Fig. 3.9 B), nitrogen quota (Fig. 3.9 C), or protein (Fig. 3.9 D) were all significantly related to growth rate (Table 3.3), but this was not true for NR activity scaled to chl \( a \) (Fig. 3.9 D)). Where regressions were significant, no significant differences were found between relationships for light-limited versus nitrate-limited cultures (\( P > 0.5 \) in all cases).

**DISCUSSION**

**Variation in cell composition with growth rate**

*Light-limited cultures*

Since few of the cultures were light-saturated (see Chapter 2, Fig. 2.13), the relationship between composition and growth rate and between composition and irradiance should be very similar (this would not be true in light-saturated cultures, since irradiance could increase without an increase in growth rate). There was variability between species and within trials, and not all data were available for all species, but some trends emerged. Table 3.4 gives a summary of previous studies where composition and growth rate (or irradiance) have been related. It is important to note, however, that these data combine continuous light with light:dark-grown cultures. As Sakshaug and Andresen (1986) have demonstrated, diel periodicity has profound effects on trends in cell composition and must be cautiously interpreted. The state of the culture in some studies in the literature have been poorly defined. Lewitus and Caron (1990) demonstrated that the trends in senescent cultures of *Pyrenomonas*
Figure 3.9. Nitrate reductase activity scaled to different parameters versus growth rate of *Thalassiosira pseudonana* in light-limited batch (○) or nitrate-limited chemostat (●) cultures. A) Per cell volume B) per g carbon, C) per g nitrogen, D) per g chlorophyll *a* and E) per g protein. Each point represents the mean of two assays from a single culture. Lines represent least squares regression fits. Parameters are given in Table 3.3.
Table 3.3. Comparison of first-order linear regression parameters for nitrate reductase activity scaled to different parameters versus growth rate in *Thalassiosira pseudonana* in light-limited batch cultures (L), nitrate-limited chemostats (N) or both types of cultures together (both). P-values represent the probability that the slope is equal to zero.

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Table 3.4. Changes in composition with increasing growth rate (irradiance or nutrient supply) for various species under light or nutrient limitation. D = hours of day light (i.e. 24 means continuous light), N SOURCE= the nitrogen source used (NO3 = nitrate, NH4= ammonium), VOL = cell volume, C =carbon quota, N = nitrogen quota, C:N = carbon:nitrogen ratio, CHL = chlorophyll a quota, C:CHL = carbon:chlorophyll a ratio, CHO = carbohydrate quota, PRO = protein quota. Responses are defined as increases (+), decreases (-), no change (nc), or complex behavior (c).

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<th>N</th>
<th>C:N</th>
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<th>C:CHL</th>
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<td>-</td>
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<td></td>
<td></td>
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<td></td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Goldman et al. 1979</td>
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<tr>
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<td>NO3</td>
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<td>Goldman et al. 1979</td>
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<td>Goldman et al. 1979</td>
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*salina* often differed from those found during logarithmic growth, and thus, this may be another source of variation in the reported results. The composition data can also be compared to those found in Chapter 1, since experimental conditions were identical.

Volume increased with growth rate in most cases, as was seen in Chapter 1. This has been found for several other species (Table 3.4). Thompson *et al.* (1991) came to a similar conclusion in a multi-species study (including *T. pseudonana*), and an extensive review of the literature. Thompson *et al.* (1991) also noted that the relationship of cell volume to irradiance and growth probably becomes species-specific above saturating irradiance, since the onset of photoinhibition is quite species-specific. Cell volumes may be under the control of internal ions (Riisgard *et al.* 1980), low molecular weight metabolites (Ahmad and Hellebust 1985b) or carbon content (Claustre and Gostan 1987), all of which may increase with photosynthetic rate at higher irradiances. The size decreases observed in the present study are very similar to those reported by Thompson *et al.* (1991), who measured volume changes on a scale of hours. This is surprising since diatom cells are bounded by a rigid silica frustule. There was evidence from one species, *Ditylum brightwellii*, that the size difference was due to addition of intercalary bands, resulting in a longer cell of similar width. Hitchcock (1983) found that at saturating irradiance and continuous light, the relationships between cell volume and other components was quite strongly conserved between species; thus volume may follow other trends. Thompson *et al.* (1991) speculated about the ecological advantages of smaller cell size, although on the contrary, larger cells may have a superior ability to absorb light (the so-called "package effect", see Raven 1986), and a proportionally lower respiratory rate (Taguchi 1976, Geider *et al.* 1986). Thus there may be equal physiological advantages to increasing cell size at low irradiance. Larger cells have increased capacities to store carbon (which could later be catabolized for energy) and this may be an advantage for cells that inhabit environments with fluctuating irradiance (see discussion in Thompson *et al.* 1991).

It is important to recognize that in diatoms there is a size change associated with sexual cycles; since cells decrease in width due to repeated asexual division, cells undergo sexual reproduction which restores their cell width (see Werner 1971a, 1971b). Over the course of
experiments in this thesis, such cycles in cell size were not noted for *T. pseudonana*, but between different experiments, maximum cell volume varied from 25 to 60 μm³. Costello and Chisholm (1981) have shown cyclic trends in cell volume in the diatom *Thalassiosira weisflogii*, but these volume changes do not appear to correspond to cycles of sexual reproduction. Armbrust and Chisholm (1992) also found changes in cell volume with growth rate in *T. weisflogii*, but noted that these changes occurred only in maximal, light-saturated growth rates. There was also a high degree of variability between clonal cultures. As noted in Chapter 1, it is also important to consider that the absolute volumes determined by the Coulter Counter are probably not correct. Changes in volume may still be real, but they might also result from cell shape changes.

An increase in carbon with growth rate was seen in the present study for only one species, *T. pseudonana*, and this was not seen in the experiments in Chapter 1. Cell volumes and carbon quotas in Chapter 1 were also greater than those in the present chapter; since growth conditions were identical, the reason for these differences is not known. A general increase in carbon quota with growth rate has been seen in many studies (Table 3.4), although there are exceptions. Thompson et al. (1991) recently reviewed the literature and reported similar results for a wide range of species. They noted that cell carbon versus growth rate relationships were more variable between species than those found for cell volume versus growth rate. As discussed in the preceding paragraph, the differences in carbon may drive the differences in volume, or may be a consequence of them. The increase in carbon has been attributed to increases in carbohydrate or lipid (e.g. Claustre and Gostan 1987), but with the exception of Post et al. (1985) this is not borne out by the few available studies where either no change, or decreases in lipid and carbohydrate with growth rate were noted (Table 3.4). For some of these studies where measurements were made on a light:dark cycle, this difference may explain the discrepancy. Several authors including Laws and Caperon (1976), Smith and Geider (1985), and (Geider 1992) have noted that at lower growth rates, respiration is a larger fraction of carbon fixed. If this was true, it would suggest that carbon quotas should decrease at lower growth rate, as observed.
Nitrogen quotas were generally more variable than carbon quotas in all species examined. Changes in nitrogen quotas were significantly related to growth rate in only one case; N quotas increased with growth rate in one experiment with Skeletonema costatum. There are a range of responses of nitrogen to growth rate reported in the literature (Table 3.4), with no change or an increase commonly found. The decrease in chl a seen with increasing growth rate would probably not produce a change in nitrogen, since very little nitrogen is accounted for in chlorophyll (Dortch et al. 1984), although there may be more nitrogen associated with light harvesting complexes (Prezelin 1981). Cell protein quota was not related to growth rate in the present study, which is in agreement with the data for nitrogen quota versus growth rate. Protein quota might be expected to vary with growth rate in the same way as nitrogen quota, since a substantial proportion of cell nitrogen is found in protein (see Appendix A). However, Morris (1981) in a review of protein synthesis showed that protein synthesis and nitrogen metabolism are often uncoupled.

The C:N ratio is a function of both carbon and nitrogen quotas and has been used frequently as an index of cell nutrient status. As summarized in Goldman (1980), the observed Redfield ratio, representing idealized elemental ratios in balanced growth, predict a C:N ratio of 6.6:1 (mol:mol). Given a 50% minimum protein content in a cell, it is unlikely that C:N ratios could fall much below 3.7 (Goldman 1980). Given that increases in carbon quota but not in nitrogen quota were observed in the present study, an increase in the ratio would be predicted, but this was seen only for A. carterae. According to a survey of the literature, the relationship between C:N ratio and growth rate varies between studies (Table 3.4). Turpin (1991) argued that C:N should correlate with irradiance (and therefore growth rate), because protein synthesis mechanisms saturate at lower irradiances than photosynthesis, and N assimilation outcompetes CO₂ fixation for reducing power. This may be what is happening in A. carterae cultures.

Variations in chl a quota with growth rate were similar for the species considered in the present study (note that chl a data were not available for light-limited T. pseudonana cultures). As would be anticipated, cells growing at high irradiance exhibited decreases in their chl a
quotas (see Richardson et al. 1983). This is consistent with the majority of studies (see Table 3.4), although there are exceptions. It has been noted that approximately half the decreases in pigment on transition from low to high irradiance is due to dilution of chl a due to cell division, while the other half is probably due to chl a degradation (Falkowski and La Roche 1991a). The C:chl a ratio provides another way to look at these data. There was no trend for S. costatum data, but this may have been due to a limited number of data points. For A. carterae, there was a significant increase in C:chl a with growth rate. The data provided by other studies agree with this trend (Table 3.4). The absolute magnitude of the ratio varies between 16 to 285 (Banse 1977, Rieman et al. 1989) in most field samples, and the results of the present study fall well within this range.

Some of the trends noted for steady state cultures were found for transition experiments. Claustre and Gostan (1987) pointed out that the two situations are not identical and differences should be expected. For example, Thompson et al. (1991) showed that carbon quota increased with growth rate, but this was not true in a transition experiment. However, L→H cells did increase their carbon quotas and H→L cells decreased carbon quotas on transitions. As shown previously (Chapter 1), cell volume responded as predicted by steady state experiments in the transition, although this trend was not statistically significant in the present study. The response of carbon was not seen in either data set, although in the present experiment the cultures began the transition with the high light cultures having significantly greater carbon quotas. There was a significant change in cell nitrogen quotas which was not reflected in steady state nitrogen data. This would be consistent with a decrease in nitrogen content with growth rate increases and might be mediated by decreases in pigment/protein complexes. It is possible that in a transition these differences are magnified. C:N ratios responded as would be expected from the A. carterae data set, indicating an increase in C:N ratio with growth rate. This was largely driven by the changes in nitrogen quotas.
**Nitrate-limited cultures**

The responses of cell constituents to changes in growth rates under nitrate limitation differed markedly from those found for light limitation.

Cell volume decreased as growth rate increased, a finding which is not supported in the literature. Cultures were examined microscopically for evidence of cell clumping, and Coulter Counter size distributions were studied for evidence of increasing size spread which might indicate clumping. Based on these examinations, no obvious clumping of cells was seen, but a relatively low percentage of clumped cells might still have caused an apparent increase in cell volumes, and a decrease in cell numbers, which in turn would have increased calculated cell quotas. In the marine alga *Heterosigma akashiwo*, Thompson *et al.* (1991) showed that nutrient limitation over-rode the effects of irradiance on cell volume; ammonium-limited cultures showed no change in cell volume with growth rate increases. With limitation by iron, increases in cell volume have also been found in a marine dinoflagellate (Doucette and Harrison 1990).

Carbon quotas decreased as growth rate increased. A similar trend towards increasing carbon quota with increasing nutrient limitation has been noted by Rhee (1980), but the literature also documents many variations in the relationship (Table 3.4). There are likely interspecific differences in these relationships (Rivkin 1989). As well, Laws and Caperon (1976) point out that some of this variation may come about because of differences in methodology; some chemostat studies have been run as cyclostats with a light:dark cycle, and the effects of this on composition have been documented. Furthermore, as reviewed in Darley (1977), the nitrogen source makes a significant difference; cells grown in nitrate have higher chl *a*, phosphorus and ATP quotas than those grown in ammonium (see also Zevenboom 1986, Thompson *et al.* 1989). In theory, nitrogen-limited cells would have sufficient energy to continue to fix carbon, but could not incorporate nitrogen, and thus, an increase in carbon or lipid would be expected. Such an increase is not well supported in the literature (Table 3.4). Cullen *et al.* (1992) suggest that carbon storage products in nitrogen-limited cells will increase
until a steady state is reached. Thereafter, all components will increase at the same exponential rate.

Surprisingly, the nitrogen quota of cells was apparently independent of growth rate (and therefore nitrogen limitation). This is at odds with the literature which unanimously agrees that nitrogen quotas increase with growth rate (Table 3.4, Goldman and Mann 1980, Zevenboom 1986, Turpin 1991). It may be that the significant differences in cell volume found in the present study play a factor. Certainly, if there were clumping of cells this would have produced such results; the most nitrogen-limited cultures would appear to have larger cells with more nitrogen; however, as previously noted, clumping could not be confirmed. Nitrogen per unit cell volume did significantly increase with growth rate. A lack of relationship between protein and growth rate was also seen. Given the expectation that nitrogen quotas increase with growth rates (Turpin 1991), this is also at odds with the findings of the present study. A clumping problem might again be invoked.

In contrast, C:N ratios fell with increasing growth rate (i.e. as cells became less N-limited), in good agreement with the literature (Table 3.4, Rhee 1979, Goldman 1980, Morris 1981, Laws and Chalup 1990). Marsot et al. (1991) did report a positive relationship between cell C:N ratio and growth rate, but their cultures were also at markedly different densities, suggesting that true steady-states may not have been achieved. This decrease in C:N ratio is normally attributed to a decrease in nitrogen quota, which was not seen. However, if clumping did occur, the C:N ratio would not be affected by it. Goldman et al. (1979) found that C:N ratios approached the Redfield ratio (6.6) only under nutrient sufficiency, a result reflected in the chemostat data in the present study.

Chl a quotas in nitrate-limited cultures increased with growth rate, as indicated in the majority of studies (Table 3.4, Turpin 1991). Herzig and Falkowski (1989) have reviewed the processes of pigment reduction under nitrogen limitation. If cell clumping had occurred, it might have been anticipated that cell chl a would rise at low growth rate. However, if this did occur, it may only have decreased the slope of the chl a versus growth rate relationship. C:chl a ratios decreased with growth rates, again agreeing well with the majority of studies
(Table 3.4, Goldman 1980), and the model of Laws et al. (1985). Sakshaug et al. (1991) found that this trend persisted regardless of the light level used, or the daylength, but noted that the precise relationship changed.

Variation in NR activity with growth rate

Light-limited cultures

NR activity in the present study was positively correlated with growth rate, and very strongly and quantitatively related to nitrogen incorporation rate. Thus, the NR activity is much more strongly related to growth than to factors such as cell size or composition. There are virtually no systematic laboratory studies of variation in NR activity with growth rate; most authors have chosen to investigate simple presence or absence of NR activity (e.g. Everest et al. 1986), NR activity in field situations (e.g. Packard et al. 1971, Blasco et al. 1984) or NR activity in cultures in transient states (e.g. Dortch et al. 1979, Clayton 1986, Smith et al. 1992). Data from studies in which nitrate incorporation rates and NR activity were compared are summarized in Table 3.5. It is evident that few other studies have found strong relationships between NR and nitrogen incorporation rates. In fact, only Morris and Syrett (1965) and Kristiansen (1987) found NR activity sufficient to account for observed nitrate incorporation rates, and relatively few studies have compared cultures at different light-limited growth rates. The good correlations found in the present study are likely the result of improvement to the NR assay (see Chapter 2).

A good agreement between NR activity and growth rate under light limitation might not have been anticipated. If cultures were light-limited, this would be evident in a limitation of energy, and a decreased ability to fix carbon, but this would not necessarily affect nitrogen uptake or incorporation. However, there is extensive evidence that nitrogen and carbon metabolism are very tightly coupled (Sawhney et al. 1978, Bassham et al. 1981, Geider 1992). As Turpin (1991) points out, because cell protein contents are high in algae, over 50% of all algal carbon is integrally coupled with nitrogen metabolism. Pace et al. (1990) and Kaiser and
Table 3.5. Relationship of nitrate reductase activity with increasing growth rate, and percentage of nitrate incorporation accounted for by NR (% NR/N) in various species under different limitations. Light is continuous and chemostats are nitrate-limited unless otherwise noted.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>CONDITIONS</th>
<th>RELATIONSHIP</th>
<th>% NR/N</th>
<th>REFERENCE</th>
</tr>
</thead>
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<tr>
<td><strong>Light-limited</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Chaetoceros affinis</td>
<td>nitrate spike</td>
<td>--</td>
<td>50</td>
<td>Slawyk and Rodier 1986</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
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<td>--</td>
<td>50</td>
<td>Smith et al. 1992</td>
</tr>
<tr>
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<td>--</td>
<td>&lt; 80</td>
<td>Clayton 1985</td>
</tr>
<tr>
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<td>12:12 light:dark</td>
<td>--</td>
<td>10 - 80</td>
<td>Kristiansen 1987</td>
</tr>
<tr>
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<td>positive</td>
<td>100-200</td>
<td>present study</td>
</tr>
<tr>
<td>Ditylum brightwellii</td>
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<td>--</td>
<td>25</td>
<td>Eppley et al. 1969</td>
</tr>
<tr>
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<td>positive</td>
<td>100</td>
<td>present study</td>
</tr>
<tr>
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<td>--</td>
<td>&gt; 100</td>
<td>Morris and Syrett 1965</td>
</tr>
<tr>
<td>Gonyaulax polyedra</td>
<td>12:12 light:dark</td>
<td>positive</td>
<td>50</td>
<td>Harrison 1976</td>
</tr>
<tr>
<td>Amphidinium carterae</td>
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<td>positive</td>
<td>20</td>
<td>present study</td>
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</tr>
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<td>--</td>
<td>338</td>
<td>Slawyk and Rodier 1986</td>
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<td>positive</td>
<td>83-190</td>
<td>present study</td>
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<td>--</td>
<td>Eppley and Renger 1974</td>
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<tr>
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<td>positive</td>
<td>87-176</td>
<td>Dortch et al. 1979</td>
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<td>--</td>
<td>Everest et al. 1986</td>
</tr>
<tr>
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<td>--</td>
<td>10-12</td>
<td>Morris and Syrett 1965</td>
</tr>
<tr>
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<td>starvation</td>
<td>positive</td>
<td>&lt; 10</td>
<td>Harrison 1976</td>
</tr>
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</table>
Brendle-Behisch (1991) have shown that there is a close coupling between photosynthesis and nitrate reduction (but not necessarily nitrate uptake) in higher plants. Work by Turpin and colleagues (Elrifi and Turpin 1986, Turpin et al. 1988, 1990) has demonstrated that when N-limited cultures of *Selenastrum minutum* receive nitrogen, photosynthesis is repressed. This is probably due to a shortage of ribulose 1,5 bisphosphate (RUBP), brought about by the removal of intermediates in the tricarboxylic acid (TCA) cycle in order to provide so-called "carbon skeletons" with which to combine nitrogen to produce amino acids. Dark respiration also increases under these conditions as carbon stores are metabolized to replenish TCA cycle intermediates. Interestingly, in cells that are limited by the supply of carbon dioxide, nitrate can be taken up, but it will not be reduced until carbon is available (i.e. futile nitrate reduction does not occur). This has also been demonstrated in higher plants such as maize seedlings (Pace et al. 1980). Flynn (1991) has suggested that the glutamine:2-oxoglutarate (α-ketoglutarate) ratio may be an important parameter in these processes, since this appears to be true in bacterial systems. If carbon became limiting the ratio would fall, but in a nitrogen-limited situation the opposite would happen. Sensitivity of biochemical and gene regulatory mechanism to this ratio could direct carbon towards nitrogen incorporation in times of nitrogen sufficiency. When nitrogen was limiting, cells would store carbon as carbohydrate for later use (Flynn 1991). This theory also makes predictions consistent with ammonium inhibition of nitrate uptake, which will be discussed in Chapter 4. Alternatively, it has been proposed that nitrate itself may activate cytosolic protein kinases. These in turn would inhibit sucrose phosphate synthase and activate phosphoenolpyruvate carboxylase, resulting in diversion of carbon from sucrose synthesis to amino acid synthesis (Van Quy et al. 1991, Campigny and Foyer 1992).

Transient experiments showed that the adaptation of nitrate reductase activity to new photosynthetic regimes and growth rates occurred quickly and within a day. There is evidence that it took up to 3 days after the transition in irradiance before a new steady state was reached (but note the apparent over-compensation of L→H cultures), but throughout the period, NR and nitrate incorporation rates were closely coupled. For culture work, where transitions
between steady states are made, this implies that "prehistory" of cells may not be so important (contrast with Dortch et al. 1979, Blasco et al. 1984). Most previous work on transitions has involved spike additions of nitrate or nitrogen starvation (see Clayton 1986), and frequently it is not clear that cells were initially in a steady state. Under these conditions any time-dependent measurements (i.e. 3 h nitrate uptake) might not be expected to correlate well with an instantaneous enzyme measurement. Measurements of NR activity during irradiance transitions have not previously been made.

**Nitrate-limited cultures**

As was the case under light limitation, NR in nitrate-limited chemostat cultures was well correlated with growth rate. However, the relationship was not 1:1 with calculated nitrate incorporation rates; NR at low dilution rates was much higher than the calculated rates, while NR at higher dilution rates was numerically lower than the calculated rate (although activity was variable, and in individual cultures NR was rarely statistically different from the nitrate incorporation rate at dilution rates > 0.5 d⁻¹). There was always some nitrate and nitrite detected in the outflow of the chemostats run at highest dilution rates. This could have occurred if the dilution rate was very close to the growth rate; minor fluctuations in either the pump rate or the growth rate of the cells may have resulted in periods when dilution was greater than growth. This would mean that there was a small loss of cells and as a result growth rates calculated from dilution rates might have been over-estimated. This might help explain the lower than expected NR activities in these cultures.

In the literature, there is wide variability in the trends in NR activity found in nitrogen-limited cultures. The chemostat experiments of Eppley and Renger (1974) and Everest et al. (1986) both showed negative relationships; NR increased as growth rate decreased. Although not strictly applicable in this chapter, Morris and Syrett (1965) and Harrison (1976) both reported that NR could not account for observed rates of nitrate reduction in starved cultures of microalgae. Results of Dortch et al. (1979) and Slawyk and Rodier (1986) are more similar to the present study. Although results from these two studies are based on only three cultures
in total, they indicated that NR activity at low dilution rates exceeded calculated incorporation rates (174 and 338%), while at high dilution rates NR was closer to the calculated rate (87%). Some authors have invoked an alternate nitrate reduction mechanism to account for discrepancies (e.g. Eppley et al. 1969, Clayton 1986, Slawyk and Rodier 1986), but this remains dubious.

NR activity in the present study exceeded that needed to account for observed rates of nitrate incorporation at low nitrate-limited growth rates. Nitrogen-limited cells develop the ability to rapidly take up limiting nutrients (so-called "surge uptake", see Conway et al. 1976, Conway and Harrison 1977, McCarthy and Goldman 1979, Dortch et al. 1991a). It is possible that cells at low growth rates maintain NR at higher levels than needed in anticipation of periods of rapid uptake (see Slawyk and Rodier 1986). Alternatively, Ingemarsson (1987) suggest that at low growth rates in the duckweed, *Lemna*, it is the flux of nitrate (i.e. the transport step) and not the NR activity that is limiting. This would be in accord with data from Dortch et al. (1979) showing a correlation between NR activity and internal nitrate concentration, but not with those of Collos and Slawyk (1977) where this relationship was not seen.

For diatom species, long periods of nutrient limitation may not be commonly experienced in the field. Typically, diatoms are first in successional patterns; they dominate at high nutrient concentrations due to their rapid division rates (see Guillard and Kilham 1977). Later, as nutrients are exhausted, other species such as flagellates replace the diatom community, which often sinks to the pyconocline. Thus, nutrient-limited chemostats run at low dilution rates may have little relevance for diatom species (see also Rhee 1979, Zevenboom 1986).

**Scaling of NR activity**

Given the range of differences in cell composition, and the different responses of cell constituents to different limitations, it might have been anticipated that NR activity scaled to a given biomass variable would correlate poorly with growth rate. In fact, this is not so; in all
cases except chl $a$, scaled NR activity was significantly and positively related to growth rates and the light-limited cultures were no different from the nitrate-limited chemostat cultures. The variability was high, however, typically only 50-60% of the variance in scaled NR activity was explained by growth rate. As discussed in Chapter 1, this may be the result of the variation in the biomass measurement increasing variability in the scaled enzyme data. The scaling problem is not an issue in the laboratory, but it becomes critical in the field. Scaling of NR to carbon is problematic because of the large amounts of detrital carbon found in marine waters (see Banse (1977) for a discussion of the problem with reference to C:chl $a$ ratios). Nitrogen potentially suffers the same problem, although these may not be insurmountable; Dugdale and Wilkerson (1991) found that nitrogen could be used as a scaling factor for nitrogen uptake without apparent interference from non-phytoplankton nitrogen. Chl $a$ is easily measured and correlates well with living phytoplankton biomass. However, it varies with irradiance and nitrogen level, and it is the one scaling variable in the present study where a significant relationship was not found. This is not surprising; cells growing slowly would have low NR in both light- and nutrient-limited cases, but under light limitation chl $a$ quota would be high (due to light acclimation), while under nitrate limitation it would be low (i.e. cells would be chlorotic). Scaling to protein seems logical and is frequently done, but as discussed in Chapter 1 and Appendix A, it is uncertain what different spectrophotometric assays actually measure. The relationship between NR scaled to protein and growth rate was also one of the poorer relationships found. Cell volume may offer an alternative, but this would requires tedious microscopic measurements, which have large errors associated with them. Light microscope measurements can be affected by halos around small particles; this resulted in volume estimates of 2 $\mu$m diameter latex bead standards that were up to 50% greater than the true values (Montagnes et al. submitted) and may constitute a significant bias in cell volume measurement. If only bulk measurements of processes are required, it is possible to scale measurements per litre or m$^3$ of seawater, but this will provide no information about the physiological state of organisms in water masses with different
biomasses. As is the case for nitrate uptake rates, scaling NR activity to particulate nitrogen seems to be the most practical course.

It is important to note that these conclusions apply, for the moment, only under steady states, or perhaps light transitions between steady states. Some non-steady states will be considered in Chapter 4. There is ample evidence that different relationships between growth and cell composition can result from day:night cycles (Sakshaug and Andresen 1986), culture senescence (Lewitus and Caron 1990), and temperature (Thompson et al. 1992). As well, limitation other than light or nitrate probably results in different patterns, as demonstrated for ammonium and phosphate (e.g. Laws et al. 1985) and iron (e.g. Doucette and Harrison 1990). However, the fact that NR activities in selenium-limited cultures were related to nitrate incorporation rates in the same way as other cultures suggest that this may not be such a problem.

In a review of composition and metabolism, Madraiga and Joint (1992) concluded that changes in composition vary with the specific limiting factor, but that physiological measurements are more related to growth rate differences. Thus, to make the method as applicable as possible, it may ultimately be more useful to scale NR activity to another physiological measurement, perhaps one that changes minimally or predictably with growth rates. At the present time, too little is known to make a recommendation, but indices such as electron transport system (ETS) activity (see Packard 1985, Martinez 1992) might have potential.

In summary, in this chapter, strong relationships between NR and growth rates and rates of nitrate incorporation have been demonstrated under steady state culture conditions. The relationship in *Thalassiosira pseudonana* is better under light limitation than nitrate limitation, where NR activity tends to exceed nitrate incorporation rates at low growth rates. Low nitrate-limited growth may not be a common situation for marine diatoms and thus may not be ecologically relevant and of less importance to the use of NR activity in the field. These findings suggest that the control of nitrate reduction may well be at the level of the enzyme under steady state conditions. The 1:1 relationship between NR activity and nitrate
incorporation (particularly under light limitation) implies a control coefficient ($C_i$) near 1.0 (Crabtree and Newsholme 1985), which suggests that NR activity can indeed be used to quantitatively predict metabolic rates *in vivo*. Enzyme scaling to biomass parameters is somewhat problematic since cell composition changes with growth rate are different depending on the specific limiting factor. However, this appears to be severe only in the case of chl $a$. It is suggested that NR be scaled to particulate nitrogen, based on the problems found in accurately measuring alternatives such as carbon, cell volume, or protein.
CHAPTER 4: EFFECTS OF LIGHT: DARK CYCLES, DIFFERENT LIGHT SPECTRA, NITRATE EXHAUSTION, AND AMMONIUM ON THE RELATIONSHIP BETWEEN NITRATE REDUCTASE ACTIVITY AND NITRATE INCORPORATION RATES IN THALASSIOSIRA PSEUDONANA

INTRODUCTION
In this chapter, the effects of several environmental influences that have been shown to play a role in the regulation of NR activity will be considered. These include diel periodicity in irradiance, different light spectra, nitrate exhaustion, and the influence of ammonium. As Guerrero et al. (1981) point out, these features (and others) that regulate NR activity also affect the capacity of cells to assimilate nitrate. In each case, the goal of these experiments is to determine how these factors influence the relationship between NR activity and nitrate incorporation rate, and whether they pose problems for the use of NR as an index in the field.

Effects of Diel Periodicity in Irradiance
With the exception of polar regions in certain periods of the year, diel periodicity is the most noticeable feature of irradiance cycles in the ocean (see Parsons et al. 1984b). Such cycles have profound influences on aquatic algae including effects on division cycles, taxis, photosynthesis, cell composition, and enzyme activity (Chisholm 1981, Prezelin 1992). There are many different diel patterns displayed, and these often appear to be taxa-specific. For example, dinoflagellates generally appear to have cell division phased near the light-dark transition, but diatoms such as Thalassiosira weisflogii display diel peaks in division frequency at midday and midnight (Chisholm 1981). Diel periodicity of nutrient uptake has been frequently demonstrated in microalgae in culture (e.g. Eppley and Renger 1974, Syrett 1981), and in field populations (see MacIsaac 1978, Manasneh and Basson 1987, Cochlan et al. 1991, Vincent 1992). Of course, photosynthesis is light dependent. From the relationships between NR and nutrient incorporation, and NR and carbon assimilation in photosynthesis previously demonstrated and discussed (Chapter 3), it is not unexpected that diel periodicity will also...
influence NR activity. This has been demonstrated in higher plants at the level of the enzyme activity (see Lillo 1983, Campbell 1988), the abundance of the enzyme protein (e.g. Oaks et al. 1990) and the rates of transcription and translation (see Lillo and Ruoff 1989, Deng et al. 1991). As well, similar results have been found for green algae (see Velasco et al. 1989), macroalgae (e.g. Gao et al. 1992) and other microalgae (e.g. Eppley et al. 1971, Packard et al. 1971a, Hersey and Swift 1976, Harrison 1976, Smith et al. 1992), as well as in field populations (e.g. Packard and Blasco 1974, Collos and Slawyk 1976). Specific light-activating mechanisms for NR are also known (see Hug and Hunter 1991, Kaiser and Brendle-Behisch 1991, Riens and Heldt 1992, Kaiser et al. 1992, Huber et al. 1992a, 1992b).

Effects of different light spectra

Unlike the full white light spectrum common in laboratory experiments and in the terrestrial environment, the light spectrum in the ocean is biased towards the blue because long wavelength light is effectively absorbed by water. This shift towards the blue increases in the deep ocean, depending on the water clarity and the abundance of suspended matter (see Parsons et al. 1984b). It has often been observed that long-term growth under blue light leads to an increase in total protein content in higher plant (Duke and Duke 1984, Barro et al. 1989) and algal cells (Wallen and Geen 1971, Morris 1981, Rivkin 1989, Kowallik et al. 1990, Apparicio and Quinones 1991). This may well influence nitrogen metabolism and thus affect nitrate incorporation. However, blue light may also have specific and possibly different effects directly on the NR molecule, perhaps mediated through blue light receptors involving phytochromes or flavin (see Azura and Aparicio 1983, Duke and Duke 1984, Ninneman 1987, Solomonson and Barber 1990, Hug and Hunter 1991, Lopez-Figueroa and Rueliger 1991).

Effects of nitrate exhaustion

In natural populations, as in cultures, microalgae pass through several growth phases; an initial period of slow growth (lag phase), a period of logarithmic growth (log phase), a plateau of biomass (stationary phase) and a later period of decline (senescence) (Fogg 1975).
The transition to stationary phase is caused by a limitation of some necessary requirement, often a nutrient. In this case, the effect is similar to that of nutrient starvation. Up to this point in the thesis, care has been taken to see that all cultures have been in logarithmic growth phase, where experiments are most reproducible (see Rhee 1979). However, in the field, cells may face periods of nitrate starvation and thus may be in different growth phases. It becomes critical to understand how NR will respond when cells become nitrogen-starved. There are data suggesting that there is a rapid decline in NR which occurs in step with decreases in nitrate assimilation (e.g. Morris and Syrett 1965, Hersey and Swift 1976), or a gradual decline in NR, which occurs more slowly than the decrease in nitrate assimilation (e.g. Syrett and Peplinska 1988). There are even reports of transient increases in NR when nitrogen runs out (e.g. Kessler and Oesterheld 1970, Slawyk and Rodier 1986, Watt et al. 1992).

**Effects of ammonium**

In previous chapters of the thesis, experiments involved cultures which had been grown with nitrate as the sole nitrogen source. This is not true in the natural environment where sources such as ammonium (Wheeler 1983) and organic nitrogen (Antia et al. 1991) are often present. Since ammonium is more reduced than nitrate, it has been argued that ammonium should be a preferred nitrogen source since it requires less energy to use, and thus confers a growth advantage (Syrett 1981, 1989). However, Thompson et al. (1989) failed to demonstrate such an advantage in *T. pseudonana* cultures. Ammonium has been shown to inhibit the uptake of nitrate in some studies (e.g. Syrett 1981, Dortch et al. 1991b, Cochlan and Harrison 1991a) but not in all cases (see Dortch 1990). There is strong evidence that ammonium is able to suppress NR activity in higher plants (Ingemarsson 1987, Solomonson and Barber 1990) and algae (Morris and Syrett 1965, Serra et al. 1978b, Dortch et al. 1979, Flynn et al. 1993), although there are exceptions (Harrison 1976, Collos and Slawyk 1980). Whether the inhibition by ammonium of nitrate uptake and the inhibition of NR activity are coordinated is an important question if NR is to be used as an index of nitrate incorporation. In higher plants, the two processes appear to be uncoupled in the short term. Lee and Drew
(1989) reported that nitrate influx to barley roots was inhibited within 3 min, a much shorter response time than is typically found for NR activity. Larsson et al. (1985), for example, argued that the inhibition of uptake was much faster than the inhibition of NR in the green alga *Scenedesmus*. Blasco and Conway (1982) suggested that the inhibitory effects of ammonium on the two processes were independent in natural populations. How the inhibitions of nitrate uptake and NR activity are mediated remains unclear, but it is generally thought that some product of ammonium assimilation, such as glutamine, is responsible (Syrett 1981, 1989, Clarkson and Luttge 1991). Other mechanisms that have been proposed include a direct influence of ammonium on NR (Florencio and Vega 1982), or an interaction between ammonium and nitrate mediated by the links to carbon metabolism (see Flynn 1990).

In this chapter nitrate reductase activity and nitrogen incorporation rates are compared in cultures of *T. pseudonana* that have been: a) grown on light:dark cycles, b) grown under white, blue or red light, c) starved of nitrogen, or d) grown on (or in the presence of) ammonium. The goal of the study was to determine whether these conditions prevent the use of nitrate reductase as an index of nitrate incorporation.

**MATERIALS AND METHODS**

**General culture conditions**

Cultures of *Thalassiosira pseudonana* were obtained from the NEPCC and maintained on artificial seawater (ESAW, modified as before) as described previously (Chapter 1). As before, cultures were grown at 17.5°C, stirred and bubbled with filtered air.

**Light:dark cycle experiments**

Cultures were grown in 6 L glass flat-bottomed boiling flasks at 16°C in an environmental chamber. Irradiance on a 14:10 h light:dark cycle was provided by fluorescent lights (Vitalites). Four cultures were grown through a minimum of eight generations, two at 45 μmol quanta m⁻² s⁻¹, two at 6 μmol quanta m⁻² s⁻¹. Growth rates were monitored by
fluorescence measurements taken within 1 h of 10 00h each day, or by cell counts using a Coulter Counter (see Chapter 1). Culture medium was identical to that previously described (Chapter 1), except nitrate concentrations were reduced from 550 to 225 μM. Cultures in logarithmic growth phase were sampled every 3 h over a 24 h cycle. At each sampling, 25 ml samples were filtered (25 mm GF/F) and frozen for nutrient analyses later. Dissolved nitrate, ammonium, silicate, and phosphate were analyzed within one month using a Technicon AutoAnalyzer II® and nutrient chemistry as described by Freiderich and Whitledge (1972). Nitrite was measured as described previously (Chapter 2). Samples were also taken and analyzed for fluorescence, cell numbers, cell volumes, and carbon, nitrogen, protein and chl a cell quotas, as previously described (Chapters 1, 2 and 3). Molar ratios of carbon:nitrogen and weight ratios of chlorophyll a:carbon were calculated. NR activity was determined at each sampling as previously described (Chapter 2). Nitrate incorporation rates were calculated from the change in particulate nitrogen in the cultures over each 3 h sampling interval, and compared with NR activities.

**Light spectra experiments**

Six cultures of *T. pseudonana* were grown in 1 L glass flasks in a water bath, as previously described (Chapter 1). Nitrate concentration in the medium was full ESAW enrichment, 550 μM. Two cultures were screened with blue-coloured filters (Roscolux #69), two with red-coloured filters (Roscolux #19), and two remained in full white light. Continuous irradiance was adjusted with neutral density filters and distance so that each culture received equal quantum irradiance of 45 μmol quanta m⁻² s⁻¹. Cultures were allowed to acclimate for a minimum of 8 generations, and they were sampled in logarithmic growth phase for cell numbers, cell volumes, and carbon, nitrogen, protein and chl a cell quotas, as previously described (Chapters 1, 2 and 3). Molar ratios of carbon:nitrogen and weight ratios of chl a:carbon were calculated. NR activity was determined as previously described (Chapter 2). Nitrate incorporation rates were calculated from the nitrogen cell quotas and growth rates, as described before (Chapter 2). For cell composition, growth rate and NR data, blue-, white-
and red-light treatments were compared using one-way ANOVA designs, followed by Tukey multiple comparison tests, with $\alpha$ set at 0.05.

**Nitrate exhaustion experiment**

Three 1 L cultures of *T. pseudonana* were grown under continuous irradiance at 115 $\mu$mol quanta m$^{-2}$ s$^{-1}$. Culture medium was as described previously, but nitrate concentration was one-fifth normal, or 110 $\mu$M. Cells were maintained in logarithmic growth phase for 8 generations, then allowed to grow into stationary phase. Beginning on day 3, for 5 days, samples were taken daily for nutrients (nitrate, ammonium, phosphate, silicate, and nitrite), cell numbers, cell volume, carbon, nitrogen, protein and chl $a$ quotas. C:N and C:chl $a$ ratios were also calculated. On days 3, 4, and 6, NR activity was measured as before (Chapter 2). For day 3, during logarithmic growth, nitrate incorporation rate was calculated as before (Chapters 2 and 3); for days 4-7 it was estimated from depletion of nitrate from the medium, or increase in particulate nitrogen in the culture. Changes in cell composition over time were evaluated by performing linear regression analyses of composition versus time and comparing the slopes of these regressions with zero using t-tests (Steel and Torrie 1980, Wilkinson 1990). Thus, increases in cell composition would be represented by regressions with slopes greater than zero and decreases by regressions with slopes less than zero. This is a conservative technique, since non-linear changes may also have occurred that might not be detected in a linear model, but the relatively few points in time meant that more complex models could not be judged statistically.

**Effects of ammonium and ammonium pulsing**

Six 1 L cultures of *T. pseudonana* were grown under continuous irradiance at 115 $\mu$mol quanta m$^{-2}$ s$^{-1}$. Cultures were grown on ESAW as described previously, except that the nitrogen source was either 75 $\mu$M nitrate for two cultures (NO3 treatment), or 75 $\mu$M ammonium (added as ammonium chloride) for two cultures (NH4 treatment). Two additional cultures were grown on nitrate-enriched medium (75 $\mu$M), but each day a pulse of ammonium
sufficient to bring the ambient concentration to 2 \( \mu M \) was added (P treatment). This treatment was chosen because 2 \( \mu M \) is a common level of ammonium in many areas of the ocean (see McCarthy 1980), and because a level of 1-2 \( \mu M \) ammonium is generally thought to affect nitrate uptake and nitrate reductase activity (see Syrett 1981, but see also Dortch 1990). Cells were maintained in logarithmic growth phase for 8 generations, then sampled for cell numbers, cell volumes, and carbon, nitrogen, protein and chlorophyll \( a \) quotas, as previously described (Chapters 1, 2 and 3). Molar ratios of carbon:nitrogen and weight ratios of chlorophyll \( a \):carbon were calculated. NR activity was determined at each sampling as previously described (Chapter 2). Nutrients were sampled at 16 h before the experiment and immediately before NR samples were taken. From the changes in nitrate or ammonium concentrations, rates of nutrient uptake were calculated and expressed as specific daily rates (i.e. \( d^{-1} \), as for growth rate). Nitrogen incorporation rates were calculated from the change in particulate nitrogen in the cultures over each 3 h sampling interval. For composition, growth rate and NR data, NO3, NH4 and P treatments were compared using one-way ANOVA designs, followed by Tukey multiple comparison tests, with \( \alpha \) set at 0.05.

RESULTS

Light:dark cycle experiments

Prior to the experimental period, growth rates based on increases in fluorescence or on cell numbers were identical: 0.90 (±0.02) \( d^{-1} \) for high-light-grown cultures, and 0.13 (±0.02) \( d^{-1} \) for low-light-grown cultures. Figure 4.1 A shows the increase in cell numbers in each of the four cultures. Over the 24 h sampling period, it was intended that culture density remain \(< 6 \times 10^5 \text{ cells ml}^{-1} \) so that cultures would remain in logarithmic growth phase. For one of the high-light grown cultures, cell density approached this limit, so after the 9 h sampling, the culture was diluted approximately by half. Despite this disturbance, growth rates and compositional trends in this culture were no different than its replicate. When measurements were made once a day at the same time of day, growth rates using \textit{in vivo}
Figure 4.1. Growth characteristics of log-phase cultures of *Thalassiostra pseudonana* grown on 14:10 h light:dark cycles. A) Culture densities, B) relative fluorescence, C) relative fluorescence per cell. Cultures were grown at high light (●, ■) or low light (○, □). Points in A) and B) represent single determinations; points in C) represent means of two cultures, with standard errors of the mean. Note that the vertical axes for A) and B) are logarithmic. The solid black bar indicates the dark period.
fluorescence were identical to those based on cell numbers. However, if a shorter time scale were used, growth rates were very different between the two methods. As illustrated in Figure 4.1 A and B, the increases in cell numbers during the day were much less than the increases in fluorescence. Conversely, at night, fluorescence changed little, but cell numbers increased. This resulted in a clear pattern in which fluorescence per cell increased in the light period and decreased in darkness (Fig. 4.1 C). This pattern was much less pronounced in the low-light-grown cultures. No pattern in cell division was found; cell numbers increased evenly in light and in darkness at similar rates.

In terms of cell composition, there was little variation in low-light-grown cultures for any parameter measured (Fig. 4.2), although protein quotas of low light cells tended to be higher in the dark (Fig. 4.2 E). For high-light-grown cultures, however, distinct diel patterns were seen in cell volume, cell carbon quota, and in the C:N and C:chl a ratios (Fig. 4.2 A, B, F, and G). Cell volume showed peaks in the middle of the light period, and at the beginning of the dark period (Fig. 4.2 A). Cell carbon increased during the light period and decreased in the dark (Fig. 4.2 B). C:chl a ratio followed a pattern similar to cell volume (Fig. 4.2 G), while C:N ratio followed a pattern similar to carbon quota (Fig. 4.2 F). Comparing low- and high-light cultures, cell volume and carbon quotas tended to be higher in the low light cultures, but the peak values of the high light culture were equal to those in cells grown under low light. Cell nitrogen and chl a quotas were uniformly higher in the low-light cultures, and C:chl a ratios were higher in the high-light cultures.

In terms of NR activity per cell, there was a diel periodicity in both low and high-light cultures (Fig. 4.3). There were two peaks, one at the middle of the light period, and a second before the beginning of the light period. NR activities at these peaks were higher in high-light than in low-light grown cultures, but NR fell to nearly equal levels at other times. In high-light cultures, NR activities per ml of culture matched rates of particulate nitrogen increase throughout the diel cycle extremely closely in one culture (Fig. 4.4 A). In the other culture, the match was good, except in the first three sampling periods when NR activity exceeded particulate nitrogen increases (Fig. 4.4 B). This was during the period when the culture
Figure 4.2. Changes in cell composition in cultures of *Thalassiosira pseudonana* grown on 14:10 h light:dark cycles at low (6 μmol quanta m\(^{-2}\) s\(^{-1}\), ○) or high (45 μmol quanta m\(^{-2}\) s\(^{-1}\), ●) irradiance. A) Cell volume, B) cell carbon quota, C) cell nitrogen quota, E) cell chlorophyll a quota, E) cell protein quota, F) cell C:N ratio, and G) cell C:chlorophyll a ratio. Each point represents the mean of duplicate determinations from two separate cultures. Error bars represent standard errors of mean values, or where absent are smaller than the symbols.
Figure 4.3. Nitrate reductase activity in log-phase cultures of *Thalassiosira pseudonana* grown on 14:10 h light:dark cycles at low (○) or high (●) irradiance. Each point represents the mean of two separate cultures. Error bars represent standard errors of mean NR activity.
Figure 4.4. Nitrate reductase activity (○) or calculated nitrate incorporation rate (●) in two log-phase cultures (A and B) of *Thalassiosira pseudonana* grown on 14:10 h light:dark cycles. Each point represents the mean of two enzyme assays. Error bars represent standard errors of mean values.
densities exceeded $6 \times 10^5$ cells ml$^{-1}$. It was hoped that nutrient concentration in the medium could be used to provide an independent estimate of nitrate incorporation rates, however, owing to the high dilutions necessary in order to measure nitrate in these cultures, the resulting concentrations varied widely and were not suitable for this purpose (e.g. For nitrate, samples had to be diluted about 1:20 to bring them within the linear range of the colourimetric reaction. Since the routine resolution of the AutoAnalyzer during the experiment was approximately 1 $\mu$M, only differences greater than about 20 $\mu$M could be reliably detected; this is on the same order as the nitrate concentration changes observed in the cultures). Nitrate, phosphate and silicate never neared depletion, and only low levels ($<0.5$ $\mu$M) of nitrite or ammonium were recorded. For the low-light cultures, particulate nitrogen varied widely as well (Fig. 4.5 A, B). As a result it became difficult to compare NR activity directly to increases in particulate nitrogen. As an alternative, NR activity and particulate nitrogen for each culture in each sampling period were used to estimate the particulate nitrogen concentration at the next sampling period. As shown in Fig 4.5, these predictions were certainly within the ranges of increases observed, given the high variability of the data. This suggests that NR activities were reasonably close to those necessary to account for the particulate nitrogen increases observed.

**Light spectra experiments**

Cell composition differed between blue-, white- and red-light treatments (Fig. 4.6). Cell volumes were higher in blue light than in white light and higher in white light than in red light (Fig. 4.6 A). Carbon quotas were greater in blue light than red light, but white light cultures were not different from either blue of red light cultures (Fig. 4.6 B). No significant differences in nitrogen quota, protein content, or C:N ratios were seen (Fig. 4.6 C, D, F). In the cases of chl $a$ quota and C:chl $a$ ratio, blue-light cultures were significantly higher than white- or red-light cultures (Fig. 4.6 E and G).

Under equal quantum irradiance, blue light cultures grew significantly faster than white or red light cultures (Fig. 4.7 A). As well, NR activities and calculated rates of nitrate
Figure 4.5. Particulate nitrogen concentration measured (○) or predicted from NR activity (●) in two log phase cultures (A and B) of *Thalassiosira pseudonana* grown on 14:10 h light:dark cycles. Each point represents the mean of duplicate determinations.
Figure 4.6. Cell composition in log-phase cultures of *Thalassiosira pseudonana* grown under equal quanta (45 μmol quanta m⁻² s⁻¹) of blue, white, or red light. A) Cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell protein quota, E) cell chlorophyll *a* quota, F) cell carbon:nitrogen ratio, and G) cell carbon:chlorophyll *a* ratio. Error bars represent standard errors of mean determinations from two separate cultures. Treatments not significantly different from one another at P = 0.05 are joined by lines.
Figure 4.7. Effects of blue, white and red light on: A) specific growth rate, and B) nitrate reductase activity (■) or calculated rates of nitrate incorporation (■■) in log-phase cultures of *Thalassiosira pseudonana*. Error bars represent standard errors of the mean of two separate cultures. Treatments not significantly different from one another at $P = 0.05$ are joined by a line.
incorporation were higher for blue light (Fig. 4.7 B). Under blue and white light, NR activity and calculated nitrate incorporation rates were not different (P > 0.5 in both cases). In red light cultures, NR activities were significantly lower than the calculated rates (P < 0.03).

Nitrate exhaustion experiment

Cultures entered stationary phase in terms of fluorescence data after the third day of the experiment (i.e. the log-normal plots of fluorescence or cell numbers versus time ceased to be linear, see Fig. 4.8 A), but cell numbers continued to increase until day 5 (Fig. 4.8 B). This resulted from a decrease in fluorescence per cell (Fig. 4.8 C). pH over the experimental period remained constant at about 8 (Fig. 4.9 A). Nitrate was the first nutrient exhausted on day 5 (Fig. 4.9 B; note that in this case and in the cases following nutrient exhaustion appears to occur earlier on the figures, due to the wide scale); low levels of silicate persisted until day 6 (Fig. 4.9 C) and phosphate was never exhausted (Fig. 4.9 D). Low levels of nitrate and ammonium were seen (Fig. 4.9 E, F), but nitrite had disappeared by the end of the experiment.

Cell volume was relatively constant over the experiment, although there was a slight, but not statistically significant decline (Fig. 4.10 A, P > 0.05). Cell carbon quota increased (Fig. 4.10 B, P < 0.001), which coupled with decreases in nitrogen quotas (Fig. 4.10 C, P < 0.05), resulted in an increase in the C:N ratio over time (Fig. 4.10 E, P < 0.001). Chl a quotas declined slightly (Fig. 4.10 D, P < 0.05), and C:chl a ratios increased with time (Fig. 4.10 F, P < 0.001).

NR activity fell over the course of the experiment (Fig. 4.11). NR activity generally followed rates of nitrate incorporation calculated from depletion or increase in particulate nitrogen, but NR was still detectable on day 6, at which point nitrate had been exhausted and there were no further increases in particulate nitrogen.
Figure 4.8. Changes in A) cell number, B) culture fluorescence, and C) fluorescence per cell, in cultures of *Thalassiosira pseudonana* entering stationary phase (indicated by the vertical line). Each point represents the mean of three replicate cultures. Error bars represent standard errors, or if not seen, are less than the size of the symbol. Note that the vertical axes for A and B are logarithmic.
Figure 4.9. pH and ambient nutrient concentrations for cultures of *Thalassiosira pseudonana* entering stationary phase as indicated by the vertical line. A) Culture pH, B) nitrate, C) silicate, D) phosphate, E) ammonium, and F) nitrite. Each point represents the mean of three cultures. Error bars represent standard errors of mean values, or if not seen are smaller than the symbols.
Figure 4.10. Cell composition for cultures of *Thalassiosira pseudonana* entering stationary phase, as indicated by the vertical line. A) Cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell chlorophyll a quota, E) cell carbon:nitrogen ratio, and F) cell carbon:chlorophyll a ratio. Each point represents the mean of three separate cultures. Error bars represent standard errors of the mean, and if not seen are smaller than the size of the symbol. Note that not all measurements were made at each time.
Figure 4.11. Nitrate reductase activity (●) or rate of nitrate incorporation calculated from growth rate and nitrogen quota (○), increase in particulate nitrogen (□), or depletion of nitrate from the medium (△). Each point represents the mean of determinations from three separate cultures of *Thalassiosira pseudonana* entering stationary phase. Error bars represent standard errors of mean values, or if not seen are smaller than the symbols. Note that not all measurements were made at each time.
Effects of ammonium and ammonium pulsing

In cultures grown on ammonium, cells were significantly greater in volume and carbon quota than either those grown on nitrate or pulsed with ammonium (Fig. 4.12 A, B). No significant differences in nitrogen or protein quotas were found, but ammonium-grown cultures tended to have numerically higher quotas (Fig. 4.12 C, D). There were no differences found in chl a quotas, or in C:N or C:chl a ratios (Fig. 4.12 E, F, G).

Ammonium-grown cultures grew at significantly higher rates than nitrate-grown or ammonium-pulsed cultures (Fig. 4.13 A). In terms of nutrient use and NR activities, the two ammonium-grown cultures behaved differently, and so are presented separately (Fig. 4.13). Ammonium was exhausted in one culture (NH4-1), but remained above 6 μM in the other (NH4-2). Nitrate levels in the ammonium-grown cultures were on the order of 1 μM, due to background contamination in the NaCl in ESAW. For the same reason, ammonium levels in the nitrate grown culture were up to 0.5 μM. Nitrate-grown cultures used only nitrate, and did so at rates consistent with their growth rates (Fig. 4.13 B). Both ammonium-grown cultures used ammonium, but a significant use of nitrate was seen in the culture in which ammonium was exhausted (NH4-2). NR activity and calculated nitrogen incorporation rates were not different for nitrate-grown and ammonium-pulsed cultures (Fig. 4.13 C, P > 0.5 in both cases). In the ammonium-grown culture where ammonium was not exhausted, no NR activity was detected, however, in the other ammonium culture, NH4 had significant NR activity (Fig. 4.13 C).

DISCUSSION

Effects of Diel Periodicity in Irradiance

The finding that in vivo fluorescence and cell number show different patterns of increase within a diel cycle is important for determination of culture growth rates. Clearly,
Figure 4.12. Cell composition in log-phase cultures of *Thalassiosira pseudonana* grown with 75 μM ammonium (NH4), 75 μM nitrate (NO3), or 75 μM nitrate with daily pulse of 2 μM ammonium (P). A) Cell volume, B) cell carbon quota, C) cell nitrogen quota, D) cell protein quota, E) cell chlorophyll a quota, F) cell carbon:nitrogen ratio, and G) cell carbon:chlorophyll a ratio. Error bars represent standard errors of mean determinations from two separate cultures. Treatments not significantly different from one another at P = 0.05 are joined by lines.
Figure 4.13. Effects of growth on 75 μM nitrate (NO3), 75 μM ammonium (NH4) or 75 μM nitrate plus daily 2 μM pulses of ammonium (P) on cultures of *Thalassiosira pseudonana*. A) Specific growth rate, B) specific nutrient uptake rates for nitrate (■) and ammonium (□), and C) nitrate reductase activity (■) and calculated nitrogen incorporation rate (□). Each bar represents the mean and standard error of two cultures, except ammonium cultures, which are shown separately in B and C because the replicates behaved differently.
fluorescence measurements must be made at the same time each day, or erroneous estimates may result. Interestingly, there appeared to be no diel periodicity in the division cycle of Thalassiosira pseudonana under these conditions. As Chisholm (1981) and Prezelin (1992) point out, division patterns are very specific to the taxonomic group considered. Chisholm (1981) found that the diatom T. weisflogii had two daily "bursts" of growth centered at midday and midnight. If this is also true for T. pseudonana, it is possible that the peak of division was simply spread out enough that individual peaks could not be discerned. Nelson and Brand (1979) have studied cell division patterns in 13 species of marine phytoplankton, including 7 clones of T. pseudonana. They found that periodicity of division was species and clone specific. In at least 4 clones of T. pseudonana division rates were nearly constant throughout the light:dark cycle.

Patterns of cell volume over diel cycles have been investigated previously. As Chisholm (1981) reports, patterns in diatoms are often complex (see discussion of cell volume in Chapter 3). For T. weisflogii, there was a bimodal pattern with maximum volumes occurring at mid-morning and just before the light-dark transitions. This was correlated with cell division cycles. A similar bimodal pattern, with different timing was seen here, but there were no associated division cycles; if a division cycle was involved, the cell volume might be expected to nearly double. The increases observed were only 20%, but as previously mentioned, Coulter Counter volumes may be suspect (see Chapters 1 and 3). In the flagellates Heterocapsa sp. and Heterosigma akashiwo, cell volume monotonically increased in light, peaked just into the dark period, then decreased until the next light period began (Latasa et al. 1992, Berdalet et al. 1992). Eppley and Coatsworth (1966) reported a similar pattern in Dunaliella tertiolecta, which appeared to correlate with cell division, as have Marsot et al. (1992) in dialysis cultures of Phaeodactylum tricornutum. There are few data for other cell composition parameters. It is interesting to note that cell carbon data, in the present study, closely follows the pattern reported for cell volumes in the majority of studies. Morris (1981) summarized data showing that for several species of green algae, carbohydrate content increased during the day, while protein increased at night because nitrogen is incorporated with
carbon into protein. Such a pattern would correlate well with carbon quotas in the present study. Increases in protein at night in low light cultures in the present study also tend to support this idea, but a similar pattern was not seen at high light. Working with *S. costatum*, Smith *et al.* (1992) reported that there were midday peaks in C:N ratios. However, these authors also exposed the algae to transitions in nutrient availability, which may have caused differences. Eppley and Coatsworth (1966) reported diel variation of up to 20% in C:chl *a* ratios in *D. tertiolecta*, similar to the present study.

Differences between high and low light grown cultures were consistent with previous results for chl *a* and C:chl *a* data (Chapter 3), but were quite different for carbon, nitrogen and cell volume data. Cell volume and carbon were previously found to increase with light-limited growth rate, but here they were either not different, or were lower at high light than at low light. There were also differences in nitrogen quotas that were not previously observed. The reasons for these different findings are unknown, but clearly there are dangers in making inferences about cell composition in cells grown on a light:dark cycle from cultures grown on continuous light.

NR activity per cell showed a double peak in the diel cycle. This has not previously been noted, although a similar pattern is visible in data from cultures of *Isochrysis galbana* in a recent study (Flynn *et al.* 1993). Three patterns have been commonly found. The first, where activity shows a peak in the light period and very low levels at night, has been demonstrated in higher plants (Deng *et al.* 1991), macroalgae (Gao *et al.* 1992) and microalgae (Packard *et al.* 1971a, Hersey and Swift 1976, Collos and Slawyk 1976, Harrison 1976, Velasco *et al.* 1988, Smith *et al.* 1992). Martinez *et al.* (1987) found a variation on this pattern in natural populations of marine phytoplankton; NR activity showed two peaks in the middle of the light period, with a decrease in activity at solar noon. This was attributed to photoinhibition of nitrate uptake during the period of highest irradiance (Martinez *et al.* 1987). The second pattern is a monotonic increase in the light and a decrease in the dark, reported in barley leaves (Lillo 1983) and natural phytoplankton populations (Manasneh and Basson 1987). Finally, increases in activity just before dawn and declines during the day have been found in
Emiliania huxleyi in chemostats (Eppley et al. 1971), and in natural marine phytoplankton populations (Packard and Blasco 1974). There is also a report of this pattern in tobacco plants (Roth-Berjerano and Lips 1970), although the pattern varied with the season and sampling was too infrequent to provide good resolution. There may be at least two reasons for these differences. Lillo (1983) found that the particular assay used (e.g. in situ versus in vitro) could give very different patterns; there is certainly great diversity among assays in the literature (see Chapter 2). In the present study, use of high EDTA may have activated dark-inactivated NR (see Kaiser et al. 1992), but this is unlikely to have made much of a difference because such a mechanism only operates on very short time scale (i.e. minutes). Secondly, the sampling frequency of many studies may be insufficient to catch both the peaks. For example, Eppley et al. (1971) sampled irregularly every 5-6 h, Smith et al. (1992) sampled every 4-8 h, Manasneh and Basson (1987) sampled at 6 h intervals, and Harrison (1976) sampled only every 12 h. Packard and Blasco (1974) also had restricted time series and samplings. From data in the present study, and these considerations, the seemingly conflicting results of many of these reports can be reconciled. It is also interesting that Collos et al. (1993) have shown diel, midday peaks in RUBISCO activity in natural flagellate populations. This concurs with the nitrogen-carbon coupling discussed in Chapter 3.

NR correlated very well with calculated rates of increase of particulate nitrogen in most cases. Apparently, NR activity exceeded calculated rates as culture densities became high (see Fig. 4.4 B), although the reasons for this are unknown. As cultures reached high densities, pH increased, indicating a possible carbon limitation (nitrate remained above 400 μM and silicate and phosphate were both in excess). Compared with activity measured during logarithmic growth, NR activity in these cultures nearly doubled (data not shown). Although such circumstances are not likely to occur in natural waters, it is an interesting result which should be pursued. The correlation between NR and calculated rates agrees with the findings of Eppley et al. (1971), but although they found a similar pattern, they could not account for more than 25% of calculated incorporation rates with NR activity. This was probably due to a poor extraction of the enzyme. Similarly, Collos and Slawyk (1976) found a correlation
between increase in particulate nitrogen and NR activity on a diel cycle, but NR activity could only account for 12% of the particulate nitrogen increase. As previously discussed, the calculated incorporation in this study may involve both uptake and assimilation (see Chapter 3), but it does clearly suggest that cells are taking up nitrate in the dark. It has been argued that nitrate reduction would not proceed at night because the energy must be derived from photosynthesis (Morris 1981) or because photosynthesis must provide carbon skeletons to attach nitrogen (Syrett 1981, 1989). However, breakdown of cell storage products could provide both these requirements (see Turpin et al. 1988, Turpin 1991). The case for nitrate uptake being restricted to the light is better established in higher plants (see Oaks et al. 1990), but it is much less certain in the algae. In dinoflagellates, Hersey and Swift (1976) reported that *Amphidinium carterae* and *Cachonina nei* did not assimilate nitrate in the dark, but Harrison (1976) and MacIsaac (1978) found that *Gonyaulax polyedra* did; under nitrogen starvation, cells were capable of meeting 50% of their nitrogen requirements by dark uptake. Because Packard and Blasco (1974) found little periodicity in NR for *G. polyedra*, they hypothesized that this species may have competitive advantages in terms of being able to take up nitrate in the dark. There are likely species-specific differences: Eppley et al. (1971) reported that the diel periodicity of nitrate and ammonium uptake was more pronounced in the diatom *S. costatum* than in the prymnesiophyte *Emiliania huxleyi*. As Cochlan et al. (1991) discuss, the absence of nitrate uptake at night may be largely a misconception. They showed that nitrate was taken up at night by natural populations at 15-16% of the maximum daytime rate. Marsot et al. (1992) found that in dialysis cultures of *Phaeodactylum tricornutum*, nitrate uptake in the dark was almost half of that in the light. It has also been suggested that NiR should be less active in the dark because it relies on ferredoxin for reducing power, and ferredoxin is apparently only available when photosynthesis proceeds (see Guerrero et al. 1981, Martinez 1991). However, Huber et al. (1992a) found that changes in the activity of NR from spinach leaves were far greater than changes in NiR over a day-night cycle.

There has been a great deal of work specifically on the activation/inactivation of NR following transitions between light and darkness. It is probable that more than one mechanism
is involved in this process. There are reports that NR activation requires synthesis of new protein (Lillo and Ruoff 1989, Velasco et al. 1988), but there are also reports of increases in NR activity occurring in a matter of minutes following a transition from dark to light (see Kaiser et al. 1992, Riens and Heldt 1992). Such rapid transitions have been shown to involve phosphorylation (Huber et al. 1992a, 1992b, MacKintosh 1992, see also Budde and Randall (1990) for a review of phosphorylation mechanisms). On a longer time scale, however, the degradation and synthesis is almost certainly involved (see Lillo 1991). Smith et al. (1992) found that in S. costatum, there was a diel cycle of NR synthesis in which peaks in NR-mRNA were followed by peaks in NR protein. However, a peak in NR activity preceded these peaks, suggesting that an activation of pre-existing NR enzyme was also involved. Deng et al. (1991) suggested that these synthesis/degradation patterns may be controlled by the presence of glutamine, or another nitrogen metabolite. Regardless of the precise mechanisms involved, on the 3 h time scale measured, NR activity was an adequate predictor of nitrate incorporation rates in the present study.

**Effects of different light spectra**

The results of the light spectra experiment are difficult to compare with other literature results because different authors have combined the light treatments with day:night cycles, or have used either equal energy, or equal quanta of light. As well, some have worked with saturating irradiances, while others chose lower irradiances. As Morris (1981) points out, the effects of irradiance level probably have a greater influence than the spectral composition of the light.

In terms of composition, most work supports the idea that cells grown under blue light accumulate protein, while cells grown under red light accumulate carbohydrate (Morris 1981, Barro et al. 1989, Kowallik et al. 1990, Grotjohann and Kowallik 1989). Rivkin (1989) demonstrated this in Dunaliella tertiolecta and Thalassiosira rotula, and found in addition that carbon quotas were higher in red and blue light-grown cells than in white light-grown cells. This contrasts with results obtained here, but note that Rivkin (1989) used equal
photosynthetically usable radiation (PUR), a measurement that is lower than photosynthetically available radiation (PAR) which is what was measured in the present study (see Person et al. 1984b). In addition, Rivkin grew cells on a 12:12 light:dark cycle. Grotjohann et al. (1992) found that *Chlorella kessleri* grown in blue light had up to 50% more protein than cells grown in red light, and 60% more reaction centres per chl *a*. Higher protein quotas in blue light-grown cells were not found in the present study. There is also some disagreement on the effects of different light spectra on photosynthetic pigments. Rivkin (1989) found that chl *a* was greatest in white or blue light-grown cells and lower in red light-grown cells. This is the opposite of what was found in the present study. Wallen and Geen (1971) found that blue light grown cells of *D. tertiolecta* and *T. pseudonana* had higher chl *a*, as did Senge and Senger (1991) in three species of green microalgae, and Hermesmeier et al. (1991) in *Scenedesmus obliquus*. However, Barro et al. (1989) found that soybeans grown in blue or white light had less chl *a* than those grown in red light. Thus, there appears to be a lack of consensus on this point. The significantly greater cell volume and carbon quotas of blue light cells may be consistent with the general tendency of cells growing at higher light-limited rates to have higher volumes and carbon quotas (see Chapter 3), since blue light cells also grew faster.

The finding that cells grown in blue light grew significantly faster than cells grown on equal quanta of other light spectra is in good agreement with the findings of Wallen and Geen (1971) where growth of *D. tertiolecta* and *T. pseudonana* was about 20% greater on blue versus white light, but it contrasts with studies by Rivkin (1989) and Grotjohann et al. (1992) where no differences were found. One reason for these differences may be the irradiance level used. For *T. pseudonana*, irradiance in the present study was 45 μmol quanta m⁻¹ s⁻¹, which is probably not saturating for growth (see Fig. 1.3, Chapter 1). Although it is difficult to compared since Wallen and Geen (1971) measured irradiance in energy versus quantum units, using approximate conversions in Parsons et al. (1984b) given irradiances of 30-40 μmol quanta m⁻¹ s⁻¹. Rivkin (1989) grew some of his cultures at saturating irradiance (120 μmol quanta m⁻¹ s⁻¹), but he also provides data from much lower irradiances (12 and 40 μmol
quantum m\(^{-1}\) s\(^{-1}\)); the trends in growth rate at low irradiance are no different from those at high irradiance. However, the possibility remains that the effect is species specific. Such growth rate differences may be reflected in other metabolic rates. In macroalgae, blue light has been found to increase photosynthetic rates by causing a surface acidification of plants leading to increases in CO\(_2\) transport and thus photosynthesis (Forster and Dring 1992, Schmid and Dring 1993). It is uncertain whether such a mechanism operates, or would be useful in a unicellular organism. Photosynthesis has also been shown to increase under blue light in microalgae (Wallen and Geen 1971, Senge and Senger 1991). In addition, blue light appears to enhance rates of cell respiration (Kowallik et al. 1990), although this has not been found in all cases (Wallen and Geen 1971). It has been proposed that the lower carbohydrate quotas sometimes seen in blue light-grown cells are due to these increased respiration rates, which are in turn the result of increased glycolytic activity. Increased glycolytic activity may be due to light activation of enzymes such as phosphofructokinase (PFK), as has been demonstrated in *Chlorella kessleri* (Grotjohann and Kowallik 1989, Kowallik and Grotjohann 1988).

NR activity correlated very well with calculated incorporation rates, except in red light grown cells, where NR was too low to account fully for observed rates. The reason for the discrepancy is unknown, but it may relate to the absence of blue light; there are indications that blue light has particular effects on NR. Duke and Duke (1984) and Ninneman (1987) have reviewed the specific effects of blue light on NR. These effects appear to involve increases in NR synthesis and are mediated by phytochrome (especially at low irradiance), or by flavin (at higher light). In green algae, where a cyanide-based inactivation mechanism has been demonstrated, blue light appears to reverse this inactivation (Solomonson and Barber 1990). Azaña and Apparicio (1983) proposed that the NR activation had to do with balancing cellular redox levels under higher energy blue light. In this scheme, NR would have a secondary non-assimilatory role in using NADH, a process which would be reflected in nitrite excretion from the cell. Work with the green alga *Monoraphidium braunii* has established that: a) blue light activation of NR is connected with the cyanide inactivation mechanism in this species and apparently involves flavin (Navarro et al. 1991), b) that activation of nitrate
and nitrite transport also occurs (Aparicio and Quinones 1991), and e) that effects similar to those of blue light can be seen under low CO₂ availability (Quinones and Aparicio 1990). Low CO₂ availability would also imply that reductant could not be used to fix carbon, and thus NR activity increases could be used to control redox levels under these conditions. Since no evidence of a cyanide-inactivation mechanism was apparent in diatoms (see Chapter 2), this may not be a feature of NR activity in the present study. The blue light induction of NR in the green macroalga *Ulva rigida* has been shown to be dependent on photosynthesis (Corzo and Neill 1992b) raising the possibility that it is a response to increased growth rate and therefore nitrate incorporation rates. In any case, blue light does not affect the close relationship between NR activity and incorporation rate. It is unlikely that the disagreement between NR in red-light-grown cells and nitrate incorporation rates poses a serious problem in the field, since it is difficult to envision a set of circumstances where cells would be exposed to red light alone.

**Effects of nitrate exhaustion**

As was the case for *T. pseudonana* grown on light:dark cycles, fluorescence and cell numbers in the cultures moving into stationary phase did not agree. There was a gradual decline in fluorescence per cell until a minimum was reached, and this did not correspond to a change in chl *a*. Thus, the usefulness of fluorescence as a biomass indicator in other than log phase cultures is questionable.

The lack of increase in pH suggests that carbon dioxide did not become limiting to the growth of the cultures; had this occurred, pH would have risen as CO₂ levels were reduced and the carbonate equilibrium shifted (Riley and Chester 1971). The nutrient data showed that at the onset of stationary phase, first nitrate and then silicate were exhausted.

In terms of cell composition, the increase in C:N ratio was a clear indication of nitrogen starvation, and has been noted in several species of diatoms (Dortch *et al.* 1984), and in *Phaeodactylum tricornutum* cultures (Syrett *et al.* 1986). In the case of the *T. pseudonana*, the increase of C:N ratio found by Dortch *et al.* (1984) was from 8.3 to 18, almost exactly the
same as in the present study. The ratio was driven largely by an increase in carbon quota. As previously suggested (Chapter 3), this is indicative of cells which are still actively photosynthesizing, but can fix no more nitrogen (see also Syrett 1981, Davidson et al. 1993). Interestingly, however, comparable changes in cell volume were not seen. This may be an acclimation to nutrient stress, as discussed in Chapter 2. Hersey and Swift (1976) noted slight decreases in protein on nitrate exhaustion in two dinoflagellate species, and a similar result has also been found in *Chlamydomonas reinhardtii* (Watt et al. 1992). Protein data were not collected in the present study, but a decrease in nitrogen quota was seen which would be consistent with a decrease in protein. Decreases in nitrogen quota on starvation for nitrate were also seen in *T. pseudonana* by Parslow et al. (1984), in several diatom species (Dortch et al. 1984), and in *Micromonas pusilla* upon nitrogen starvation (Cochlan and Harrison 1991b). In terms of chl *a*, little change was noted in the starving cultures. This was also true of nitrogen-starved *C. reinhardtii* cells (Watt et al. 1992). According to data reviewed by Syrett (1981), a decrease in chl *a* in nitrogen-starved cells would be expected. It is possible that this may have occurred over longer time periods than were used in the present study.

NR activity fell as stationary phase was reached, and this decline was comparable to declines in the rates of nitrate incorporation and nutrient depletion. Three patterns of NR activity in response to nitrogen starvation have been noted in the literature. In some cases, NR activity increases after nutrient exhaustion. This has been found in *Chlorella* (Kessler and Osterheld 1970), *C. reinhardtii* (Watt et al. 1992), cyanobacteria (Bednarz and Schmid 1992) and in a survey of six species of marine phytoplankton (Hipkin et al. 1983). In some cases, this increase even occurred in cultures that had previously been grown on ammonium and had shown no NR activity previously. It is unclear why this would occur, but suggestions range from a simple de-repression of nitrate reductase synthesis once ammonium is removed, to the presence of oxidative pathways within the cell that provide nitrate in the absence of a nitrogen supply (Funkhouser and Garay 1981, Watt et al. 1992). A second pattern of response to nitrogen starvation is a slight increase in NR activity in the first hours of nitrate exhaustion,
followed by a decline. This has been observed in *Chaetoceros affinis* (Slawyk and Rodier 1986), and *P. tricornutum* (Syrett and Peplinska 1988). In the present study, this may have happened, but daily sampling would not detect such a short term change. Many cells develop a "rapid uptake" ability in this time period (see Hipkin et al. 1983, Slawyk and Rodier 1986, Cochlan and Harrison 1991b, Dortch et al. 1991a, Martinez 1991). An increase in NR activity may be part of this response, however, Parslow et al. (1984) reported that development of nitrate uptake in *T. pseudonana* took 24 to 48 h after nitrate exhaustion, so the time scales of these two processes may not match. Parslow et al. (1984) also report nitrite excretion into the medium following nitrate exhaustion, but this was not observed in the present study. Clayton (1986) also documents a high degree of cellular reprocessing of nitrogen after nitrate exhaustion in *S. costatum*, in which NR might play some role. A third pattern observed is a constant decline in NR activity after nitrogen depletion. This can be a gradual process that happens on a longer time scale than the cessation of nitrate uptake, as seen in *Gonyaulax polyedra* (Harrison 1976), or in natural marine phytoplankton populations (Eppley et al. 1969), or it may be more rapid, and correspond to decreases in uptake rates as seen in *Chlorella vulgaris* by Morris and Syrett (1965), two dinoflagellates by Hersey and Swift (1976), or *Chlorella sorokiniana* (Tischner and Lorenzen 1980). The decrease in NR activity is thought to be the result of enzyme degradation (Syrett 1981). Hersey and Swift (1976) hypothesized that NR might be less stable in the absence of nitrate and so be susceptible to degradation. In a cyanobacterium, Hererro et al. (1984) suggested that there were two stages to NR degradation: an oxidation of the enzyme in the absence of nitrate, followed by protease attack. They noted that this was occasionally accompanied by an actual short term increase in NR synthesis. Aside from Morris and Syrett (1965) and Hersey and Swift (1976), NR activity in cultures depleting nitrate has usually not been well correlated with decreases in nitrate incorporation rates. As previously noted, however, in the case of Morris and Syrett (1965), NR activity was only sufficient to account for 10-12% of the actual incorporation. In the present study, declines in incorporation and NR activity are closely and quantitatively matched.
Effects of ammonium and ammonium pulsing

In general, the ammonium pulsed cultures behaved exactly as the nitrate grown cultures in all respects. It is possible that the ammonium pulses were too low to have any effect, since there appears to be a threshold for inhibition effects (Syrett 1981, Dortch 1990). At the growth rates observed, ammonium would have been taken up within 2-3 h after the addition.

Ammonium grown cells had larger cell volumes and carbon quotas. Since ammonium grown cultures also grew more quickly, these increases in cell volume and carbon quotas may be a reflection of increased growth rate, as previously discussed (Chapter 3). Nitrogen quotas and C:chl a ratios were no different. These results are identical to those found for ammonium-grown versus nitrate-grown cultures of *T. pseudonana*, grown at saturating irradiance by Thompson et al. (1989). However, Thompson et al. (1989) also found higher C:N ratios and higher chl a quotas at light saturation in ammonium grown cells, results that were not observed in the present study. In contrast, Darley (1977) summarized data from the diatom *Ditylum brightwellii* showing that nitrate grown cells contained more carbon, nitrogen and lipid than ammonium grown cells, although growth conditions in the majority of these experiments were not well documented. Paasche (1971) reported higher protein quotas in ammonium- versus nitrate-grown *Dunaliella tertiolecta*, but although numerically larger protein quotas were seen in ammonium-grown cells than in those grown on nitrate in the present study, they were not significantly different. In higher plants, increases in protein content when grown on ammonium has also been found (Barraro et al. 1989). Flynn (1990) suggests that nitrate grown cells are more nitrogen-stressed than those grown on ammonium. The data from the present study suggests that nitrate grown cells do share some of the characteristics of nitrate-starved cells, when compared with ammonium-grown cells.

Ammonium grown cultures also grew faster than nitrate or pulsed cultures. This was also found by Paasche (1971) in *Dunaliella tertiolecta*, and Thompson et al. (1989) in *T. pseudonana*, and is consistent with the idea that because ammonium is a more reduced form of nitrogen, it is therefore less energetically costly to grow on than nitrate (Syrett 1981).
However, Thompson et al. (1989) found that growth rate differences were only seen at saturating irradiance, and in the present study irradiance was close to, but probably not saturating. Arguments about energy advantages of ammonium over nitrate should not hold at high light when energy is no longer limiting. Thompson et al. hypothesized that there may be competition between photosynthesis and nutrient uptake for reductant within the cells, which could result in slower growth and perhaps larger carbon quotas if photosynthesis were more successful at using reductant. NR activities were very close to nitrate use rates and calculated rates of incorporation in both nitrate and ammonium pulsed cultures. The ammonium pulses appeared to have little effect. In one ammonium culture (NH4-1), where ammonium did not become depleted, no NR activity was observed. This is consistent with the vast majority of the literature that suggests that ammonium completely inhibits NR activity above 1-2 μM (see Syrett 1981, 1989). However, Zehr et al. (1989) reported that cultures of *T. pseudonana* and *Dunaliella tertiolecta* grown on ammonium were able to take up and reduce nitrate, which suggested that these species possess a constitutive for of NR. Zehr et al. (1989) speculated that they were able to detect nitrate reduction where other studies had not because their method used the radioisotope $^{13}$N, as opposed to less sensitive assays for NR activity. In the other culture (NH4-2), ammonium was exhausted and significant NR activity was found. Larsson et al. (1985) found similar results in *Scenedesmus obtusiusculus*: after ammonium exhaustion NR activity rapidly appeared. However, in this case, there was also excess nitrate in the medium. Alternatively, Morris and Syrett (1965) and Zeiler and Solomonson (1989) found that in *Chlorella vulgaris*, an increase in NR activity followed ammonium exhaustion even when there was no nitrate present. Solomonson and Barber (1990) concluded, based on several species, that nitrate may not be necessary to induce synthesis of NR, but that removal of ammonium repression is sufficient.

Although not considered in the present study, many other authors have investigated the effects of ammonium additions to cultures growing on nitrate. It appears that in the short term, ammonium may shut down nitrate uptake before it has an effect on NR activity. Pistorius et al. (1979) found that in *Chlorella*, nitrate uptake ceased within 5 min of the
ammonium addition, but NR was still active up to 60 min later. Similar results have been found by Tischner and Lorenzen (1979), and for higher plants as well (see Ingemarsson (1987), Lee and Drew 1989). Serra et al. (1978a) working with *S. costatum*, showed that the decrease in NR activity was not simply an arrest of enzyme synthesis; additions of cyclohexamide to block protein synthesis did not show the same degree of inhibition. Alternatively, Hersey and Swift (1976) found that the decrease in NR was very well correlated with loss of nitrate uptake ability in two dinoflagellates pulsed with ammonium. In other cases, the situation is not so clear. Larsson et al. (1985) found a rapid cessation of nitrate uptake, but a slow decrease in NR activity in *Scenedesmus obtusiusculus* only when cultures were bubbled with CO₂. When cultures were bubbled with air instead, NR activity and nitrate uptake closely paralleled one another. No satisfactory explanation could be found, but Collos (1989) also reported that ammonium inhibition was more severe in CO₂-bubbled versus air-bubbled cultures. Flynn (1990) has proposed that an interaction with carbon, as previously discussed (Chapter 3) may be involved. Furthermore, as McCarthy (1981) points out, simultaneous use of nitrate and ammonium has been noted in many cases, including dinoflagellates (Harrison 1976). In the macroalga *Ulva rigida*, ammonium did not eliminate nitrate uptake or NR activity even when it was supplied at 3-4 times the concentration of nitrate (Corzo and Neill 1991). Tischner and Lorenzen (1979) reported that ammonium additions could actually induce NR activity in *Chlorella sorokiniana*. There are even reports of nitrate inhibition of ammonium uptake (see Collos and Slawyk 1980), and nitrate preference over ammonium (Proctor 1957, Dortch 1990). Although it has been held that ammonium concentrations greater than 1-2 μM will inhibit nitrate uptake (see e.g. Packard and Blasco 1974, Syrett 1989), Dortch (1990) extensively reviewed the field data from marine environments and concluded that the evidence did not support this point of view. Moreover, the distinction between "inhibition" and "preference" has rarely been adequately assessed because the uptake rates with either ammonium or nitrate alone and both together must be measured. True cases of ammonium inhibition (i.e. a specific direct effect on nitrate uptake
versus indirect effects of ammonium preference) were most often seen at low light, or when nitrogen was sufficient (Dortch 1990).

It appears that the inhibition of nitrate incorporation by ammonium (whether at the level of uptake or reduction) is reflected in reduction in NR activity, although inhibition of nitrate uptake can occur more rapidly than inhibition of NR activity. Changes in NR activity are likely mediated by changes in enzyme protein at longer time scales, but perhaps by an inactivation mechanism at scales of minutes to hours. Although at short time scales nitrate-uptake inactivation mechanisms may operate, which would be poorly reflected in NR activity, most measurements in the field are on much longer time scales. Thus, even under these circumstances, NR activity may be a useful index of nitrate incorporation rates.

**Implications of regulatory mechanisms**

There is clear evidence that NR activity is regulated by more than one mechanism. There is the temptation to assume that all of these mechanisms are important and that regulation must necessarily be more complicated than it appears. However, as pointed out by Ottaway (1988), many enzyme regulatory mechanisms may be redundant (i.e. a "belt and braces" situation), or they may be part of a "fossil record" of the way the enzyme was controlled at different stages in evolution. For nitrate reductase, an enzyme which may have evolved for using nitrate as a terminal electron acceptor before becoming involved in assimilatory pathways, and especially if NR retains additional functions, these regulatory considerations are legitimate concerns.

In summary, despite temporal changes in nitrate incorporation rates caused by periodicity in irradiance and nitrate starvation, NR activity closely followed these changes, at least on a scale of hours to days. This suggest that NR activity could be useful in natural environments with similar scales of variability. Different light spectra did not alter the relationship between nitrate incorporation and NR activity, except under red light, which is a condition unlikely to be found in ocean environments. Finally, although ammonium does inhibit NR activity, the inhibition appears to be instep with changes in nitrate uptake. Pulses
of ammonium, which may be common in the marine environment (see Goldman 1986), do not appear to influence the relationship either. Based on the results of these experiments, it appears that in the majority of cases, NR activities can adequately predict rates of nitrate incorporation.
CHAPTER 5: ACTIVITY AND CHARACTERISTICS OF NITRATE REDUCTASE IN NATURAL PHYTOPLANKTON POPULATIONS FROM MONTEREY BAY, CALIFORNIA

INTRODUCTION

In previous chapters, work has focused on nitrate reductase activity in phytoplankton mono-cultures. With some confidence in the NR assay (Chapter 2), and the relationship between NR activity under steady-state and non-steady state conditions (Chapters 3 and 4), the results of studies in which the NR assay was applied to field situations are described in this chapter. The question of where to try NR assays in the marine environment deserves careful consideration. Ideally, a prime location would have relatively few species, and preferably diatoms, since there is interspecific variability in the NR assay, and the best relationships between NR activity and nitrate incorporation rates were found in diatoms. The biomass and growth rates should both be high to permit the greatest analytical sensitivity. Furthermore, high nitrate and low ammonium concentrations would simplify experiments at this stage. Coastal upwelling zones, and the California current upwelling system in particular, as discussed below, meet these criteria very well.

Characteristics of coastal upwelling zones

The general physical mechanisms of upwelling have been well described (Boje and Tomczak 1978, Codispoti 1983, Parsons et al. 1984b, Valiela 1984, Mann and Lazier 1991). Coastal upwellings arise due to an interaction of winds, Coriolis force and coastal morphology. The California coast provides an excellent example. Wind circulation patterns in summer are from north to south, that is, along the coast. Due to Coriolis and frictional forces, an Ekman spiral develops; as depth increases, ocean water is displaced at an angle to the wind direction, i.e. offshore (Pond and Pickard 1978, Mann and Lazier 1991). As a result, surface water is moved offshore which results in a change in barotropic forces and results in deep off-shore water being forced up along the coast (Codispoti 1983). This is not simply a dilution of
surface waters, as in other types of upwelling, but an actual replacement and can be recognized by a decrease in surface temperatures. Typically, waters rise from depths less than 250 m, and are confined to relatively narrow coastal regions, on the order of 25 km, although the biological effects of upwelling may influence a much wider region. In a review of the California current system, Bernal and McGowan (1981) showed that based on over 20 years of data, the area of nitrate enrichment is up to 300 km wide. Upwelling intensities are generally on the order of $10^{-3}$ cm s$^{-1}$, but vertical velocities of $10^{-2}$ to $10^{-1}$ cm s$^{-1}$ have been measured. In temperate zone upwellings, water is colder and is rich in nutrients; often nitrate levels of $>25 \mu$M are measured. Ammonium and nitrite levels are usually low ($<2 \mu$M), although in specific cases they may be higher where there are areas deficient in oxygen (e.g. in the Peru upwelling, Codispoti and Packard 1980), or where turbid waters lead to reduced photosynthesis and higher grazing and ammonium regeneration rates (e.g. Whitledge 1981). Because of the variability in wind intensity and direction, there is a very high degree of spatial and temporal variability in upwelling, which makes such regions very difficult to study. Furthermore, in addition to the coastal upwelling itself, there is evidence that cyclonic eddies can form in the California current system, which result in localized upwellings (see Pond and Pickard 1978) and increased spatial variability (Traganza et al. 1981). Other localized phenomena include shear-induced turbulence, and island and seamount effects that cause vertical mixing (Bernal and McGowan 1981). The importance of smaller scale upwellings has only been recognized since synoptic coverage using satellite images have been available (e.g. Traganza et al. 1981).

Upwellings may be particularly important in global nitrogen and carbon cycles. Since nitrate is high and ammonium is low, new production (i.e. the primary production based on nitrate, see Introduction and Dugdale and Goering 1967) is high, with f-ratios on the order of 0.7 to 0.75 (Eppley and Peterson 1979). Furthermore, they are critical regions for world fisheries. The abundance of large diatoms (see Semina 1968, Parsons and Takahashi 1973, Hecky and Kilham 1974, Guillard and Kilham 1977), and the spatial variability, which prevents grazing zooplankton populations from controlling primary producers, may result in
shorter food chains where energy transfer to higher trophic levels (e.g. fish) is very large (Ryther 1969).

In biological terms, upwelling areas show distinct characteristics. Margalef (1967) first described in detail the pattern of species succession. MacIsaac et al. (1985) modeled this pattern by dividing upwelling regions into four zones, each further from the upwelling centre. In newly upwelled water (zone 1), nutrients are high, but phytoplankton biomass and growth rates remain low for some period after reaching the surface. Factors such as metal availability or toxicity are thought to play a role in this delayed response of cells to increased light (see Sunda et al. 1981). In the second zone, cell division rates and photosynthetic rates have increased dramatically. The species found in this zone are principally diatoms, particularly *Chaetoceros, Thalassiosira, and Skeletonema* species (see also Guillard and Kilham 1977 for an excellent review of the particular species present in different geographic regions). In zone 3, nutrients begin to become depleted, particularly nitrogen, although there is also evidence that silicate may become limiting (see Dugdale 1972). The number of species increases, but the abundance of individual species falls by a factor of 10-100. At this stage, large chain-forming diatom species dominate (e.g. *Chaetoceros*). In the last zone, nutrients are very low, biomass decreases and growth rates are again low. Diatoms decline in importance and are replaced with large motile dinoflagellates, although some diatom species persist (e.g. *Rhizosolenia, Hemiaulus* and *Mastogloia*).

Biological adaptation rate is thought to play a significant role in these systems. Wilkerson and Dugdale (1987) advanced a conceptual "conveyor belt" model, represented in Figure 5.1. In this model, the ability of species to "shift-up" (i.e. increase their specific rates of nutrient uptake and growth) from previously limiting conditions on exposure to resources is a critical parameter. Cells from deep water have abundant nutrients, but are light-limited. They undergo a shift-
Environmental conditions such as low light or low nutrients cause a decrease in the rates of phytoplankton physiological processes ("shift-down"), while high light and high nutrients cause an increase in rate processes ("shift-up").

Figure 5.1. Diagram of phytoplankton processes in a coastal upwelling zone (after Wilkerson and Dugdale 1987).
up as light increases after upwelling, then face a "shift-down" (i.e. a decrease in rates of nutrient uptake and growth) as nutrients are depleted. After this, cells also undergo a second shift-down in light as they sink from the euphotic zone (Fig. 5.1). The shift-up sequence has been studied off Point Conception, California, where the entire cycle is completed in 5 to 7 days (e.g. Dugdale and Wilkerson 1989). Using the stable isotope $^{15}$N as a tracer, biomass-specific nitrate uptake rates were at first low, but increased dramatically after upwelling. Carbon fixation rates followed nitrogen uptake increases but only after a slight time lag. The phenomenon has also been demonstrated off the coast of Peru (e.g. MacIsaac et al. 1985), and Washington and Oregon (e.g. Dortch and Postel 1985, Kokkinakis and Wheeler 1987). However, Garside (1991) demonstrated, using a simple model, that the shift-up phenomenon could also occur simply because uptake rates are normalized to particulate nitrogen (note that Dugdale and Wilkerson (1991) argue that this does not happen in practice; chl $a$-specific rates show the same pattern).

The high spatial and temporal variability of upwelling zones constitute a serious disadvantage for study; repeated monitoring of populations over time is difficult. One solution is to mark discrete parcels of waters ("drogues") with drifter buoys, and follow them for some period of time (e.g. Wilkerson and Dugdale 1987). However, such an approach is rarely convenient, and some exchange with surrounding waters is inevitable. As an alternative, samples can be collected and maintained on deck under suitable temperature and light conditions (e.g. Wilkerson and Dugdale 1987). Not only does this ensure that the same phytoplankton assemblage is being sampled, but it makes repeated, frequent sampling very simple. There are dangers that natural populations may respond differently when they are contained than they do in situ (see the General Introduction), but it has previously been shown that the trends in populations that have been contained follow those of cells in tracked drogues reasonably well (Wilkerson and Dugdale 1987).

In this chapter, a field study was conducted in the upwelling region of Monterey Bay, California to test how well the newly modified NR assay (Chapter 2) worked in the field with a natural phytoplankton assemblage. This was achieved by comparing rates of nitrate uptake
and incorporation with NR activity in natural populations under near-natural conditions. Characteristics of NR in these populations, and the effects of diel periodicity and ammonium additions were also examined.

**MATERIALS AND METHODS**

Data were collected aboard the R.V. *Point Sur* during May 1993, in conjunction with the second cruise in the Shift-Up-93 program. Samples for assay optimization and NR activity characterization were taken during an initial survey off the coast of California in Monterey Bay (Fig. 5.2). Sampling for time series experiments was conducted on 11 May, 1993 at Station 41 (36° 47.77' N, 121° 54.75' W), indicated by the cross in Figure 5.2. At each sampling site, vertical profiles of temperature, salinity, and *in vivo* chlorophyll *a* fluorescence were made using a SeaBird CTD probe. Ten L Niskin water bottles equipped with silicone rubber springs and fittings were used to sample water from the 50% light penetration depth (usually 3 m).

**Modifications to NR assays**

The constraints of ship-board equipment and the low biomass relative to laboratory cultures made changes to the previous NR assay necessary. Filtration and homogenization were performed as described before, and the same extraction buffer was used (Chapter 2). A refrigerated centrifuge was not available, and thus, homogenates were used directly. As a result, the homogenates contained glass fibres from the filters. Homogenate volumes used in assays were corrected for the volume occupied by the filter fibres (see below). Time-stopped assays measuring nitrite production were performed as described in Chapter 2, and samples were incubated at the *in situ* temperature in flowing seawater incubators on deck.

**Assay validation and enzyme characterization**

In order to determine whether the use of uncentrifuged homogenates made a difference, a preliminary experiment was conducted. The stainless steel rotor tubes of a clinical
Figure 5.2. Study site, Monterey Bay, California. The map on the left shows where Monterey Bay (surrounded by box) lies in relation to the coast of California. The "X" symbol marks the site of the major sampling location.
centrifuge, and 15 ml glass centrifuge tubes were held on ice until immediately before use. Six 500 ml samples were filtered and homogenized. Three were used directly for assays, while three were centrifuged for 5 min at full speed, and the supernatant then used in NR assays. Temperature in the chilled tubes remained below 4°C during this procedure. Since the homogenates of the uncentrifuged samples contained glass fibres from filters, a volume correction for these samples was necessary. The volume of glass fibres was estimated from the scale on the glass centrifuge tubes in the three centrifuged homogenates, and averaged 0.2 ml for a filtered sample homogenized in 1 ml of extraction buffer. The activities in centrifuged and uncentrifuged samples were compared using Student t-tests (Steel and Torrie 1980).

For subsequent experiments, assays were conducted on uncentrifuged homogenates. As in Chapter 2, the effect of different substrates and activators of NR were verified. Three separate homogenates were prepared and assayed with: a) 0.2 mM NADPH in place of NADH, b) with addition of 0.1 mM flavin adenine dinucleotide (FAD), or c) after incubation with 0.2 mM ferricyanide (FeCN). Results were compared using a one-way ANOVA procedure, followed by Tukey's multiple comparison technique at the 95% confidence level.

To verify linearity of the assay with time, replicate samples from a single homogenate were assayed for periods of 0, 15 or 45 min. Nitrite produced was plotted against the incubation length and the data was analyzed by linear regression. To test linearity of the assay with the amount of homogenate added, NR activity was measured in samples with between 0 and 800 µl of homogenate added. Again, results were analyzed using linear regression. In subsequent experiments, 30 min incubations were used and homogenate additions were 500 µl.

Enzyme kinetics were studied by performing assays of NR activity in replicate samples from single homogenates, and varying NADH concentration (0-0.4 mM) or KNO₃ concentration (0-20 mM). Enzyme kinetic constants were estimated as before (see Chapter 2, Appendix C).

To investigate possible degradation of NR activity with time, three individual samples were assayed immediately after homogenization, and at 15, 30 and 60 min after
homogenization. NR activities were compared using a one-way repeated measures ANOVA, followed by Tukey's multiple comparison technique at the 95% confidence level.

**Containment experiments**

At Station 41, samples were collected from the 50% light penetration depth and placed into four acid-cleaned 20 L polyethylene containers (LMG Reliance). Two of the containers received no additions (control). The other two received additions of ammonium chloride to bring ambient concentrations up to 5 µM.

Initial samples (t = 0 h) were taken directly from the Niskin sample bottles. Containers were placed in a seawater-cooled deck incubator and irradiance was adjusted to 50% of surface irradiance using neutral density screening. Sampling was repeated at approximately 4 h intervals for 32 h.

At each time, samples for nutrient analyses (nitrate and ammonium), particulate nitrogen, and chl a were taken. Ammonium samples were analyzed by hand within 24 h using the method of Parsons et al. (1984a) and measuring samples in a 10 cm cuvette in a Hewlett Packard model 8452A spectrophotometer. A 50°C water bath was used to accelerate colour development. Samples for nitrate were frozen for later analysis using a Technicon AutoAnalyzer II (see Freiderich and Whitlege 1972). Single 460 ml samples were taken from each container and NR assays performed within 1 h. Nitrate uptake experiments were performed with samples collected in 280 ml polycarbonate bottles. Incubations for 15N uptake experiments were started by adding 2.0 µM 15N-labeled nitrate (99 atom %, Cambridge Isotope Laboratories). Enriched samples were placed in deck incubators for 4-5 h. Incubations were terminated by filtration onto 25 mm pre-combusted GF/F filters, and frozen for later analyses. Dried filters were analyzed for 15N enrichment and particulate nitrogen (PN) content with a Europa Scientific RoboPrep Tracermass mass spectrometer. Particulate nitrogen-specific uptake rates ($V_{NO3}$) were estimated according to Dugdale and Wilkerson (1986). NR activity was normalized to particulate nitrogen, and particulate nitrogen-specific
rates of nitrate disappearance (Δ NO₃⁻) and increase in particulate nitrogen (ΔPN) were also calculated.

As well, samples for species determination were taken before and after the experiment, preserved in 2% formalin, and qualitatively examined to determine the dominant taxa.

**RESULTS**

The cruise took place during an upwelling event; there were high winds for the first two days, then conditions calmed. Figure 5.3 A shows a vertical profile of the water column typical of the early part of the cruise. Thermal stratification was weak and the water column was relatively well mixed. There was a small peak in biomass, indicated by *in vivo* fluorescence, near 10 m. By the sixth day, there was evidence of stratification (i.e. warmer, less dense water near the surface) and structure in both salinity and temperature profiles (Fig. 5.3 B). The fluorescence peak was greater, and had deepened to almost 20 m, indicating an increase in phytoplankton. Microscopic examination showed that the species in this bloom were predominantly diatoms, with approximately 80% of cells being (in order of abundance) *Chaetoceros, Rhizosolenia, Skeletonema,* and *Nitzschia* spp.

**Assay validation and enzyme characterization**

NR activity in uncentrifuged homogenates was not different from activity in samples that had been centrifuged (Fig. 5.4 A, P > 0.5). NR activity using NADPH was approximately 15% of the activity with NADH as electron donor (Fig. 5.4 B). FAD additions gave numerically higher NR activity, but this was not significantly greater than NR activity without FAD (Fig. 5.4 B, P > 0.2). On the other hand, FeCN addition gave significantly lower NR activity (Fig. 5.4 B, P < 0.001).

In the NR assay, nitrite production was linear up to at least 45 min (Fig. 5.5 A). Homogenate additions of up to 400 µl gave linear results, but there was evidence of non-linearity at additions of 800 µl (Fig. 5.5 B).
Figure 5.3. Profiles of temperature (•-----), salinity (——) and relative fluorescence (-----) at sampling sites in the vicinity of the main sampling station (indicated by the "X" symbol on Fig. 5.2) in Monterey Bay, CA. Profiles were taken: A) on day 3, early in the bloom, and B) on day 6 at the height of the bloom.
Figure 5.4. Effects of different assay conditions on nitrate reductase activity in natural phytoplankton populations sampled from Monterey Bay, CA.

A) NR activity in homogenates used directly, or centrifuged to remove filter fibres. B) Effects of additions of NADPH in place of NADH, 0.1 mM FAD, or 0.2 mM ferricyanide (FeCN). Each bar represents the mean of three replicate homogenates. Error bars represent standard errors of the mean.
Figure 5.5. NR assay validation in natural phytoplankton samples taken from Monterey Bay, CA. A) Linearity of assay with time. B) Linearity of assay with homogenate addition. Lines represent least squares regression fits to the data. Note in B) the open point (○) is not included in the regression. Each point represents a single determination.
Enzyme kinetic analyses showed inhibition of NR activity at NADH levels of 0.4 mM, and also lower NR activity at 0.2 mM levels (Fig. 5.6 A). K_m values for NADH were 0.021 mM, whether calculated with or without the 0.2 mM point. For KNO_3, a K_m of 0.307 mM was calculated (Fig. 5.6 B).

NR activity had significantly declined by 30 min after homogenization (Fig. 5.7). Since the assay itself took 30 min, the resolution of this decline was poor. No difference was found between the initial NR activity and activity in assays performed after 15 min (P > 0.5).

**Containment experiments**

Rates of increase of chl a and particulate N were identical in control and ammonium-spiked containers over the 36 h of the experiment (Fig. 5.8 A, B). Growth rates (μ) were estimated at 0.82 d^{-1} and were the same whether based on increases in chl a or particulate N. Over the same time period, nitrate concentrations decreased in both sets of containers, but nitrate fell to lower concentrations in control containers (Fig. 5.8 C). Ammonium gradually increased with time in control containers, reaching 0.45 μM by 36 h (Fig. 5.8 D). Ammonium declined steadily in containers with ammonium added until 28 h when concentrations were no different from control containers.

NR activity showed a diel periodicity in both control and ammonium-spiked containers, but because ammonium also had an effect on NR activity, the trend was clearest in control containers (Fig. 5.9 A, B). Activity was low at the beginning of the light period, rose to a peak and then declined by the beginning of the dark period (Fig. 5.9 A). Towards the end of the dark period, NR activity increased, but fell once again by the first sampling of the next light period.

NR activity in ammonium-spiked containers decreased relative to activity in control containers. With the exception of the 8 h sampling time when NR activity was not different from the control, NR activity in the ammonium-spiked containers was always lower than controls, and declined continuously to undetectable levels by 20 h (Fig. 5.9 B). This
Figure 5.6. Kinetic curves for nitrate reductase activity in natural populations of phytoplankton from Monterey Bay, CA. A) NR versus NADH concentration, B) NR versus nitrate concentration. Curves are fit to rectangular hyperbolae. $K_m$ values are 0.021 mM for NADH and 0.307 mM for nitrate. Each point represents a single determination.
Figure 5.7. Nitrate reductase activity in natural phytoplankton populations assayed at different times after homogenization. Each point represents the mean of two enzyme assays from different homogenates. Error bars represent standard errors of mean values.
Figure 5.8. Changes in biomass and ambient nutrient concentrations in contained natural phytoplankton populations from Monterey Bay, CA for control cultures (●) and cultures with 5 μM ammonium added (○). A) Chlorophyll $a$, B) particulate nitrogen, C) nitrate, and D) ammonium. Each point represents the mean of two separate contained cultures. Error bars represent standard errors of the mean, or where absent, they are smaller than the symbols. Cultures were grown under natural light and the black bar on the time scale indicates the dark period.
Figure 5.9. Changes in nitrate reductase (NR) activity, and specific rates of nitrate incorporation calculated from changes in particulate nitrogen, changes in ambient nitrate concentration, or saturated uptake of $^{15}$NO$_3^-$ ($V_{NO_3^-}$) for contained natural populations of phytoplankton from Monterey Bay, CA. A) Control cultures without any nitrogen additions, and B) cultures with 5 μM ammonium added at $t = 0$ h. Symbols represent mean values of determinations in two separate cultures. Error bars represent standard errors of the mean, or where absent are smaller than the size of the symbols. Cultures were grown under natural light, and the black bar on the time scale indicates the dark period.
corresponded to the time when ammonium concentrations fell below 2 μM (Fig. 5.8 D). After this, NR activity increased and was not different from activity in control containers at the last two sampling times.

Diel patterns of ΔPN, ΔNO₃⁻, and VₕNO₃ were somewhat different from those found for NR activity in control containers. These rates were highest at the first sampling of the day and fell continuously during the light period (Fig. 5.9 A). There were increases in rates during the dark period, but activities tended to reach peaks at the beginning of the light versus the end of the dark period; the same pattern was observed in NR activity.

Estimates of nitrate incorporation rates (ΔPN, ΔNO₃⁻, and VₕNO₃) generally agreed well with each other in the control containers, although ΔNO₃⁻ rates did exceed other estimates between 20-30 h. In ammonium-spiked containers, different estimates of nitrate incorporation rates also agreed at most points in time. There was significant nitrate incorporation in the presence of ammonium until the 12 h sampling. ΔNO₃⁻ and VₕNO₃ were zero at 12 and 16 h samplings. ΔPN rates did not fall to zero, indicating that growth was being supported by ammonium incorporation over this period. After 16 h, when ammonium was still greater than 2 μM, rates of nitrate incorporation rose again.

NR activity was equal to or greater than nitrate incorporation rates in all but one case for control containers (Fig. 5.9 A), and in all but 2 cases for ammonium-spiked containers (neglecting ΔPN rates, Fig. 5.9 B). On average, NR activity was 254% of ΔPN rates, 285% of ΔNO₃⁻ rates, and 285% of VₕNO₃ rates in control containers, while in ammonium-spiked containers, NR activity averaged 139% of ΔPN rates, 166% of ΔNO₃⁻ rates, and 233% of VₕNO₃ rates. However at individual sampling times (e.g. t = 0 h), the rates agreed considerably better (Fig. 5.9 A). In fact, at the first sampling of each day (t = 0, t = 24 h, both occurring at 10: 00 local time), the NR activity agreed well with the other estimates.
DISCUSSION

Adequacy of the NR assay

NR activity and assay characteristics in natural populations showed many similarities to results using laboratory cultures. Use of uncentrifuged samples probably resulted in some loss of precision because the correction of homogenate volumes for the presence of filter fibres was somewhat crude, but despite this problem, assay results were no different whether homogenates were centrifuged or not. The assay was linear with time and linear with homogenate added for all but the highest additions. Since high concentrations of NADH and KNO3 were added, this non-linearity was unlikely to be the result of substrate depletion, but could be due to end product inhibition. Particularly in dense blooms, attention must be paid to this factor or NR assays will underestimate activity. Activity of homogenates held on ice declined in 30 min. This suggests that assays, which were conducted over 30 min, may have underestimated true NR activity. The magnitude of this error would be difficult to judge, but the fact that activities at 0 and 15 min were not different gives some confidence in the assay.

The \( K_m \) for NADH estimated in this chapter is very similar to that calculated for *Thalassiosira pseudonana* cultures in the present study and the majority of algal species examined by others (see Chapter 2 and Table 2.5). For nitrate, \( K_m \) values appear to be substantially higher than those found for *T. pseudonana* (Chapter 2), but closer to values found for *S. costatum* in culture in the present study, and by other researchers (see Chapter 2 and Table 2.5). NR kinetic constants have previously been determined in field populations, but only for nitrate. Packard and Blasco (1974) reported \( K_m \) values of 0.314 mM in a mixed population of *Gonyaulax polyedra* from an upwelling off Northwest Africa. This is close to the value found in the present study, but the Monterey Bay population was diatom-dominated. \( K_m \) values for nitrate in natural diatom-dominated populations averaged 0.082 (±0.052) mM, with a range of 0.042 to 0.201 mM (Packard and Blasco 1974). There is a possibility that intracellular nitrate may bias the calculation of kinetic constants, but in the present study, NR
activity in samples without added nitrate was no different from blanks, suggesting that this did not happen. In terms of the assay, 10 mM nitrate additions appear adequate, but there may be a problem with NADH levels used; 0.2 mM apparently caused some degree of inhibition. Inhibition of NR activity in certain species was seen previously at concentrations of NADH 0.4 mM or greater (see Chapter 2), but it may be that inhibition occurs at lower levels in different taxa. Based on data presented in Figure 5.6 A, the problem of NADH inhibition does not appear to be severe, but it is critical that this be verified in each field study. This has not been done in the past.

**Diel Periodicity**

There was strong evidence of diel periodicity in NR activity in control containers. The pattern was remarkably similar to that found for laboratory cultures of *T. pseudonana* in Chapter 4, despite the fact that sampling frequency in the field study was 4-5 h versus 3 h in the laboratory study. Diel periodicity has previously been investigated in natural populations. Packard and Blasco (1974) noted a 100% increase in NR activity just before dawn in natural populations from a California upwelling, and Epplle *et al.* (1970) found evidence of a midnight low in NR activity and a pre-dawn rise, but, as discussed in Chapter 4, these different patterns are likely due to different or irregular sampling times. For example, evaluating the data from Epplle *et al.* (1970) more closely, there is some evidence of a noon peak in activity as well as the pre-dawn rise, but the sampling was intermittent, with gaps of more than 6 h. Manasneh and Basson (1987) found that diel periodicity in natural populations in the Red Sea could be accounted for purely on the basis of biomass changes, evidently coupled to diel vertical migration of dinoflagellates. This was not the case in the present study (note that NR activity in Figure 5.9 is normalized to particulate N). Packard *et al.* (1971a) also found that diel periodicity in populations from upwelling areas did not correlate with biomass. Irregular sampling cannot be invoked as an explanation for the pattern seen by Martinez *et al.* (1987). In this study, samples were collected every 1-2 h over a diel cycle, but
no pre-dawn rise in NR activity was seen, and a double peak in activity was found on either side of solar noon.

Collos and Slawyk (1976) showed that diel periodicity in NR activity was well correlated with internal nitrate concentration, and nitrate uptake, although these were greater in magnitude than NR activity. Diel periodicity in nitrate uptake and incorporation were also found in the present study. The patterns were somewhat different from those found for NR activity, but comparisons are difficult; NR activity is based on an assay at a single point in time, while the other rates are averages of 4-5 h periods.

The diel pattern was less obvious in ammonium-spiked containers. Collos and Lewin (1974) could not detect a diel periodicity in NR activity in populations of surf zone diatoms, and they attributed this to a diel periodicity in ammonium concentration which may have obscured the pattern of NR activity.

Effects of ammonium

Ammonium additions at the 5 μM level caused a decrease in nitrate incorporation rates; however, the effect was relatively minor compared to laboratory results (see Chapter 4); nitrate uptake rates in ammonium-spiked containers were only slightly lower than those in control containers after 4 h, and only at 12 h did rates drop to near zero. Furthermore, at 20 h nitrate uptake rates began to rise again, when there was still 2 μM ammonium present. These results re-enforce the conclusions of a review by Dortch (1990), and argue against the concept that nitrate uptake is always inhibited by ammonium in natural populations (Syrett 1981). As has been shown in many studies with higher plants, the responses of nitrate uptake to ammonium depend to a high degree on previous growth conditions and prior exposure to ammonium (Clarkson and Luttge 1991).

NR activity was apparently slower to respond to the introduction of ammonium than was the rate of nitrate uptake (see discussion in Chapter 4), but this was difficult to resolve due to the different time scales involved (i.e. the instantaneous enzyme measurement versus 4-5 h average uptake rates). Evidence of ammonium inhibition of NR in natural populations is
mostly limited to observational, rather than experimental data. For example, Packard and Blasco (1974) present data showing that a decline in NR activity correlated with a decline in nitrate uptake with increasing ammonium concentrations above 0.2 μM in samples from coastal Greece. However such correlations do not prove a causal relationship. Furthermore, as Dortch (1990) has pointed out, true ammonium inhibition of nitrate uptake (i.e. an indirect interaction in which decreases in nitrate uptake vary with ammonium concentration) cannot be distinguished from a simple preference for ammonium (i.e. a direct interaction in which decreases in nitrate uptake are independent of ammonium concentration) under these conditions.

NR activity and nitrate incorporation rates

Studies comparing NR activity and nitrate uptake or nitrate incorporation rates have generally shown reasonable correlations, but poor quantitative relationships. In most cases, NR activity is too low to account for observed nitrate incorporation rates. For example, Eppley et al. (1970) found good correlations between uptake and NR activity, but NR activity only accounted for an average of 15% of the nitrate uptake rates. Collos and Slawyk (1976) found reasonable correlations between uptake, incorporation and NR activity, but NR averaged only 12% of the other rate estimates. In other cases, results are less consistent. Collos and Lewin (1974) found NR activity was between 5 and 90% of nitrate uptake rates measured in surf zone diatoms. Working in freshwater lakes, Wynne and Berman (1990) demonstrated that NR activity ranged from 1 to 50% of nitrate uptake rates. In a study using large shipboard-contained populations, Wilkerson and Dugdale (1987) found that NR activity was almost an order of magnitude lower than rates determined by □N uptake, while Dortch and Postel (1989) showed that □N-nitrate uptake rates in coastal Washington and Oregon waters always exceeded NR activity by at least a factor of 2. The sampling location may also play a role. Blasco and Packard (1974) showed that in data from Californian and Northwest African upwellings, NR activity and uptake rates measured with □N were only well correlated in upper waters; the relationship became poorer with increasing depth.
Other authors have demonstrated correspondence between uptake and NR activity, but have also shown variability in the relationship. Collos and Slawyk (1977) found that the ratio of nitrate uptake to NR activity ranged from 0.03 to 10.8 in natural populations in the Costa Rica dome upwelling. They compared 12 h average rates of $^{15}$N uptake versus NR activity measured at noon, so the mis-match of time scales may be responsible for some of the variability. Collos and Slawyk (1977) also tried to correct for internal nitrate pools by estimating an in vivo reduction rate. This gave a range of nitrate uptake:NR activity ratios of 0.1 to 11.2, most of which were greater than 3 (i.e. NR activity only accounted for a third of observed nitrate uptake rates). Comparing $^{15}$N uptake to NR activity in hundreds of samples from Peru and California upwellings gave significant relationships (Blasco et al. 1984, MacIsaac et al. 1985). Furthermore, regressions analyses indicated that NR activity (on average) exceeded nitrate incorporation rates. This is encouraging, but the relationship only explained 44 to 69% of the variance, and NR activity only equaled or exceeded uptake rates in 10 of 23 cases. In contrast, in the present study, NR activity was almost always in excess of that needed to account for nitrate uptake and incorporation by a factor of 2, on average.

There are a number of possible explanations for the differences between NR activity and incorporation rates. As previously noted, the difference in time scale between NR activity and the other methods is a critical factor. Trying to determine uptake rates on shorter time scales would not be practical; differences in PN or nitrate concentrations would quickly approach limits of detection. However, NR assays could be performed more frequently (perhaps hourly or even half-hourly), allowing a better comparison to be made. Changes in ambient nitrate and $^{15}$N uptake may not be equivalent to NR activity if internal pools were being formed. However, if this were true, uptake rates would be expected to exceed NR activity instead of what was actually observed. It could be hypothesized that nitrate uptake was a limiting factor. However, given the facts that ambient nitrate in the present study was always high, and that half-saturation constants for nitrate uptake are very low (see Packard et al. 1979), this explanation seems less likely. Furthermore, several previous studies in
upwelling zones have demonstrated the formation of internal nitrate pools (e.g. Collos and Slawyk 1976), indicating that nitrate uptake exceeded nitrate incorporation.

In previous studies, a wide variety of other explanations have been considered based on the premise that rates of NR activity are incorrect. Most of these studies have been forced to reconcile lower NR activity with higher nitrate uptake rates. It has been proposed that NR is more a function of external nitrate concentration (Kristensen 1987), internal nitrate concentration (Collos and Slawyk 1976, Dortch and Postel 1989), or simply biomass (Manasneh and Basson 1987) than of nitrate incorporation rates. Others have argued that ammonium inhibition (Blasco et al. 1984) or light limitation (Blasco and Packard 1974) affect the relationship. Kristensen (1987) has also pointed out the high degree of species variability. From the results of the laboratory experiments (Chapters 2-4) and the diatom community found in the present field study, these possibilities appear to be less likely.

The effects of "nutrient prehistory" are often invoked (e.g. Wynne and Berman 1990), but there is no clear understanding of the potential magnitude of such effects, or of the time scales of adaptation in NR activity. Blasco et al. (1984) concluded that NR activity was an index of nitrate assimilation before the time of sampling while $^{15}$N provided an estimate of nitrate assimilation in the 6 hours following sampling. In the present study this cannot be supported; there is no evidence of consistent time lags between $^{15}$N uptake rates, NR activities and increases in particulate N.

Another factor that should be addressed is the potential for interference from organisms that reduce nitrate in a dissimilatory pathway. For example, Packard et al. (1978) noted that NR activity from deep water (250 m) of the Peru upwelling where oxygen was low was due to bacterial populations. However, this is unlikely to constitute a significant interference for a number of reasons. Dissimilatory nitrate reduction occurs when bacteria use nitrate in place of oxygen as terminal electron acceptor; it does not occur under aerobic conditions. All laboratory cultures were bubbled and photosynthesis should ensure that culture medium was supersaturated with oxygen. Due to mixing, the surface waters of Monterey Bay were also oxygen-rich. Even if dissimilatory nitrate reduction were occurring, it is unlikely that the NR
assay would detect bacterial nitrate reductase. NADH cannot be directly used by bacterial NR (see Stouthamer et al. 1980), and although assays exist where NADH is added, these techniques rely on an intact bacterial respiratory electron transport chain to provide electrons from NADH to the cytochrome moiety of the NR enzyme (see Stouthamer et al. 1980). It has been demonstrated that when cells are homogenized with a detergent such as Triton X-100 (as was the case in the present study), the electron transport system is not functional, and NADH cannot support nitrate reduction (Stouthamer et al. 1980). Thus, the potential for bacterial interference in the NR assay is minimal.

Finally, it must also be noted that there are likely errors in the estimated rates of nitrate incorporation and uptake. As pointed out in Dugdale and Wilkerson (1986), there are several potential problems with the $^{15}$N methods used in the present study. Additions of nitrate are saturating; thus rates must be regarded as representing a maximum potential rate, but this is unlikely to be a problem in the present study because ambient nitrate levels were always high. The incubation period is a problem, since containment effects become important, or a surge uptake phenomenon may cause rates to be over-estimated, but incubations were short (4-5 h) and cells were not nutrient-deficient, so these may not be serious problems. There is also a potential loss of labeled nitrogen (see Kokkinakis and Wheeler 1987, Bronk and Glibert 1991, 1993). This has been attributed to increases in the pool of dissolved organic nitrogen, and bacterial uptake, and appears to be a particular problem in oligotrophic waters when incubations longer than 1 h are used (Bronk and Glibert in press). There is also the question of whether normalization to particulate nitrogen is biased because of particulate detrital pools of nitrogen. This was not a problem for comparison in the present study, since both NR activity and uptake rates can be normalized to the same variable, but it may result in an error in the absolute rate. Chl $a$ has been suggested as an alternative variable, but this may also be problematic (see Dugdale and Wilkerson 1991, and Chapter 3). Given that different methods of measuring uptake and incorporation agree substantially, it is likely that the difference between uptake rates and enzyme activity actually lies with the measurement of NR activity. One possible explanation for the overestimate of nitrate incorporation rate by NR activity
involves the addition of FAD to the assay mixture. Work with NR activity in S. costatum showed that the enhancement of NR activity with FAD was highly variable (Chapter 2). In field assemblages, NR activity showed no increase with FAD addition, but these experiments were performed early in the light period, when NR activity and other estimates of nitrate incorporation rate agreed. It is possible that FAD addition at different points in the diel cycle has different effects on NR activity, potentially giving rate estimates which are too high. This could be resolved by comparing the effects of FAD on NR activity at different times of day.

In summary, although a consistent quantitative relationship between nitrate incorporation and NR activity remains elusive, field experiments successfully applied the modified NR assay to field assemblages of phytoplankton, and supported data from laboratory experiments using unialgal cultures. NR activity was easily and conveniently measured in the field using a modification of the assay described in Chapter 2, providing assays were performed within 30 min and homogenate volume was kept low enough to ensure linearity of the assay. NR kinetic constants and substrate preferences in a natural diatom assemblage were similar to those found for diatoms species in laboratory culture experiments, but it is recommended that appropriate concentrations of nitrate and NADH be verified in each new field situation. Patterns in diel periodicity of NR activity were very similar between laboratory cultures and natural populations; better resolution will result from more intensive time series. Ammonium "inhibition" in natural populations was apparently less complete and occurred on a much longer time scale than is generally appreciated. The effects of ammonium on NR activity generally correspond to the effects on nitrate uptake, but are perhaps slightly delayed in time. In contrast to the majority of previous studies, NR activity was usually in excess of that necessary to account for observed nitrate uptake and incorporation rates. Better temporal resolution of uptake rates and more frequent sampling of NR activity are needed before definite conclusions about the relationship between these variables can be drawn, since at present it is necessary to compare instantaneous NR activities with 4-5 h averaged uptake or incorporation rates.
GENERAL DISCUSSION AND CONCLUSIONS

This thesis has described a systematic laboratory-based approach to using enzymes to estimate in situ growth and nitrate incorporation in marine phytoplankton. The general conclusions from this thesis are best summarized by chapter.

In Chapter 1, the enzyme NDPK has been measured in marine phytoplankton for the first time. Due to the enzyme's central role in biosynthetic energy metabolism, it may be related to growth rate. From general kinetic and thermodynamic characteristics, the enzyme appears very similar to that found in a variety of other organisms, which argues in favor of its general use as a growth rate index. However, the relationship between NDPK and growth rate is complex and shows very high variability. Furthermore, scaling enzyme activity to different estimates of biomass may be problematic. As a result, NDPK activity assays are not likely to be of use in estimating in situ growth rates of marine phytoplankton.

The remaining chapters consider NR activity and its relationship to nitrate incorporation rates in marine phytoplankton. The enzyme has previously been proposed as an index of nitrate incorporation rate, but field work (and limited laboratory studies) have failed to find consistent relationships. An assay for NR activity, based on NADH oxidation, has been developed in Thalassiosira pseudonana, and the results are presented in Chapter 2. Assay conditions have been adapted from previous work, an important innovation being the addition of BSA to combat cellular proteases and perhaps stabilize the NR enzyme in additional ways. NR activity has been measured in a variety of species and shows several species-specific features, including the ability to use NADPH in place of NADH, requirements for FAD, possible stimulation by FeCN, and inhibition by high NADH levels. A good relationship between NR activity and nitrate incorporation rate was demonstrated in light-limited diatom species, but NR activity (as currently assayed) cannot fully account for nitrate incorporation rates in all taxa; cyanobacteria and dinoflagellates appear to be particularly problematic.

In Chapter 3, the relationship of NR activity to nitrate incorporation rate was extended to the case of steady state nitrate limitation as well as light limitation in T. pseudonana. The relationship was substantially poorer under nitrate limitation; NR activity exceeded nitrate
incorporation rates at low growth rates. Such low growth rates may not be relevant to diatoms in the field, because diatom species tend to dominate under nutrient-replete conditions and sink out of the photic zone under nutrient-deplete conditions. Combining data from light- and nitrate-limited cultures, the problem of scaling of NR activity was reconsidered. Cell composition varied with growth rates with opposite effects under light or nitrate limitation, i.e. chl \(\alpha\) in light-limited cells decreased with increasing growth rate, while chl \(\alpha\) in nitrate-limited cultures increased with increasing growth rate. Despite this, most biomass measurements were acceptable for scaling NR activity, with the exception of chl \(\alpha\). Based on the serious problems with measuring cell volume, carbon quota and protein quota in the field, it is recommended that NR activity be scaled to particulate nitrogen, when necessary.

In Chapter 4, cultures of \(T. pseudonana\) were used to test the relationship between NR activity and nitrate incorporation rate under a number of special situations with relevance to the field: diel periodicity in irradiance, the effect of different light spectra, the effect of nitrate starvation, and the effect of ammonium additions. NR activity matched changes in nitrate incorporation rates on a diel cycle very well. Peaks in activity and nitrate incorporation were found in the middle of the light period and just prior to the beginning of the light period. This pattern has not been demonstrated before, but the irregular and widely spaced sampling times in previous studies are probably responsible. Cells grown under sub-saturating blue light grew faster than cells grown under equal quanta of white or red light. NR activity was correspondingly higher under blue light, but was too low to account for nitrate incorporation rates under red light. This underestimate is probably not important in the field, as there are few situations in aquatic environments where red light dominates. Changes in nitrate incorporation rates were reflected very well in changes in NR activity during nitrate starvation, at least on a scale of days. Steady state growth on ammonium inhibited nitrate uptake and nitrate reductase activity to the same degree. Ammonium-grown cells grew faster than those grown on nitrate, but had no detectable NR activity. Nitrate-grown cultures, given daily pulses of 2 \(\mu\)M ammonium, did not show a different relationship between NR activity and nitrate incorporation rates than nitrate-grown cells, at least on a daily scale.
In Chapter 5, the NR activity assay was applied to natural populations in an upwelling zone in coastal California. NR activity in natural populations showed characteristics very similar to those found for diatoms in culture. NR activity showed the same pattern of diel periodicity as demonstrated in cultures. Ammonium additions (5 μM) decreased nitrate uptake and NR activity, but this effect took over 4 h and NR activity began to increase while there was still 2 μM ammonium present. NR activity was always equal to or greater than the nitrate incorporation rates estimated from changes in ambient nitrate, particulate nitrogen, or 15N incubations. This is the first time that this has been achieved for field populations. A precise comparison of rates was difficult because the enzyme measurements are instantaneous, while the other rates are 4-5 h averages. It appears, however, that NR activity may often overestimate nitrate incorporation rates.

**EXPERIMENTS FOLLOWING FROM THE THESIS**

Before NR activity can be used to estimate in situ nitrate incorporation rates, a number of further experiments must be done.

**Applicability to different species**

Since there are species (e.g. dinoflagellates) in which NR activity did not correspond to nitrate incorporation rates, a detailed investigation of these cases is required. However, this thesis has not addressed species differences, but rather, clonal differences have been considered, since each species is represented by only a single clone (but note that this is also the case for the overwhelming majority of studies concerning phytoplankton "species" comparisons; see Wood and Leatham, 1992). As has previously been documented, variation within a series of clones of a single species may exceed that found between species (see Murphy and Guillard, 1976, Gallagher 1980), although the ecological relevance of these differences remains unclear (see Murphy 1978).

In future, however, the issue of clonal and of true species variability must be addressed systematically, perhaps by comparing stock and fresh isolates of species across broad taxa.
Non-nitrogen starvation effects

In this thesis, the effect of nitrogen starvation was considered; NR activity appeared to track the decline in nitrate uptake rather well. This time series should be repeated at a greater resolution (i.e. < 1 day sampling), now that these experiments have provided a better idea of the time course of changes. This will give a better indication of whether short-term variations in NR activity match patterns of nitrate incorporation, and may help reconcile results of the present studies with those in the literature (see Chapter 4 discussion). The results of preliminary experiments in which nitrate remained high are compelling. In the preliminary study, NR activity increased as cells moved into stationary phase. It would be interesting to repeat the nutrient starvation experiment with exhaustion of carbon, silicate and phosphate and see if this is a repeatable phenomenon. There may be another NR response to nutrient starvation under these conditions (see Chapter 4). On the other hand, experiments in which selenium may have been limiting apparently did not affect the NR-nitrate incorporation relationship (see Chapter 3), so it may also be important to perform the experiment with a trace element limitation, e.g. iron. These may not be simply physiologically interesting phenomena, but are ecologically relevant (see General Introduction re: limitations). Furthermore, different responses to different nutrient exhaustions may also provide some basis for distinguishing different limitations.

Ammonium inhibition time series

Experiments with steady-state ammonium versus nitrate growth were enlightening, but additional experiments should consider the short-term effects of addition of ammonium to nitrate grown cultures. Such experiments should monitor internal pools, as well as ambient nutrients on a scale of minutes to hours, rather than days. The degree of inhibition and the time course of inhibition of NR activity and nitrate uptake must be clarified, as well as the minimum levels of ammonium having an effect. The use of $^{13}$N would provide a very sensitive method to examine very short time scales of nitrate uptake.
Temperature effects

All cultures in the present study were grown at a single temperature. The response of the NR enzyme to temperature would be of interest, particularly if species that experience a wide temperature variation in situ, or those that have extended geographical distributions, are to be considered. For example, responses to temperature may involve the sequential expression of different isozymes (see examples in Hochachka and Somero 1984), and the optimal assays for these isozymes may be different. Laboratory studies initially characterizing the enzyme by measures such as activation enthalpy, but moving to gel electrophoretic separations and detection of enzyme by activity would be useful.

Metabolic control analyses

Evidence from these studies suggest that under steady state conditions, control of nitrate incorporation rests either with NR, or the adaptation of NR to prevailing metabolic fluxes is very rapid. This should be properly assessed in a systematic manner. Enzyme assays for all the enzymes in nitrate assimilation, i.e. NR, NiR, GDH, GS and GOGAT should first be optimized, as should assays for internal and external pools of nitrate, nitrite, ammonium, glutamate and glutamine. Such work would be aided by better quantification of intracellular localization of nutrient pools, perhaps using centrifugation to separate organelles, or intracellular dyes. These experiments should first be considered under steady state, then perturbations should be made, e.g. a shift-up in light (in a light-limited case) and a shift-up in nutrients (in a nutrient-limited case), or a spike of ammonium in a nitrate grown culture. The results of these experiments can be interpreted in the context of metabolic control analysis. This system might very well provide an ideal test of metabolic control theories, since it is one of the few cases where the individual parameters can be measured, while global parameters such as growth rate can also be monitored.
Optimization of assays in natural populations

Another problem to be considered is the diversity of NR enzymes. The three species (actually the three clones) considered in Chapter 2 showed different characteristics. This is problematic for natural populations where a mixture of species will be the norm. It may be possible to selectively determine parameters for different segments of the population by using selective assays, e.g. NADH-specific NR for diatoms and dinoflagellates, NADPH-NR for green algae, and ferredoxin-NR for cyanobacteria. Then these could be added up for a total community assay. Alternatively, there are now cell sorting technologies involving flow cytometry (e.g. Chisholm et al. 1986) that might eventually allow cell separations and assays of different fractions of the population.

Purification and analysis of NR proteins from different taxa might also provide information about the bases of differences in NR characteristics.

FUTURE DIRECTIONS

Ultimately, once experiments following from the thesis have been completed, a number of future studies are suggested.

Assay sensitivity and single cell analyses

Given the constraints in sensitivity of NR assays, for the present NR activity, application to the natural environment must remain a population assay. There is, however, evidence that there may be significant diversity in cell responses within a population. Bulk measurement will not distinguish these phenomena. The activity assay could conceivably be scaled down perhaps to the level of individual cells (see e.g. Wulff and Paigen 1974), but this would be extremely tedious and would not allow rapid broad scale surveys of variability in response. An alternative might involve adapting immunoassays to the problem. NR antibodies for different species are available (e.g. Balch et al. 1988, Falkowski and LaRoche 1991b, Smith et al. 1992), and using different detection methods, single cell analyses are
possible (see Balch et al. 1988, Orellana and Perry 1992). As previously discussed, however, immunoassays have their own associated problems (see Chapter 2), and may not be applicable to more than a very small segment of the population.

**Automated assays**

As pointed out by Parsons and LeBrasseur (1968) and more recently by Platt et al. (1989), marine ecology suffers because of the scales on which processes must be measured. Sampling on these synoptic scales is impossible, and the degree of temporal and spatial variability means that the oceans are infinitely under-sampled. In terms of NR assays, one approach to resolving the problem might be the development of automated sampling techniques. The AutoAnalyzer method of Slawyk and Collos (1976) is an improvement, but it still requires manual homogenization of samples. Ideally such an automated method might involve continuous flow sampling, with flow cytometric detection and sorting, in-line homogenization by sonication, pressure (but note the problems found here, see Chapter 2), or even rapid freeze-thawing, and then automated assays. Such a system could ultimately be installed on selected ships of opportunity or routinely on oceanographic cruises to provide more synoptic coverage. Furthermore, if such a method could be developed and miniaturized, there is the potential to use it on long-term moored arrays which would help give temporal coverage.

**Coupling hydrodynamic and biological scales**

As Legendre and Demers (1984) pointed out, biological processes in the marine environment are controlled by hydrodynamics to a very high degree. Oceanographers are now beginning to resolve physical processes on the temporal scale of seconds and on the spatial scale of centimetres. The conclusions are generally that these scales of variability are important, but may be difficult to study in conventional ways (see Platt and Denman 1975). For biologists, the scales of resolution remain very much coarser. The potential of enzymes is not simply to provide a point estimate, but perhaps to provide a measurement of a small-scale
biological integrator. Ultimately, such measurements and the scale of changes in enzymes in response to environmental shifts might provide an estimate of their biological importance. For example, while microscale changes in nitrate and ammonium have been shown, their importance to phytoplankton remains uncertain. Measurements of NR in these situations could ultimately provide convincing support to clarify such questions.

Field application: important regions

Currently, there are regions of the world oceans where dynamics are particularly poorly understood. In particular, certain areas are noted for having high nutrients (particularly nitrate), yet low chlorophyll (see Miller et al. 1991, Mitchell et al. 1991). Hypotheses to explain these findings include elements of grazing control, limitation by other trace elements (e.g. iron), or nutrient interactions. Under these conditions, incubation methods face serious problems (see General Introduction). Thus, the use of NR assays may help to resolve questions which in the past could only be addressed by using incubations. It is critical at this stage to have a thorough appreciation of potential problems. It is imperative that a number of issues be resolved in the laboratory before attempting to apply the NR assay in the field in any but the most preliminary form.

Central oceans

Vast areas of the open ocean are under-surveyed and there is great debate over what kinds of processes are important. One example is the subarctic Pacific ocean, typified by Station P (see Miller et al. 1991). Before application of NR assays to these regions is possible there are several issues that must be resolved. The plankton of these regions is often dominated by picoplankton, many of which are cyanobacteria or prochlorophytes. Suitable NR assays must be developed and tested. Sampling may present a problem if a significant fraction of production in these regions is due to large, rare diatoms (see Goldman 1989). Sampling techniques including fine mesh nets must be considered in addition to bulk water collections. In these regions in particular necessary biomass for assays may not be available
conveniently. Thus, increasing assay sensitivity and perhaps moving to immunoassays and single cell analyses may be important. Finally, the importance of recycling of nutrients in these regions means that ammonium inhibition of nitrate uptake may be a major factor. This must be carefully considered in laboratory studies before considering large-scale field sampling.

*Arctic and Antarctic*

In these regions of the ocean the caveats noted for central oceanic areas apply (see Nelson and Smith 1991), but in addition the issues of low temperature effects must be addressed (Smith 1991), as previously described. As well, since these regions receive nearly continuous light in the summer, or nearly continuous darkness in winter, the effects of seasonality in these species must be considered (see Mitchell *et al.* 1991). It would be of interest to know if diel patterns persist in the absence of cyclic irradiance. Finally, in these waters, due to thinning ozone, ultraviolet radiation may be significantly higher. The issue of what effects this short wavelength radiation has specifically on the NR enzyme should also be addressed.

If carefully applied, the NR assay developed and tested in this thesis has great potential for becoming a useful investigative technique. However, careful and cautious application of the assay at this stage is still required.
LITERATURE CITED


APPENDIX A: A COMPARISON OF LOWRY, BRADFORD AND SMITH PROTEIN ASSAYS USING DIFFERENT PROTEIN STANDARDS AND PROTEIN ISOLATED FROM THE MARINE DIATOM THALASSIOSIRA PSEUDONANA

INTRODUCTION

This appendix describes a comparison of protein assays using different purified proteins, and protein from the diatom Thalassiosira pseudonana. Protein is commonly selected as a variable to scale enzyme activity in marine and freshwater algae (e.g. Morris and Syrett 1965, Eppley et al. 1969, Dortch et al. 1979, Bressler and Ahmed 1984, Kristiansen 1987). In theory, it provides a good index of biomass which is not biased by lipid or carbohydrate storage, and is less sensitive to short-term environmental changes than are variables such as chlorophyll a (Glover and Morris 1979, Dortch et al. 1979).

Although absolute quantification of protein requires fractionation followed by Kjeldahl nitrogen determination (e.g. Whyte 1987), the difficulties with obtaining sufficient material, and the time consuming nature of this method have lead researchers to use spectrophotometric analyses (see Rausch 1981). Ultraviolet methods are available (Stoscheck 1990) and modifications to the classical Biuret assay have been used (e.g. Dorsey et al. 1978), but in general, these methods lack the required sensitivity to be applicable to marine phytoplankton. Commonly used assays include the alkaline copper assay ("Lowry" assay, Lowry et al. 1951) and the Coomassie dye binding assay ("Bradford" assay, Bradford 1976). A substantially simpler modification of the Lowry assay, using bicinchoninic acid in place of the Folin-Ciocalteu reagent has been proposed by Smith et al. (1985; "Smith" assay).

It is recognized that spectrophotometric methods are, at best, relative measurements because it is necessary to use a purified protein standard, most commonly bovine serum albumin (BSA). However, it is a frequent practice to infer absolute protein content from such data, despite suggested precautions (e.g. Zamer et al. 1989). In such cases, it is necessary to have an indication of the reactivity of the protein standard relative to the protein being assayed. It is also important to be able to compare the results of different assays. A growing
body of evidence suggests that the Lowry and Bradford assays give different measures of protein relative to BSA for marine invertebrates (Zamer et al. 1989), higher plants (Eze and Dumbroff 1982) and marine phytoplankton (Clayton et al. 1988). Differences between the assays might be expected because the Lowry and Bradford procedures use distinctly different principles. The Lowry assay detects protein through a copper-catalyzed reduction of Folin phenol reagent. This reaction will detect peptide bonds, but it is also highly sensitive to specific amino acids such as tyrosine and tryptophan (Legler et al. 1985). In the Bradford assay, Coomassie Brilliant Blue dye is bound by protein, primarily by arginine residues, but also to a lesser degree by histidine, lysine, tyrosine, tryptophan and phenylalanine (Compton and Jones 1985). The reactivity of either of the assays to a particular protein will be a function of that protein’s composition as well as any other compound which might oxidize the Folin phenol reagent, or bind the Coomassie dye (Stoscheck 1990). In fact, disagreements between the Lowry and Bradford assays have been ascribed to different reactivities of the assays to the specific protein being measured, and to interferences by compounds such as chlorophyll a (Eze and Dumbroff 1982). The Smith assay has seldom been compared to the other assays and has only recently been applied to marine phytoplankton (Smith et al. 1992). If different protein reactivity relative to a standard is responsible for the discrepancy, it is also plausible that protein reactivity (e.g. changes in specific cell proteins, or amino acid composition) may vary in a single species under different growth conditions (see Lohrenz and Taylor 1987). This would be particularly problematic if protein was being used as a variable to scale other measurements, but the possibility has never been examined. There are also many minor variations in methods. For example, some researchers have chosen to precipitate proteins with trichloroacetic acid (TCA) in order to remove free amino acids (e.g. Clayton et al. 1988), while others (e.g. Mayzaud and Martin 1975) have not.

The objectives of this study were to: a) compare Lowry, Bradford and Smith protein assays using a typical marine diatom, *Thalassiosira pseudonana*, b) to purify proteins from *T. pseudonana* grown under either high or low light conditions and compare their reactivity to BSA and other common protein standards, c) to assess the potential for chlorophyll a to
interfere with each assay, and d) to test whether precipitation of protein using TCA affects estimated protein content or the relationship between the results of the different assays.

MATERIALS AND METHODS

Culture conditions

The marine diatom Thalassiosira pseudonana (Hustedt) Hasle and Heimdal (3H clone) was obtained from the Northeast Pacific Culture Collection, Department of Oceanography, University of British Columbia. Cultures were grown in enriched artificial seawater (ESAW) as previously described. Temperature was maintained at either 17.5 ± 0.5°C using a circulating water bath, or at 22 ± 1°C in a cold room. Cultures were grown in glass flasks ranging from 1 to 12 litres, stirred at 60 rpm with Teflon-coated stir bars and bubbled with air filtered through a 0.22 μm membrane filter. Continuous illumination was provided by Vitalite™ fluorescent tubes and attenuated by distance or neutral density screening to give a range of irradiances from 6 to 120 μmol quanta m⁻² s⁻¹. Growth rates were followed by in vivo fluorescence, measured twice daily using a Turner Designs™ Model 10 fluorometer and cell counts using a Coulter Counter™ model TAI equipped with a population accessory.

Protein assay comparisons

Samples for protein determination were collected from 35 T. pseudonana cultures grown as described above and harvested in a range of growth phases. Homogenates were prepared as described by Dortch et al. (1984), grinding with 3% TCA and extracting for 30 min in 1 N NaOH at room temperature. Preliminary experiments showed that increasing NaOH to 2 N, sonicating samples, extracting for 2 h, or extracting at 80°C did not increase the concentration of protein measured in subsequent assays, and occasionally decreased it (compare with Rausch 1981).

Lowry assays were performed as modified by Dortch et al. (1984). Bradford assays were performed using the micro-assay procedure of the Bio-Rad Protein Assay kit (Bio-Rad
Laboratories, 500-0001). The Smith assay was performed using a Sigma Protein Assay kit (Sigma Chemical Co., BCA-1). Protein was calculated on a per cell basis and Bradford and Smith results were compared to those of the Lowry assay using Student t-test comparisons at the 95% confidence level.

To provide some basis for judging the accuracy of the assays, cell nitrogen content was also measured in 16 of these cultures, using 50 ml samples collected on precombusted Gelman type AE glass fibre filters and analyzed using a Carlo Erba CNS analyzer.

**Protein reactivity comparisons**

Three separate experiments were performed, the first using two 6 litre cultures, one grown at high light (120 μmol quanta m⁻² s⁻¹), the other at low light (15 μmol quanta m⁻² s⁻¹). The second and third experiments used four 12 litre cultures, two grown at high light, two at low light. For each experiment, cultures were inoculated from a common high light grown culture at a density which allowed 8 generations before harvest. Mean growth rates were 0.32 (±0.06) and 2.04 (±0.08) d⁻¹ for low and high light cultures respectively.

Cultures were harvested in late log phase, approximately one generation before stationary phase. Cells were collected by filtration onto 47 mm pre-combusted 934 AH glass fibre filters and frozen at -20 °C until protein purification could be performed.

Protein purification followed the method of Zamer et al. (1989). Each filter was ground for 2 min in ~5 ml of 1 N NaOH in a 15 ml glass/Teflon tissue homogenizer. Homogenates were pooled for each culture, extracted for 30 min and centrifuged for 15 min in a clinical centrifuge to remove filter fibres. The supernatant was decanted and its volume measured. Proteins were precipitated by adding 25% trichloroacetic acid (2.5: 1.0 v/v, TCA:homogenate). The mixture was centrifuged for 15 min and the pellet was washed with 10% TCA. The pellet was resuspended in 5% TCA (5:1 v/v TCA homogenate) and heated to 90°C for 15 min in a water bath to remove nucleic acids. The cooled supernatant was centrifuged and washed with 5% TCA. To remove pigments associated with proteins, the pellet was resuspended in 90% acetone (5:1 v/v acetone:pellet) and centrifuged for 15 min.
This was repeated until the supernatant retained no visible color. Lipids were extracted by the method of Bligh and Dyer (1959). The water-methanol fraction was collected and placed in a 50 ml beaker. This was dried in a desiccator for several days.

Purity of the protein extract was assessed by stoichiometric analysis. Ash free dry weight was determined by placing known quantities of purified protein into pre-weighed aluminum cups. These were re-weighed after ashing in a muffle furnace at 550° C for 6 h. Samples were analyzed for carbon and nitrogen content using a Carlo-Erba CNS analyzer with sulfanilamide as a standard. Purity was assessed assuming a nitrogen-protein conversion factor of 6.0. Although a ratio of 6.25 is generally used, Gnaiger and Bitterlich (1984) suggested that 5.8 may be better for a variety of aquatic organisms; a review by Laws (1991) found 6.0 to be the best value for diatoms.

Standards for protein assays consisted of bovine serum albumin (BSA, Sigma Chemical Co. A 7638), bovine gamma-globulin (BGG, Sigma G 7516) and casein (Sigma C 6905). Replicate samples and standards were individually weighed and dissolved in 1.0 N NaOH to a concentration of ~1 mg ml⁻¹. For Bradford and Smith assays, both samples and standards were diluted with distilled water to appropriate final concentrations. For each purified sample and separately weighed standard, a linear regression was performed. In no case was the intercept significantly greater than zero. Therefore the mean of these samples was taken as the best estimate of the slope of the absorbance vs. protein curve. These slopes were compared within each assay using a one-way analysis of variance (ANOVA), followed by Tukey’s multiple range test to investigate differences (Steel and Torrie 1980).

Chlorophyll interference experiments

To assess chlorophyll interference with assays, two approaches were used. The first involved preparation of two BSA standards (1 mg ml⁻¹, 4 ml total volume) with and without addition of chlorophyll a. Parsons et al. (1961) reported protein:chlorophyll a ratios between 23 and 75 for diatom species (see also Banse 1977). We selected a ratio of 40:1 for these experiments. One standard received 0.1 mg of chlorophyll, dissolved in 100 ml of 90%
acetone, while the second standard received only 100 ml 90% acetone. Each assay was performed and the slopes of absorbance-concentration curves were compared by regression analysis. The second approach used two sets of subsamples (n = 6) taken from a single culture. The first set was homogenized as described previously. The second set was extracted twice with 5 ml aliquots of 90% acetone before being centrifuged and solubilized in 1 N NaOH. Protein contents were compared within each assay using Student t-tests.

**TCA Precipitation Experiment**

As in the chlorophyll interference experiment, two sets of subsamples (n=5) were taken from a single culture. The first set was homogenized as previously described. The second set was homogenized directly in 1 N NaOH without TCA precipitation. Results were compared for each assay using Student t-tests.

**RESULTS**

**Assay Comparisons of T. pseudonana Homogenates**

For 35 different T. pseudonana cultures, a ratio of 1.23 (±0.04) was found between the Lowry and Bradford assays, indicating significantly higher protein determined by the Lowry assay (P < 0.001). The Smith assay was not significantly different from the Lowry; the ratio of Lowry:Smith was 0.97 (±0.05) and not significantly different from 1.0 (P > 0.5).

Using cell nitrogen quota for 16 different cultures and a nitrogen-protein conversion factor of 6.0, protein measured by the Bradford assay accounted for 47 ± 3% (standard error) of cellular nitrogen, while the protein measured using Lowry and Smith assays accounted for 58 ± 3%.

**Relative Protein Reactivity**

In all cases, CNS analyses (following Zamer et al. 1989) indicated that > 90% of the ash-free dry weight of purified protein from Thalassiosira pseudonana was protein. Fig. A.1
Figure A.1. Absorbance versus protein content for different pure proteins and purified algal protein from Thalassiosira pseudonana for A) Bradford, B) Lowry, and C) Smith protein assays. For clarity, only one out of six sets of bovine serum albumin (BSA, ●), bovine gamma globulin (BGG, ▼) and casein (■) data are shown. Lines represent least squares regression fits to pooled data. Fits to algal protein data for high light grown (○) and low light grown (□) cultures are not shown. Note scale changes for protein.
shows the relationship between algal protein and other pure protein standards for each assay. Statistical comparisons are summarized in Table A.1. The reactivity of algal protein was identical whether cells are grown under high or low light for every assay. BSA reactivity was not significantly different from algal protein when the Bradford or Smith methods were used, but significant differences were detected between BSA and algal protein using the Lowry method. The differences were most pronounced at high concentrations of protein (Fig. A.1).

**Chlorophyll Interference**

Fig. A.2 demonstrates that the addition of chlorophyll to a BSA standard did not result in a significantly different slope for any assay ($P > 0.20$ in all cases). There is apparently some deviation from linearity at high concentrations for the Lowry assay, perhaps due to the addition of acetone.

Fig A.3 summarizes comparisons of TCA precipitated homogenates with those which were extracted with acetone. Acetone removed all trace of color in the protein pellet, and gave a clear green supernatant. No differences were found for Bradford and Smith assays ($P > 0.2$ in both cases), but the Lowry assay gave a significantly higher protein content when acetone extraction was used ($P < 0.001$).

**TCA Precipitation**

Results of TCA precipitation experiments are summarized in Figure A.4. For the Bradford assay, no differences were found ($P > 0.4$), but both the Lowry and Smith assays gave up to 36% higher values for non-precipitated homogenates ($P < 0.001$ and $P < 0.002$, respectively). As a result, Lowry and Smith assays gave protein contents that were 50-60% higher than Bradford assays, if TCA precipitation was omitted.
Figure A.2. Comparison of absorbance versus protein curves for A) Bradford, B) Lowry, and C) Smith protein assays for BSA samples with (●) and without (○) additions of 0.1 mg chlorophyll in 90% acetone. Points represent the means of two separately prepared standards. In all cases, associated error bars are smaller than the symbols. Lines represent least squares regression fits to the data.
Table A.1. Comparison of absorbance versus protein content slopes for bovine serum albumin (BSA), bovine gamma-globulin (BGG), alpha-casein, and protein purified from *Thalassiosira pseudonana* cultures grown under either high or low light. Values represent mean and standard error of 5 different determinations from separately prepared standards. Summaries of statistical comparisons (one-way ANOVA, followed by Tukey's multiple range test) are provided below the table. Lines join proteins which are not significantly different from each other at the 95% C.I.

<table>
<thead>
<tr>
<th>Slope</th>
<th>Assay</th>
<th>BSA</th>
<th>BGG</th>
<th>Casein</th>
<th>High Light</th>
<th>Low Light</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bradford</td>
<td>0.0570 (0.0014)</td>
<td>0.0284 (0.0010)</td>
<td>0.0379 (0.0003)</td>
<td>0.0561 (0.0016)</td>
<td>0.0567 (0.0019)</td>
</tr>
<tr>
<td></td>
<td>Lowry</td>
<td>0.0026 (0.0001)</td>
<td>0.0037 (0.0001)</td>
<td>0.0021 (0.0001)</td>
<td>0.0035 (0.0001)</td>
<td>0.0036 (0.0002)</td>
</tr>
<tr>
<td></td>
<td>Smith</td>
<td>0.0092 (0.0001)</td>
<td>0.0097 (0.0002)</td>
<td>0.0052 (0.0003)</td>
<td>0.0102 (0.0011)</td>
<td>0.0102 (0.0010)</td>
</tr>
</tbody>
</table>

**BRADFORD:**
- BSA
- HIGH LT
- LOW LT
- CASEIN
- BGG

**LOWRY:**
- BGG
- HIGH LT
- LOW LT
- BSA
- CASEIN

**SMITH:**
- BGG
- HIGH LT
- LOW LT
- BSA
- CASEIN
Figure A.3. Comparison of protein content (expressed as pg cell\(^{-1}\)) for acetone-extracted (▱) versus non-acetone-extracted (◼) homogenates of *Thalassiosira pseudonana* (n = 6 for each treatment). Error bars represent standard errors of mean protein content.
Figure A.4. Comparison of protein content (expressed as pg cell$^{-1}$) for trichloroacetic acid (TCA) -precipitated (■) versus non-TCA-precipitated (□) homogenates of Thalassiosira pseudonana (n = 5 for each treatment). Error bars represent standard errors of mean protein content.
DISCUSSION

Assay Comparisons of *T. pseudonana* Homogenates

There is general agreement that the Bradford assay gives lower protein values than the Lowry assay in a variety of organisms. Chiapelli *et al.* (1979) and Manahan and Nourizadeh (1990) determined a Lowry:Bradford ratio of 1.6 for rat liver preparations and a range of marine invertebrates, respectively. Using data from Eze and Dumbroff (1982), a ratio of 1.4 for kidney bean leaves was calculated. Clayton *et al.* (1988) compared the Bradford and Lowry assays for *T. pseudonana* and reported the ratio ranged from 1.8 to 2.0. These values are higher than the ratio of 1.23 found in the present study. Alternatively, Setchell (1981) found no difference between protein content determined with Lowry and Bradford assays in either *T. pseudonana* or *Skeletonema costatum*. Unlike other researchers, however, Setchell (1981) used BGG as a standard. The reasons for differences in the ratio of protein measured by Bradford and Lowry assays are unclear. Part of the explanation may involve minor variations in the Lowry procedure, including use of detergents (Clayton *et al.* 1988) and sample treatment with TCA (Eze and Dumbroff 1982). As well, there are differences in the Bradford assays. In the present study we used a micro-volume version of the Bradford assay which has a higher dye:sample ratio than the assay used in many other studies (Bradford, 1976).

The Smith and Lowry assays gave identical results. Relatively little is known about the comparability of these assays. Smith *et al.* (1985) noted that most proteins show similar reactivities using the two assays, but there were exceptions (e.g. the protein avidin). Brown *et al.* (1989) found good agreement between Lowry and Smith assays. Because the two reactions have a common first step, this similarity is not unexpected. The Smith assay uses only one reagent and does not require precise timing and may therefore be preferable.
Protein Assays and Cell Composition

Results indicate that only 47 to 58% of cell nitrogen can be accounted for in the protein measured by spectrophotometric assays. These values appear low compared to values in the algal literature. Using acid hydrolysis and amino acid detection, protein values in the range of 75-85% of cell nitrogen have been found for the green alga *Chlorella* (Fowden 1952), and various marine phytoplankton species (Parsons *et al.* 1961; Cowey and Corner 1966; Mayzaud and Martin 1975, Laws 1991). It is important to recognize that hydrolysis techniques without fractionation cannot distinguish between protein, free amino acids and small peptides. It is unlikely that the cells in this study were abnormal in composition, since carbon:nitrogen ratios were 6.78 ± 0.14 (n = 16) which is very close to the average value for phytoplankton of 6.5 found by Laws (1991).

Alternatively, other data suggest that percentages of protein nitrogen may be lower than is generally assumed. Mayer *et al.* (1986) found cell protein accounted for 71% of the nitrogen in a mixed culture of *T. pseudonana* and *D. tertiolecta*. Using a fractionation and a micro-Kjeldahl technique, Whyte (1987) calculated that protein in *T. pseudonana* accounted for 75% of the nitrogen in early logarithmic growth phase, but only 53% of the nitrogen in stationary phase. In *T. pseudonana*, Dortch *et al.* (1984) reported 67 ± 15% of the cell nitrogen in protein. The values obtained in the present study fall into the lower end of this range, but experiments of Dortch *et al.* were conducted under blue light, which Kowallik (1978) predicts will increase protein content. Conover (1975) measured protein in the co-generic *T. weisflogii* and calculated that 55.7% of the cell nitrogen was in protein in log phase, while only 27% was accounted for in senescence. Both Conover (1975) and Whyte (1987) point out that *Thalassiosira* species have a high chitan content, which may account for substantial portions of non-protein nitrogen. As well, Lui and Roels (1972) showed that the TCA-soluble nitrogen pool in the diatom *Biddulphia aurita* accounted for 18-33% of the cell nitrogen, and that up to 58% of it was free or combined amino acids. Thus, on the basis of cell nitrogen content, it remains uncertain which method is more accurate.
Relative Protein Reactivity

BSA appears to be a suitable standard for Bradford and Smith assays because its reactivity is not significantly different from algal protein. However, this is not true for the Lowry assay. The fact that the Lowry estimate of *T. pseudonana* protein agreed with the Smith assay (where there are not differences between algal protein and BSA) may be explained by the fact that Lowry assays of *T. pseudonana* cultures typically gave absorbance values <0.20, where the curves of algal protein and BSA fall relatively close together. Selecting a suitable standard protein is critical to the accuracy of these assays. Stoscheck (1990) points out that BSA has a high reactivity with the Bradford assay, and suggests that BGG gives a more "normal" response. In addition, Mayer et al. (1986) suggests that BGG is more representative in studies of mixtures of proteins. Setchell (1981) used BGG as a standard and found no differences between protein content of *T. pseudonana* using Bradford and Lowry assays. Results indicate that the use of BGG as a standard will give inflated values for Bradford assays because BGG gives significantly lower absorbances than algal protein. This may explain why Setchell (1981) found no differences between assays when he used BGG as a standard, while researchers previously cited have noted differences. Dortch et al. (1984) also recommended caution when using BSA as a standard. Alternatively, data from Dorsey et al. (1978) show similar reactivity between protein from *T. pseudonana* and a BSA standard. As well, Dorsey et al. (1977) compared "purified protein" (details were not given in their paper) from *Chaetoceros curvisetus* and noted that the characteristics of its reactivity in a modified biuret assay were quite similar to BSA. Lowry et al. (1951) pointed out that mixtures of protein were less variable than individual purified proteins. Results of the present study suggest that BSA is not an unreasonable standard for *T. pseudonana* but they contrast with those of Zamer et al. (1989) who showed that purified protein from sea anemones was much lower in reactivity than BSA. For the Lowry assay there is the potential to overestimate phytoplankton protein at high concentrations. In this case, BGG may be a more accurate standard.
Chlorophyll Interference

Chlorophyll a added to BSA standards had no significant effect on any of the protein assays. Some differences appeared at high protein concentrations in the Lowry assay, but because the Lowry assay agreed with the values for the Smith (Fig. A.2), and the Smith assay showed no evidence of interference with chlorophyll, it seems unlikely that chlorophyll represents a real interference under these conditions.

In contrast, Eze and Dumbroff (1982) showed that chlorophyll in kidney bean leaves could increase absorbances in protein determination by up to 400%, particularly for the Lowry assay. They found that TCA precipitation was able to remove the interference, probably by denaturing the chlorophyll. In this study, it was noted that TCA-precipitated homogenates still remained green, indicating substantial chlorophyll remained intact. Comparisons of acetone extraction versus normal TCA-precipitated samples revealed no differences in protein content as determined by the Bradford or Smith assays, but significantly higher protein in acetone-extracted samples according to the Lowry assay. This result is unusual, since Eze and Dumbroff (1982) found chlorophyll a caused an increase in estimated protein. The reasons for this difference are unclear, but acetone may remove other compounds in addition to plant pigments.

Other potential interfering compounds such as amino acids (Cowey and Corner 1966), lipid (Hopkins et al. 1984, Morton and Evans 1992) and phenols and phenolases (Mattoo et al. 1987) have been identified. These compounds usually increase absorbance, but it has been speculated that they could also decrease absorbances by inhibiting the reactions with the assay reagents (e.g. Mattoo et al. 1987). In general, these authors have demonstrated these interferences can be corrected by TCA precipitation. Morton and Evans (1992) reported that interference by lipoproteins could be removed by adding 2 % sodium dodecyl sulphate.
TCA Precipitation

The fact that TCA precipitation results in lower protein estimates for Lowry and Smith assay, but makes no difference in the case of Bradford assay estimates, suggests that TCA precipitation removes compounds that are detected selectively by the Lowry/Smith assays. Chlorophyll does not seem to be a likely candidate, based on previous evidence, nor do phenols and phenolases that would also affect the Bradford assay (Mattoo et al. 1987). However, other groups of compounds known to be reactive with protein assays include amino acids and peptides. The literature suggests that microalgae have quantities of these compounds, particularly in stationary phase (e.g. Martin-Jezequel et al. 1988, Flynn 1990). Dortch et al. (1984) observed large TCA-soluble pools of nitrogen, and Whyte (1987) has shown that non-protein nitrogen is principally amino acids, peptides and amines in T. pseuodonana. Lui and Roels (1972) showed that 30% of soluble nitrogen was free amino acids, and an additional 30% was combined amino acids. The Bradford assay is known to be less sensitive than the Lowry to small peptides and amino acids. Mayer et al. (1986) found that, of the peptides tested, none smaller than 6 amino acids gave a reaction, and little sensitivity was noted in peptides smaller than 10-25 amino acids. Chiapelli et al. (1979) previously tested the hypothesis that Bradford-Lowry differences could be accounted for in terms of a class of small peptides. Most of his data, drawn from rat liver samples, indicate no significant differences. In at least one case, however, the ratio of TCA/normal homogenates is markedly lower for the Lowry (79.2%) than for the Bradford assay (91.8%). There are also reports suggesting that TCA precipitation may leave some protein in solution. Beckman et al. (1943) reported that 4-20% of total protein in human urine samples was not precipitated in 4% TCA. Such TCA-soluble proteins may react differently in Bradford, Lowry and Smith assays.

Extending this argument, if TCA precipitates a class of peptides to which the Lowry and Smith are sensitive, but the Bradford is not, this could explain the differences reported here in estimated protein, despite the similarity in pure algal protein reactivity. If this is true, the size of intracellular amino acid and peptide pools might also constitute a significant source
of variability in measurements of algal protein based on spectrophotometric assays. In a preliminary attempt to address this question, Lowry, Bradford and Smith assays were compared against BSA standards in the presence and absence of the amino acids, proline, glycine and betaine. Dickson and Kirst (1987) have found substantial levels of these amino acids in marine diatom species. Results indicate that even at concentrations as high as 100 mM, none of the amino acids listed above shows interference with any of the assays. Flynn and Flynn (1992) have also reported finding substantial levels of non-protein amines in microalgae, but the identity of these compounds remains unclear.

In conclusion, although selection of a protein standard may seem unimportant if only a relative measure of protein content is required, such data are often misleading because they are presented as absolute protein values. Despite criticism in the literature, BSA appears to be a suitable standard for algal protein measured in the Bradford or Smith assays. There is evidence that BGG is preferable as a standard for the Lowry, but the error which results from the use of BSA is probably small. Researchers must be aware that the Bradford assay gives different results from the Smith or Lowry, but such differences are not due to different reactivities of algal protein relative to a standard. In this study, the question of which assay is most accurate has not been resolved, however, Manahan and Nourizadeh (1989) have suggested that the Lowry assay (and thus the Smith assay) tend to over estimate protein. On the basis of speed, simplicity and relative insensitivity to interfering compounds, we recommend use of the Bradford assay. However, because of the volume of research previously performed using the Lowry assay, it is both useful and possible to calibrate the assays for the specific system being investigated. Researchers using spectrophotometric protein assays must make every effort to give precise details of methodology (e.g. TCA precipitation) and indicate the protein standard being used. In terms of implications for using protein as a variable for scaling enzyme activity, these results suggest caution. Until it is clear exactly which compounds the spectrophotometric protein assays are detecting, the meaning of measurements scaled to "protein" will be in doubt.
APPENDIX B: ADAPTING AN LKB ULTROSPEC II UV SPECTROPHOTOMETER FOR ENZYME KINETIC ANALYSES

INTRODUCTION

Frequently, measurements of enzyme reaction rates involve timing and stopping reactions, then measuring changes in concentration of a product or a reactant (see Rossomando 1990). There are disadvantages to this techniques, because it necessitates use of extra enzyme homogenate for a zero time blank, and because the linearity of the reaction over time cannot be confirmed with a single assay. Alternatively, a variety of enzyme reactions can be continuously monitored spectrophotometrically. For example, a wide variety of enzymes either oxidize NADH or reduce NAD or can be coupled to other reactions which do. Because NADH absorbs strongly at 340 nm, while NAD does not, recording the change in absorbance over time as the reaction proceeds will give a measure of the reaction rate (Rossomando, 1990). Often, this involves either timing the reaction and taking readings at different intervals (a tedious process), or plotting the data directly using an X-Y recorder (with the inherent problems in estimating values from the plot). In either case, it is difficult to measure more than one reaction at a time. Many modern spectrophotometers have built-in kinetics routines to perform such assays, but these units are often expensive (> $25 K Canadian), and offer limited flexibility in the handling of data.

Fortunately, a wide array of laboratory equipment manufactured today is equipped with an RS232c serial communication port and some degree of on-board microprocessing of data. This allows the user can control the equipment with simple programs and microcomputers. The availability of microprocessors means that the instruments provide data in digital form, avoiding the need for expensive analogue to digital conversion hardware and software (Jones et al. 1991).

This appendix describes a procedure for interfacing an LKB Ultrospec® II UV-Visible spectrophotometer (LKB Biochrom Ltd., Cambridge, England) with an IBM microcomputer and using its built-in microprocessor through an RS232c port. This permits enzyme kinetic
analyses, a function which could not be easily accomplished with this model of the spectrophotometer alone.

MATERIALS AND METHODS

Description of Hardware

The LKB Ultrospec® II is a low cost (less than $10 K Canadian) standard spectrophotometer that allows absorbance or transmittance readings in the ultraviolet and visible ranges from 200 to 900nm. The standard unit comes with a six-position cell holder, but accessories allowing temperature control and auto-sampling are also available (Anonymous, 1985). The spectrophotometer is equipped with a built-in microprocessor and an RS232c serial communications port, as well as its own instruction set for instrument control and output (Anonymous, 1985). The spectrophotometer was connected to an IBM XT computer equipped with 640 KB of RAM memory. Although not essential for instrument control or data logging, a Colour Graphics Adapter video card was also used for data display. The data connection was made using a null-modem cable to serial communications port 1 (logical DOS device COM1: ). In this configuration, pin #2 is used to send data to the computer and pin #3 is used to send instructions to the spectrophotometer. The DIL switches on the spectrophotometer were set to allow data transfer at 9600 baud as described in the instruction manual (Anonymous, 1985).

Description of Software

Programming Language and General Considerations

The program was written in Microsoft QuickBASIC® (version 4.0), an easily learned and widely available language, which supports serial communication. Only minor adaptations are necessary to allow the programs to run under other versions of the BASIC language (e.g. Borland TurboBASIC, or IBM BASICA). The programs use a CGA graphics adapter (screen
2). Communication is established with COM1: as an input/output device (in this case we assigned the spectrophotometer to device #1) with data transfer at 9600 baud, 8 data bytes, 2 stop bytes and no parity checking. Commands are sent to the spectrophotometer using PRINT commands and the spectrophotometer's instruction set. Each print command must terminate in a line feed and carriage return (ASCII characters 10 and 13). For example, the command "WAVE 300" when printed to device #1, will cause the spectrophotometer to move to a wavelength of 300 nm. To request and collect data from the spectrophotometer, the command "STATUS" is printed to device #1. This results in the output of a 29 character string containing wavelength, absorbance and sample number to the serial port. This data is collected in a character variable using the INPUT command. For example, the commands:

```
PRINT #1, "STATUS", CHR$(10), CHR$(13)
A$ = INPUT(29, #1)
```

will result in wavelength, absorbance and cell number data being retained in variable A$.

*Enzyme Kinetics Program*

The program prompts the user to enter a) the wavelength at which to monitor the absorbance, b) the number of cells to monitor (up to 6), c) the length of time to monitor the reaction, and d) a file to which the data will be recorded. The user may also zero the spectrophotometer using a suitable reference solution. At time intervals between 6 and 45 seconds (depending on the number of cells used), each sample is read for absorbance and a paired time and absorbance value recorded. The data is also plotted to the screen (absorbance from -1 to 2.0 relative units versus time in minutes) to allow monitoring of the progress of the reaction. To prevent data points from falling on top of each other, the results of each sample are offset by 0.1 absorbance units on the plot. During the data collection, the user may abort the data collection (the data file will still be saved), or pause in the course of the reaction to add another substrate.
Program Source Code

This program was written in June of 1989 by Carl Virtanen and John Berges. They reserve all specific rights regarding this program and its distribution.

********************

* Definitions *

DECLARE SUB delay (wtime!) 'Subroutine to allow a delay in execution
DEFINT C-D, I, N, P, X-Y: 'Integer variables
DIM hlines%(6), PLC(100, 100): 'Dimensioned variables
TIME$ = "00:00:00": 'Initialize timer

hlines%(1) = &HCCCC: 'Define patterns of up to 6 graph lines
hlines%(2) = &HFF00
hlines%(3) = &HF000
hlines%(4) = &HFFFF
hlines%(5) = &H8888
hlines%(6) = &H7777

R$ = CHR$ (13): L$ = CHR$ (10): 'Define carriage control and line feed for output

********************

* Prompt User for Initial Parameters *

PRINT "ULTROSPEC II CONTROL PROGRAM"
LOCATE 5, 1: PRINT "PRESS ANY KEY TO CONTINUE"
GOSUB HOLD

CLS
INPUT "ENTER WAVELENGTH: ", WAVES$: PRINT
INPUT "NUMBER OF CELLS BEING USED (maximum of 6) ": , cmmax: PRINT
INPUT "ENTER MAXIMUM TIME (in minutes) ": , maxim: PRINT
INPUT "ENTER FILENAME FOR DATA OUTPUT (include disk drive and extension): ", PLACE$

IF cmmax = 1 THEN
    timint = 6 / 60
ELSEIF cmax = 2 THEN
  timint = 12 / 60
ELSEIF cmax = 3 THEN
  timint = 18 / 60
ELSEIF cmax = 4 THEN
  timint = 24 / 60
ELSEIF cmax = 5 THEN
  timint = 34 / 60
ELSEIF cmax = 6 THEN
  timint = 40 / 60
ENDIF

poinmx = INT(maxtim / timint)
POIN = poinmx * cmax

'***********************************
" Input/Output Setup "
'***********************************

OPEN PLACE$ FOR OUTPUT AS #3
PRINT #3, "CELL: TIME(min): ABSORBANCE:
CLS

PRINT "TIMER WILL BEGIN AS SOON AS REFERENCE SEQUENCE IS INITIATED"
LOCATE 5, 1: PRINT "PRESS ANY KEY TO CONTINUE"
GOSUB HOLD

CLS
OPEN "COM1:9600,N,8,2" FOR RANDOM AS #1:'Set up serial port 1 for spec
PRINT "SETTING UP"

'***********************************
" Spectrophotometer Initialization "
'***********************************

IF VAL(WAVE$) > 350 THEN:'If wavelength is UV, strike deuterium lamp
  PRINT #1, "DOFF", R$, L$
ELSE
  PRINT #1, "DON", R$, L$
  CALL delax(30)
ENDIF

PRINT : PRINT "SET REFERENCE IF DESIRED"
PRINT : PRINT "PRESS ANY KEY TO CONTINUE"

PRINT #1, "SA. 1, WAVE "; WAVE$, R$, L$: 'Initialize spec on cell #1 at wavelength
GOSUB HOLD

CALL delax(5)
cell = 1
TIMES$ = "00:00:00"
CLS

GOSUB INISCR: 'Initialize output screen

GOSUB GRAB: 'Collect a data point
i = 1: p = 1: GOSUB GRAPH

'*******************************************************************************
'' Loop to Collect and Plot Absorbance versus Time Data *
'*******************************************************************************

FOR i = 2 TO POIN

    IF cell = cmax THEN
        cell = 0: p = p + 1
    END IF

    k$ = INKEY$: 'Check for keyboard input- q to stop, p to pause
    IF k$ = "q" OR k$ = "Q" THEN
        GOTO FINISH
    ELSEIF k$ = "p" OR k$ = "P" THEN
        LOCATE 1, 40
        PRINT "pausing..... (any key to continue)"
        GOSUB HOLD
        LOCATE 1, 40
        PRINT ""
    END IF

    cell = cell + 1
    GOSUB GRAB
    GOSUB GRAPH

    IF TIMER > 900 THEN
        i = POIN - 1
    END IF

NEXT i

'*******************************************************************************
'* Routine to Close Files and Terminate Program *
'*******************************************************************************

PRINT #1, "SAMPLE 1", R$, L$
CLOSE #1
CLOSE #3
LOCATE 22, 1
PRINT : PRINT "ALL DONE!!!"
END
Subroutine to Allow a Pause Until Keyboard Input

HOLD:
DO
LOOP WHILE INKEY$ = ""
RETURN

Subroutine to Collect a Data Point

GRAB:
B$ = "SAMPLE " + CHR$(cell + 48)
PRINT #1, B$, R$, L$:
'Move to next cell
CALL delay(5)
BEFORE = TIMER
PRINT #1, "::: STATUS", R$, L$:
'Request spec data
after = TIMER
a$ = INPUT$(29, #1)
tm = ((BEFORE + after) / 2) / 60:
'sCalculate time of data collection
sorb = VAL(MID$(a$, 14, 5)):'Trim spec data to provide absorbance only
PRINT #3, USING "####.#### "; cell, tm, sorb
RETURN

Subroutine to Graph a Data Point

GRAPH:
IF i > cmax THEN
xl = PLC(cell, 1)
Yl = PLC(cell, 2)
PSET (xl, Yl), cell * 2 + 1
END IF
Yl = 137 - INT(sorb * 50) - (cell * 5):'Scale abs data, offsetting each cell 0.1 units
xl = 25 + INT(tm * 550 / (poinmx * timint)) 'Scale time data
IF i > cmax THEN
LINE -(xl, Yl),cell*2+1,,hlines$(cell):'Connect points with different lines
CIRCLE (xl, Yl), 2, cell * 2 + 1
ELSE
PSET (xl, Yl), cell * 2 + 1
CIRCLE (xl, Yl), 2, cell * 2 + 1
END IF
PLC(cell, 1) = xl
PLC(cell, 2) = Yl
RETURN

Subroutine to Initialize Screen:

INISCR:
SCREEN 2, 0
LINE (25, 37)-(25, 187), 2: 'Draw X and Y axes
LINE (25, 137)-(600, 137), 2
interval = INT(500 / 10): 'Label and Scale Axes
FOR i = 1 TO 10
xl = 25 + INT(i * 50)
LINE (xl, 139)-(xl, 135), 2
xlabel! = (i * (maxtim / 10))
xtikloc = INT((3 + i * interval / 640 * 80)
LOCATE 20, xtikloc: PRINT USING "##.#"; xlabel!
NEXT i

LINE (20, 37)-(30, 37), 2
LINE (20, 87)-(30, 87), 2
LINE (20, 137)-(25, 137), 2
LINE (20, 187)-(30, 187), 2

LOCATE 24, 35: PRINT "time (min)"
LOCATE 4, 1: PRINT " 2"
LOCATE 10, 1: PRINT " 1"
LOCATE 17, 1: PRINT " 0"
LOCATE 23, 1: PRINT "-1"
LOCATE 2, 50: PRINT "Press p to pause, q to quit"
LINE (375, 0)-(630, 20), , B
LOCATE 2, 1: PRINT "ABS"

RETURN

'--------Subroutine to Allow a Specified Delay in Execution------------------
SUB delax (wtime)

deltime = TIMER + wtime
    WHILE TIMER < deltime
        WEND

END SUB

'---------------------------------------------------------------------------
**Example analysis**

To illustrate the use of the program, a reaction was prepared using the pure enzyme lactate dehydrogenase (LDH, L2500, Sigma Chemical). The enzyme reduces pyruvate to lactate, oxidizing NADH in the process. Final reaction concentrations were: 50 mM imidazole buffer, pH 7.9 (I0125, Sigma Chemical), 20 mM sodium pyruvate (P2256, Sigma Chemical) and 0.2 mM NADH. The reaction was run in 1.0 ml volumes in 10 mm methylacrylate cuvettes, and was started by the addition of between 1 and 50 mUnits of enzyme (where 1 Unit catalyzes the conversion of one µmol of substrate to product per minute). The reaction was monitored for 10 min at 340 nm. Fig. B.1 shows a screen plot of the reaction at the midpoint. The different amounts of enzyme are reflected in the different slopes of the lines. Fig. B.2 shows an example of the ASCII file created. Data analysis of this file is easily accomplished through a spreadsheet, utilizing sorting and linear regression routines.
Figure B.1. Screen output of absorbance versus time progress curves from the enzyme kinetics program. From bottom to top, lines represent additions of 50, 20, 10, 5, 1 or 0 mU of lactate dehydrogenase, respectively. Other assay conditions are as described in the text. Note that the screen image was taken only 6 min into the reaction.
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Figure B.2. Sample data file output from the enzyme kinetic program of cell (sample) number, time (min) and absorbance. Cells 1 through 6 represent additions of 50, 20, 10, 5, 1 or 0 mU of lactate dehydrogenase, respectively. Other assay conditions are as described in the text.
APPENDIX C: FITTING ECOLOGICAL AND PHYSIOLOGICAL DATA TO RECTANGULAR HYPERBOLAE: A COMPARISON OF METHODS USING MONTE CARLO SIMULATIONS

INTRODUCTION:

Rectangular hyperbolae (e.g. Michaelis and Menten 1913) are widely used to model biological oceanographic processes such as enzyme kinetics (e.g. Packard 1979), heterotrophic microbial activity (e.g. Li 1983), nutrient uptake or growth rate versus nutrient concentration (e.g. MacIsaac and Dugdale 1969), and grazing rates versus prey concentration (e.g. Mullin et al. 1975). Such hyperbolae are typically of the form:

\[ V = \frac{V_{\text{max}} S}{K_m + S} \]

where \( V \) is the biological rate, \( S \) is the concentration of some substrate required in the process, and the fitted constants \( V_{\text{max}} \) and \( K_m \) represent the maximal rate at substrate concentration and the substrate concentration at half the maximal rate, respectively.

A variety of methods exist to fit this equation to a data set including linearizing the data and iterative non-linear procedures. In the course of fitting data from enzyme kinetics, nutrient uptake and growth versus irradiance experiments, it was noted that different methods gave dramatically different results. Thus, it became important to resolve which method was best for a given data set. Although there is an extensive biochemical and physiological literature to guide the investigator in the choice of a fitting procedure (see Wilkinson 1961, Cleland 1967, Eisenthal and Cornish-Bowden 1974, Lam 1981, Nelder 1991, Johnson 1992), the data obtained in the course of thesis experiments often violated several key assumptions of fitting procedures: a) error variation was high (>10% coefficient of variation) and was either constant with \( S \) or varied in proportion with \( S \), b) \( S \) itself was not measured without error, and c) the distribution of values for \( V \) at given \( S \) was not always uniform. Depending on the procedure used, these violations of assumptions may introduce significant error into
determination of \( V_{\text{max}} \) and \( K_m \). There have been considerations of these problems in which field data have been used to assess the accuracy of different procedures (e.g. Mullin \textit{et al.} 1975, Li 1983). Ultimately, however, such an approach relies on a subjective judgment of which fit is most "correct", since the actual parameters are unknown.

Recently, Tseng and Hsu (1990) used a Monte Carlo approach to the problem, by creating data sets with known \( V_{\text{max}} \) and \( K_m \) and adding random errors. However, their study considered a restricted range of error levels, a regular spacing of data points across \( S \), and only one non-linear fitting method. In this investigation, randomly generated data sets of different error levels and distributions of points were used in order to compare six fitting procedures.

\textit{MATERIALS AND METHODS:}

\textbf{Data Generation}

Five data cases were devised (Fig. C.1): Case 1, where data were geometrically distributed; Case 2, where there were no data for \( S \) less than \( K_m \); Case 3, where there were no data for \( S \) greater than \( K_m \); Case 4, where all data had \( S \) higher then \( 2 \times K_m \) or lower than \( 0.5 \times K_m \); and Case 5, where all data fell between \( 2 \times K_m \) and \( 0.5 \times K_m \). For each case, data sets were generated with \( V_{\text{max}} = 10 \) and \( K_m = 2 \). Normally distributed random errors (generated using a SYSTAT procedure, Wilkinson 1990) were assigned in four ways: errors of 10\%, 20\%, 50\% in \( V \) alone, or 20\% (independent) error in both \( S \) and \( V \). This was done holding error constant (as a fraction of \( 0.5 \times V_{\text{max}} \)) or letting error vary with \( V \) (see Fig. C.2). In each case, 100 data sets of 10 points each were generated.

\textbf{Fitting Procedures}

Fitting procedures were implemented on an IBM personal computer using software written by the author or modified from published software, as noted below. Six fitting procedures were compared:
Figure C.1. Data cases considered. Cases represent geometrically distributed data (Case 1), data where no points are lower than $K_m$ (Case 2), data where no points are higher than $K_m$ (Case 3), data where only points higher than $2xK_m$ or lower than $0.5xK_m$ are available (Case 4), and data where all points fall between $2xK_m$ and $0.5xK_m$ (Case 5). In each case, data sets of 10 points were generated with $V_{max} = 10$ and $K_m = 2$. 
Figure C.2. Examples of error levels (as percentages of V and S) assigned, using Case 1 as an example. Constant error levels are set as percentages of 0.5 x \( V_{\text{max}} \). Errors were assigned in a normal distribution.
a) Lineweaver-Burk (LB, Lineweaver and Burk 1934)

This double-reciprocal transformation of Equation (1) gives: 
\[ \frac{1}{V} = \left( \frac{K_m}{V_{\text{max}}} \right) \left( \frac{1}{S} \right) + \left( \frac{1}{V_{\text{max}}} \right) \]
which gives a straight line with slope \( \left( \frac{K_m}{V_{\text{max}}} \right) \) and intercept \( \left( \frac{1}{V_{\text{max}}} \right) \). Slope and intercept were estimated by linear regression and parameters calculated.

b) Eadie-Hofstee (EH, Hofstee 1952)

Rearranging equation (1) gives: 
\[ V = -K_m \left( \frac{V}{S} \right) + V_{\text{max}} \]
from which parameters are estimated as for the LB method.

c) Hanes-Woolf (HW, Hanes 1932)

Another rearrangement of equation (1) gives: 
\[ \frac{S}{V} = \frac{K_m}{V_{\text{max}}} + S \left( \frac{1}{V_{\text{max}}} \right) \]
from which parameters may be estimated as above.

d) Eisenthal and Cornish-Bowden (ECB, Eisenthal and Cornish-Bowden 1974)

In this median method, the points of intersection of the lines defined for each \((V, S)\) pair by: 
\[ y = \left( \frac{V}{S} \right)x + V \]
are calculated. The median value of \(x\) corresponds to \(K_m\), and the median value of \(y\) to \(V_{\text{max}}\). The program was adapted from Myers et al. (1990), with improvements to the sorting routine.

e) Cleland-Wilkinson (W, Wilkinson 1961)

This method first estimates values of \(V_{\text{max}}\) and \(K_m\) from a linear plot (comparable to HW), then uses an iterative non-linear method to directly fit the equation to the data using least squares as a criterion.

f) Tseng-Hsu (TH, Hsu and Tseng 1989)

This method uses a least squares criterion as for W, but uses a random search technique. Estimate of the ranges in which \(V_{\text{max}}\) and \(K_m\) lie are made. Then 500 random pairs of points are picked within this range. Least squares are calculated for each estimate and
the best 10 pairs are identified. The search range is re-defined based on the variance of the best 10 parameter pairs and the process is repeated for up to 100 iterations.

Real data sets

Fitting procedures were also applied to three real data sets for comparison. These included nitrate reductase activity versus nitrate concentration in a marine microalga (see Chapter 2), representing data with low, constant error; phosphate uptake versus phosphate concentration in a marine macroalga (data from Hurd and Dring 1990), representing data with high, variable error; and growth rate versus prey concentration in a marine ciliate (data from D. Montagnes, unpublished), representing data with high, constant error.

RESULTS:

Table C.1. shows typical data generated by this procedure for Case 1 and 3. These cases were selected because they show best (Case 1) and worst (Case 3) fits. Median values were chosen for presentation because frequency distributions of estimates were often non-normal (see Fig. C.3).

Table C.2. is a visual summary of the results of fitting procedures by case and by error levels. In order to rank the success of fitting procedures, two criteria were selected: the accuracy of the estimate (i.e. how close the median estimate was to the true value) and the precision of the estimate (i.e. what percentage of estimates fell within a specified range). The choice of levels was somewhat arbitrary. Enzyme activity and growth rate data in other chapters of the thesis usually gave coefficients of variation of 20% or less, thus a 20% margin of error was allowed in terms of accuracy. Based on a normal distribution, the majority of values would be expected to fall within two standard deviations of the mean, thus a 40% range was selected for precision i.e. values between 6-14 for $V_{\text{max}}$ and 1.6-2.4 for $K_m$. In Table C.2, median estimates that fell within 20% of the true value and in which fewer that 50% of the 100 estimates outside the respective range were marked with a single asterisk. If less than 20% of the 100 estimates fell outside the range a double asterisk was used.
Table C.1. Results of model-fitting procedures (Lineweaver-Burk, LB; Eadie-Hofstee, EH; Hanes-Woolf, HW; Eisenthal and Cornish-Bowden, ECB; Cleland-Wilkinson, W; or Tseng-Hsu, TH) for Case 1 (CS1) and Case 3 (CS3) data. Notation describes error as constant (C) or variable (V) and error levels as a percentage (10, 20 or 50). Error in both S and V is denoted "XY". Bold numbers represent medians of estimated parameters of the data. The true values are \( V_{\text{max}} = 10 \), and \( K_m = 2 \). Numbers in lighter face represent the percentages of estimates which fell outside the ranges of 1.2-2.8 for \( K_m \) and 6-14 for \( V_{\text{max}} \).

### ESTIMATED Vmax

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Figure C.3. Examples of frequency distributions of $V_{\text{max}}$ and $K_m$ estimates for various fitting procedures for Case 1 data with 20\% variable or constant error. Procedures are: ○ Lineweaver-Burke, • Eadie-Hofstee, ▽ Hanes-Wolfe, ▼ Eisenthal-Cornish Bowden, □ Cleland-Wilkinson, and ■ Tseng-Hsu. Y-axis scale is relative percentage.

True values of $V_{\text{max}}$ and $K_m$ are 10 and 2, respectively.
Table C.2. Summary of the results of fitting procedures for $V_{\text{max}}$ and $K_m$. Conditions marked with "***" indicate cases where the median of the estimates was within 20% of the true parameter values (10 or 2) and less than 20% of the estimates fell outside the ranges of 6-14 for $V_{\text{max}}$ or 1.2-2.8 for $K_m$. Conditions marked with "**" indicate that the median was within 20% of the true values, and less than 50% of the estimates fell outside the specified ranges.

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Table C.2. (Continued)

### Km Estimates

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In general, linear models performed well only at low error (10%) and when error varied with the mean (variable, V). Interestingly, the EH technique seldom specified $V_{\text{max}}$ within criteria, but was more successful for $K_m$. LB and HW methods estimated both parameters equally well.

The ECB method was more successful than linear methods as error increased, but W and TH methods showed the greatest accuracy and precision. Even for these non-linear least-squares methods, however, parameters were poorly estimated at high error levels. The estimates were worst when few points at high S were available (e.g. Cases 3 and 5). In contrast to linear methods, constant error cases appeared to be fitted better than variable cases, particularly at high error levels.

The TH method was marginally better in terms of accuracy of estimates than W, but this was always in cases where initial estimates for W (derived from a linear method estimate in the procedure used) were substantially in error. The methods were exactly equivalent when suitable initial estimates were provided. It was also found that parameters estimated using these methods corresponded exactly to those found using a number of other non-linear least-squares methods (e.g. Gauss-Newton, Marquant, Simplex) which are available in packages such as SYSTAT (Wilkinson 1990), or SigmaPlot (Jandel Scientific, Santa Clara, CA) or in a range of specially written software programs (e.g. Hansoon et al. 1967, Lam 1981, Leatherbarrow 1990, Jones and Taransky 1991, Stals and Declerq 1992, Brooks 1992).

It is also of note that despite cautions about the effects of error in S (see Leatherbarrow 1990), the results of analyses with errors in both S and V (i.e. the "XY" error levels) were very similar to cases where the same error was introduced only in V.

The results of the Monte Carlo simulation were reflected in real data set analyses. Fig. C.4, A represents enzyme kinetic data with a low (10%), constant error and a distribution similar to Case 1. From Table C.2, it would be predicted that such a data set would not be fit well by linear methods. As shown in both Fig. C.4A and Table C.3, linear estimates are clearly inadequate. In the nutrient uptake data set represented in Fig. C.4B, data is close to Case 2, with high (20-50%) and variable error. Although none of the methods would be
Figure C.4. Examples of real data sets fit to rectangular hyperbolae using different fitting methods: —— Lineweaver-Burke, ⋯⋯ Eadie-Hofstee, ⋯⋯⋯ Hanes-Wolfe ⋯⋯⋯ Eisenthal-Cornish Bowden, or ⋯⋯⋯ Cleland-Wilkinson or Tseng-Hsu.

A) Nitrate reductase activity versus nitrate concentration in extracts of the diatom *Thalassiosira pseudonana*, B) phosphate uptake versus concentration in the marine macroalga *Fucus spiralis*, and C) growth rate versus prey concentration for the marine ciliate *Strombidium* sp. feeding on the marine alga *Rhodomonas* sp. Parameters for each fit are given in Table C.3.
Table C.3. Comparison of estimates of the parameters of rectangular hyperbolae ($\text{V}_\text{max}$ and $\text{K}_\text{m}$) for three real data sets, using different fitting methods. Fitting procedures are: Lineweaver-Burke (LB), Eadie-Hofstee (EH), Hanes-Woolf (HW), Eisenthal-Cornish Bowden (EBC), Cleland-Wilkinson (W), and Tseng-Hsu (TH). Data sets are pictured in Fig. C.4, and described in the text.

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<th>NR vs. [NO$_3^-$]</th>
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<tr>
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predicted to do a good job, as indicated in Table C.2, in this case, where error is variable, parameter estimates from linear techniques would be expected to come closer to parameters estimated by non-linear techniques. This is in fact the case (Table C.3). For the growth versus prey concentration data, a case of high (20-50%) and constant error, linear methods again gave the poorest results (Fig. C.4C; Table C.3), as predicted from the simulation (Table C.2).

**DISCUSSION:**

Provided adequate initial parameter estimates are given, non-linear least square methods appear to be the most useful approach to fitting data to rectangular hyperbolae. Of the non-linear fitting methods available, commercially-available non-linear fitting packages using methods such as Gauss-Newton, Simplex, or Marquant may be most suitable for routine use. The W techniques suffers from poor initial estimates in some cases, while the TH method is inefficient and difficult to apply to data sets larger than 25 points (see Cornish-Bowden 1991). This approach can also give error estimates for parameters, although these must be interpreted cautiously, particularly if statistical comparisons are to be made (see Johnson 1992). A better strategy would be replication of experiments with parameter estimates made for each replicate. The mean and standard error of these estimates would provide a better estimate of the true parameters and their error.

In considering the conclusions from this simulation two points must be kept in mind. First, the errors introduced were normally distributed and were either constant, or increasing with S. Clearly, the number of possible patterns of error distribution are enormous. It is unclear how readily the results of this simulation can be applied to a case where error decreases with S and is Poisson distributed, for example. Secondly, this study has not considered whether the rectangular hyperbola is an appropriate model for a given data set. Indeed, there is evidence that nutrient uptake kinetics, and growth versus food concentration may not fit hyperbolic kinetics perfectly (see Mullin *et al*. 1975, Hurd *et al*. 1990).
Unfortunately, in real data sets, it may often be impossible to distinguish a lack of fit due to error from a lack of fit due to an unsuitable model.

Finally, in addition to superior accuracy and precision, non-linear models provide greater flexibility in that they readily permit consideration of cases where there are non-zero intercepts (e.g. Leatherbarrow 1990), or inhibition constants (e.g. Li 1983), which are problematic to accommodate in linear fitting methods.